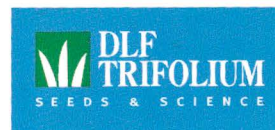# EUCARPIA

European Association for Research on Plant Breeding
Europäische Gesellschaft für Züchtungsforschung
Association Européenne pour l'Amélioration des Plantes

PROCEEDINGS

XXVIIth EUCARPIA SYMPOSIUM ON IMPROVEMENT OF FODDER CROPS AND AMENITY GRASSES

Copenhagen (Denmark), 19th to 23rd August 2007

DLF
TRIFOLIUM
SEEDS & SCIENCE

PROCEEDINGS

XXVIIth EUCARPIA SYMPOSIUM ON IMPROVEMENT OF FODDER
CROPS AND AMENITY GRASSES

Copenhagen (Denmark), 19th to 23th August 2007

Chairman of the organising committee

Thomas Lübberstedt, University of Aarhus, Faculty of Agricultural Sciences (DJF), Research Centre Flakkebjerg, Forsøgsvej 1, 4200 Slagelse, Denmark
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General meeting information

http://www.eucarpia.org/01sections/foddercrops/section_meetings2/sm2.html

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Sven Bode Andersen, KU LIFE, Denmark
Ulf Feuerstein, EURO GRASS, Germany

Editors

Thomas Lübberstedt
Bruno Studer
Sonja Graugaard

Programme

Sunday, August 19

18:00 - 20:00 — Registration, poster mounting, welcome reception

Monday, August 20

7:30 — Registration, poster mounting

Opening session

8:30 — **Ian Max Møller**, DJF, Research Director Genetics and Biotechnology

8:45 — **Beat Boller**, ART, Chairman EUCARPIA Fodder Crop and Amenity Grass section

Session 1
Seed production

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Chairpersons
Birte Boelt, Ulrich Posselt

9:00 — **Anders Mondrup**, DLF
Limitations in seed production

9:30 — **Birte Boelt**, DJF
Seed yield components and their contribution to yield

10:00 — Coffee break and poster viewing

10:30 — **Danny Thorogood**, IGER
Towards resolving vegetative and reproductive growth conflicts in turfgrasses

11:00 — **Grzegorz Żurek**, IHAR
Relation between seed yield potential and turf quality in Poa pratensis *L.*

11:20 — **Susanne Barth**, TEAGASC
Variation in seed yield and other morphological traits in a collection of Irish Lolium perenne *ecotypes and bred varieties*

11:40 — **Bruno Studer**, DJF
Genetic characterisation of seed yield and its components in perennial ryegrass Lolium perenne *L.*

12:00 — **Peter Button**, UPOV
New developments in the international union for the protection of new varieties of plants (UPOV)

12:30 — Lunch, poster viewing

Session 2a
Applied genomics

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Chairpersons
Christian Huyghe, Odd Arne Rognli

14:00   **Thomas Lübberstedt**, DJF
EU-project "Development of allele-specific markers for sustainable grassland improvement (GRASP)": overview

14:10   **Oene Dolstra**, PRI
GRASP: Candidate gene isolation

14:30   **Thomas Lübberstedt**, DJF
GRASP: SNP discovery

14:50   **Isabel Roldán-Ruiz**, ILVO
GRASP: SNP validation by association studies

15:10   **Niels Roulund**, DLF
GRASP: Technology transfer to plant breeding

15:30   Coffee break and poster viewing

16:00   **Philippe Barre**, INRA
Association studies using synthetic varieties: case study of GAI gene and leaf length in perennial ryegrass

16:20   **Lesley Turner**, IGER
Changes in allele frequency during phenotypic selection for leaf water-soluble carbohydrate (WSC) in an experimental population of perennial ryegrass

16:40   **Roland Kölliker**, ART
*Genetic characterisation of persistence in red clover (*Trifolium pratense *L.)*

17:00   **Bernt Hackauf, Hans Lellbach**, BAZ
Mapping of LmPc1, a major dominant gene conferring resistance to crown rust in Lolium multiflorum.

19:00   Social dinner at LIFE

Tuesday, August 21

Session 2b
Applied genomics

Chairpersons
Mervyn Humphreys, Roland Kölliker

8:30   **Odd Arne Rognli**, UMB
Genetic analysis of seed yield components

9:00   **Rikke Bagger Jørgensen**, Risø National Laboratory
Co-existence with GM crops: grasses, clover and fodder beet

9:30   **Heidi Rudi**, UMB
SNP haplotypes at candidate gene loci associated with frost tolerance in Lolium perenne *L.*

9:50   **Klaus Dehmer**, IPK
SNP genotyping of a large Lolium *genetic resources collection and data analysis via a diversity studies toolkit*

10:10   Coffee break and poster viewing

10:40   **Ian King**, IGER
Comparative analyses reveal the major fraction of functionally annotated gene models in monocots are located in recombination poor/very poor regions of the genome

11:10   **Birgit Hougaard**, Aarhus University
Bridging model and crop legumes using legume anchor markers

11:40   **Leif Skøt, Michael Abberton**, IGER
Using translational genomics to underpin germplasm improvement for complex traits in crop legumes

12:00   **Puthigae Sathish**, ViaLactia
Perennial ryegrass improvement through Cisgenics

12:20   **Muriel Vandewalle**, ILVO
DNA marker assisted selection for yield and nutritional traits in Italian ryegrass

12:40   Lunch and poster viewing

14:00 - 17:00   Parallel workshops
Chairpersons listed

15:30   Coffee break

Workshop 1	**Genetic resources: optimal management and quality assessment** **Beat Boller,** ART **Klaus Dehmer,** IPK
Workshop 2	**Conventional versus molecular breeding** **Niels Roulund,** DLF **Ulrich Posselt,** LSA
Workshop 3	**DUS and VCU – implementation of molecular markers?** **Ulf Feuerstein,** EURO GRASS **Isabel Roldán-Ruiz,** ILVO

17:00 - 18:30	Plenary: conclusions from the three workshops
18:30 - 19:00	Business meeting for EUCARPIA members / Poster removal
19:00	Free evening / Copenhagen tours

Wednesday, August 22

8:00	From LIFE: Excursion to Tystofte (variety testing) and Research Centre Flakkebjerg
12:30	Lunch in Flakkebjerg
14:30	Visit at DLF-TRIFOLIUM A/S
19:00	Conference dinner near DLF-TRIFOLIUM A/S

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Thursday, August 23

Session 3
Forage quality and bioenergy

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Chairpersons
Fred Eickmeyer, Iain Donnison

8:30   **Ulf Feuerstein**, EURO GRASS
NIR-spectroscopy of non-dried forages as a tool in breeding for higher quality– laboratory test and online investigations in plot harvesters

9:00   **René Gislum**, DJF
NIRS and chemometrics – exploration of grass forage quality

9:20   **Kevin Jensen**, USDA
Forage quality of irrigated pasture species as affected by irrigation rate

9:40   **Leif Skøt**, IGER
Association of candidate genes with flowering time and forage quality traits in Lolium perenne

10:00   Coffee break

10:30   **Slobodan Katić**, IFVC
Genetic and seasonal variations of fibre content in lucerne

10:50   **Ulrike Anhalt**, TEAGASC
Identification of biomass QTL in a perennial ryegrass inbred derived population

11:10   **Claus Felby**, LIFE
Biomass for bioenergy - What are the ideal properties of biomass crops

11:40   **Iain Donnison**, IGER
Quality traits for bioenergy in grasses

12:10   Closing of meeting

Other meetings/workshops

Rust workshop
August 19, 11:00 - 16:00, see separate announcement
(roland.koelliker@art.admin.ch)

AGROBASE workshop
August 19, 16:30 - 18:00, see separate announcement
(ulf.feuerstein@eurograss.com)

European Lolium & Festuca Initiative (ELFIN) meeting
August 21, 19:00 - 21:00, see separate announcement
(torben.asp@agrsci.dk)

NIRS Online Forage User Group (NOFUG) meeting
August 21, 19:30 - 22:00, see separate announcement
(ulf.feuerstein@eurograss.com)

Description of exhibitors

DLF-TRIFOLIUM A/S

Roskilde, Denmark
www.dlf.dk

With an estimated market share of 20% worldwide, DLF-TRIFOLIUM is the world's biggest seed company within the breeding, production, sale and marketing of cool season clover and grass seed species.

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The Royal Barenbrug Group is a globally operating grass seed company. We specialise in plant breeding, seed production and international marketing of forage and turf grasses.

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EURO GRASS Breeding is one of the leading companies in forage and turf grass breeding as well as in small seeded forage legumes. The main species are perennial ryegrass, Italian and annual ryegrass, timothy, meadow fescue, cocksfoot, kentucky bluegrass, red fescue and different clover species. International marketing and sales are carried out by Euro Grass, a joint venture of Deutsche Saatveredelung AG (Germany) and Hunsballe Frø (Denmark). Combining the strengths of these partners a powerful breeding network, large seed multiplication and efficient structures in sales, logistics and other services are built up to meet the customers' needs.

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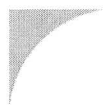
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Copenhagen, Denmark
www.landbrugsraadet.dk

The Danish Agricultural Council (the DAC) is the leading special-interest organisation for farmers and the food industry in Denmark. In 2006 our members exported agricultural products and equipment in the amount of EUR 14 billion and employed a total of 180,000 people.

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Contents

Towards resolving vegetative growth and seed production conflicts in perennial ryegrass used for turf and forage

D. Thorogood & I.P. Armstead

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ABSTRACT

Turf and forage quality is entirely dependent on vegetative characteristics, yet to produce commercially viable varieties, seed production potential must be optimised. However, selection for seed production traits that are dependent on preferable allocation of resources from vegetative to reproductive organs will inevitably be negatively correlated with turf performance. The proportion of florets that produce a seed, and seed retention are two reproductive traits that are independent of vegetative growth performance traits and are therefore worth targeting. This paper focuses on seed set in perennial ryegrass for which there is considerable variation. Our approach has been to simply identify major QTL in mapping families that influence seed setting ability. In particular, we wanted to know whether the QTL identified were determined by resource allocation factors, that might suggest some compensatory mechanisms which would lead to no increase in overall seed yield potential, or, more significantly, genetic breakdown of gamete/embryo development processes. By comparing orthologous regions in rice, we also wished to identify putative orthologues of genes underlying these QTL, the physiological basis of which may already be known. Using data from an F2 and a BC1 mapping family we identified a common genomic region on linkage group 7, which is also associated with heading date, that accounted for 22 and 18% respectively of the total variation. We also identified other regions in the F2 family on LG4 that influenced seed set that indicated the presence of a recessive gene affecting gamete development accounting for 16% of the total variation. Two further seed set QTL were identified from selfing data in the F2 family that co-located with the *S* self-incompatibility locus and the *T* self-compatibility locus and accounting for 13 and 9% respectively of the total variation for self seed set.

Key words: gamete development; *Lolium perenne*; orthologous region; quantitative trait locus (QTL); resource allocation; seed/ovule ratio; self-incompatibility; zygote development

INTRODUCTION

Seed production is an essential characteristic for breeding commercially viable grass forage and turfgrass varieties. It is a complex trait with many components all contributing to final seed yield. A major dilemma for grass breeders is to produce grass varieties with superior vegetative growth characteristics yet at the same time maintain adequate seed yield to ensure successful multiplication on a commercial scale. Competition for resources between vegetative and reproductive structures within the grass plant or sward will determine the seed setting potential which is determined by the final number of inflorescences produced, the number of spikelets per inflorescence and the number of florets per spikelet. All of these

parameters are subject to high heritability (see Elgersma, 1990). The timing of inflorescence production is also highly heritable and may have a profound influence on seed yield depending on coincident environmental conditions. Uniformity of flowering time may also be critical in determining the proportion of seed that is mature at harvest. Although heritability estimates for theses parameters indicate that seed yield can be increased by selection, this selection is almost certain to adversely influence agronomic performance.

Ultimately, seed yield is determined by the effective exploitation of the seed setting potential. A major paradigm of life-cycle evolution is that outbreeding species have lower seed/ovule ratios than inbreeders. Wiens *et al.* (1987) showed this to be the case in a wide range of congeneric species, one of the most striking examples given being that of the outbreeding willowherb (*Epilobium angustifolium*) with a seed/ovule ratio of 0.372 compared to the 0.953 of the inbreeder, *E. ciliatum*. Similarly the self-incompatible *Lolium perenne* sets significantly less seed than self-fertile *L. temulentum* (Table 1).

Table 1. Published seed setting data for Lolium perenne L. (outbreeder) and L. temulentum L. (inbreeder).

		% florets setting seed	
		Fearon *et al.* (1983)*	**Cornish *et al.* (1980)***
L. perenne	open pollinated	57.5	
	Pair-cross units	20.6	6.68
			10.33
			10.88
		Jenkin (1930)*	
L. temulentum	open pollinated	79.64	
		78.31	
		88.20	
		95.44	
	Pair-cross units	76.72	

*These three papers are not included in the list of literature.

Increasing seed setting ability is a useful strategy for increasing total seed yield because (1) there is potential in outbreeding crops to improve it and (2) under normal grassland management systems this increase will have no detrimental effect on agronomic performance. Our research therefore seeks to identify the underlying genetic control mechanisms of seed setting ability which will lead to the development of marker-based selection strategies for its improvement.

MATERIALS AND METODS

We used two unrelated *L. perenne* mapping families for our initial studies. The first was an F2 family of 188 genotypes produced by selfing an F1 plant that was in turn derived from a cross between genotypes of 'Aurora' (early flowering) and 'Perma' (late flowering) that had been produced by obligate selfing over three generations (see also Armstead *et al.*, 2002 (not included in the reference list); Thorogood *et al.*, 2005; Turner *et al.*, 2006). The second was a BC1 (ILGI) family of 183 genotypes derived from a cross between a doubled haploid anther-

culture derived plant and a heterozygous plant from the IGER Ryegrass breeding programme (see also Armstead *et al.*, 2002; Jones *et al.*, 2002; Thorogood *et al.*, 2002).

There was no clonal replication of plant genotypes as each marker genotype was represented by several plants.

Seed set was determined in the F2 family as mean number of seeds per inflorescence after both open pollination and in selfed units of between five and ten inflorescences isolated by bagging, in an unheated, unlit glasshouse. Seed set in the BC1 (ILGI) family was determined after open pollination as mean number of seeds per spikelet.

In addition, in order to gain insight into the potential underlying causes of genetic differences in seed set, flag leaf length and width from five flag leaves per genotype was determined in both mapping families. Considerable male sterility manifested through poor anther dehiscence was observed in the F2 family so anther dehiscence was scored on a subjective 0-5 scale where 0 = no dehiscence and 5 = complete dehiscence along with pollen viability scored as a proportion of viable pollen as measured by observing pollen grains under a low power (X10) light microscope that had been stained with aceto-carmine.

The marker data used was produced as described by Jones *et al.* (2002) for the ILGI family and by Armstead *et al.* (2004), Skøt *et al.* (2005) and Turner *et al.* (2006) for the F2/WSC family. Additional markers used were developed using primer sequences from LpCk2a-1 and LpVrn-1 (chromosome 4) and Hd3a, 06g10880, 06g11020 and 06g11180 (chromosome 7). QTL analyses were made using the software package mapQTL v. 4.0 (van Ooijen *et al.*, 2002) – not included in the list of literature. Candidate genes and orthologous QTL in rice were identified using the database www.gramene.org.

RESULTS

Seed set on open pollination showed a bimodal distribution in the F2 family with about a quarter of the plants being completely sterile whereas that in the ILGI family showed a normal distribution (Figure 1).
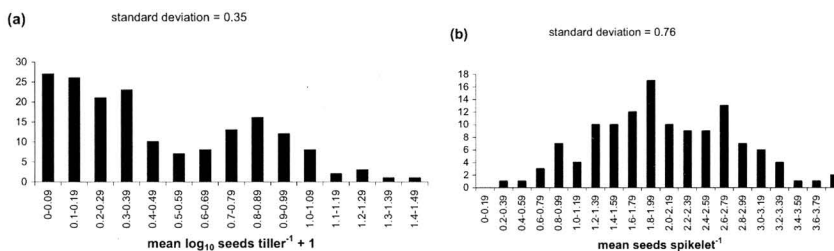
Fig. 1. Distribution of seed set in (a) F2 family and (b) ILGI family.

MQM mapping revealed a significant QTL on linkage group (LG) 4 in the F2 family but not in the ILGI family. This QTL aligned closely although separated from a QTL for heading date that was revealed in both mapping families (Figure 2). QTL analyses of pollen viability in the F2 family revealed a QTL that co-located to the seed set QTL and an anther dehiscence QTL approximately 19cM away (Figure 2).

The mean scores for the segregating marker genotypes (Table 2) indicate that low seed set and pollen viability is a recessive trait, probably controlled by a single gene underlying the QTL on LG4 that originates from the original 'Aurora' inbred parent. The fact that the QTL was revealed after open pollination indicates that female fertility is also adversely affected.

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MQM mapping also revealed a QTL on LG7 for seed setting and the positions which coincided in both cases with a heading date QTL in the *hd1/hd3* gene region were aligned for both maps (Figure 3). Again low seed set appeared to be a recessive trait associated with late heading in both families. Furthermore, flag leaf length and width associations were also significant in this region (Table 3).
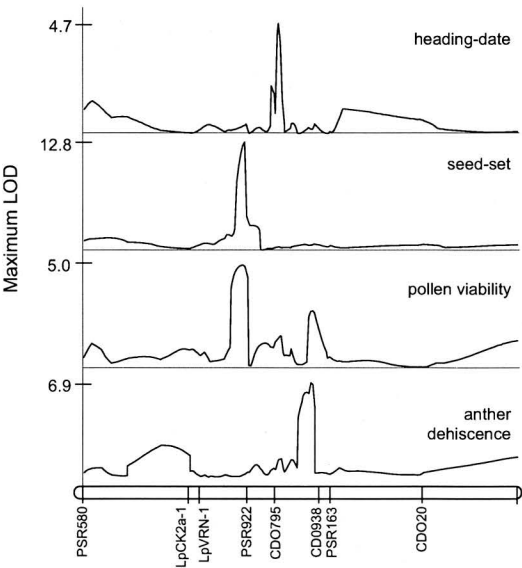
Fig. 2. MQM profiles for heading-date, seed-set, pollen viability and anther dehiscence QTL on C4 of the F2 family. Heading date QTL LOD profile is included for reference.
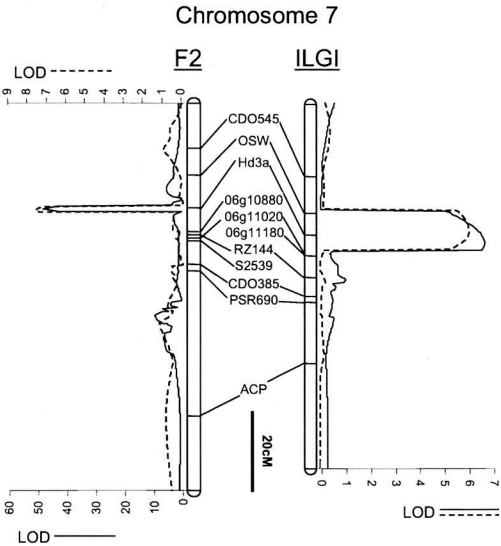
Fig. 3. MQM LOD profiles for heading date (solid line) and seed-set (dashed line) for chromosome 7 of the F2 and ILGI familes.

Table 2. Mean genotype scores for seed set and pollen viability for markers closely associated with the seed set and pollen viability QTL on LG4.

Trait	Marker	Genotype class			
		aa	ab	bb	sed
Seed set	Rye12	0.18	0.40	0.52	0.068
Pollen viability	Rye12	0.28	0.60	0.58	0.056
	R2702B	0.31	0.55	0.66	0.072
	CD0938	0.31	0.55	0.66	0.061

The F2 population was, in general, highly self-fertile although the same QTL on LG4 revealed after open pollination was also revealed after selfing. Although both parents only set a few seed when selfed, the F1 exhibited considerable heterosis for self seeding. Thorogood *et al.* (2005) demonstrated through an analysis of in-vitro self-pollinations, that could be classified as either half- or fully compatible, that self-fertility in this population was controlled by two genes which align to positions on LG1 and LG5 that correspond to the *S* incompatibility locus (Thorogood *et al.*, 2002) and the *T* self-compatibility locus (Fuong *et al.*, 1993; Voylokov *et al.*, 1993) where the *S* sf allele derives from the 'Aurora parent and the *T* sf allele from the 'Perma' parent. The QTL analysis of seed set on selfing reported here also revealed two QTL coinciding with the self-compatibility QTL identified by Thorogood *et al.* (2002) (Figure 4).

Table 3. Mean scores of marker classes for hd3a(LD) marker underlying significant QTL on C7 for F2/WSC and ILGI mapping populations.

Trait	F2 (hd3agt)				ILGI (hd3(LD))		
	aa	ab	bb	sed	aa	ab	sed
Heading date	39.2	42.7	61.1	1.52	57.8	54.8	0.76
seed set	0.57	0.50	0.14	0.06	1.61	2.21	0.13
flag leaf width	8.4	8.2	7.0	0.23			
flag leaf length	17.0	18.0	15.8	0.57	99.0	86.7	3.10
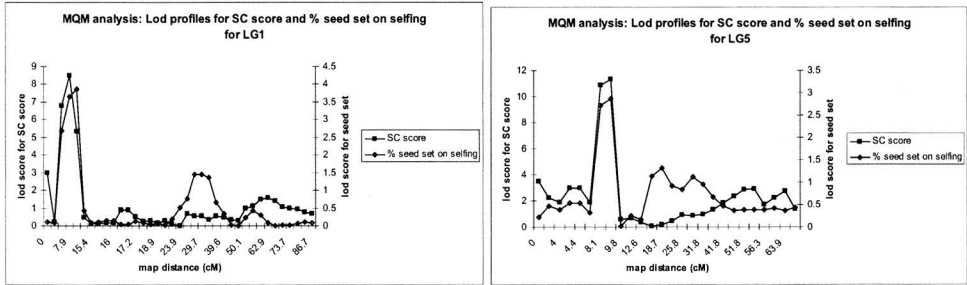
Fig. 4. Coincidence of QTL for self-compatibility and self seed set.

Table 4. Mean % seed set on selfing in the F2 family (upper case S and T = self-fertility alleles, lower case s and t = functional self-incompatibility alleles.

		cdo580 (LG1)			
		aa (SS)	ab (Ss)	bb (ss)	Total
	aa (tt)	34.11	27.34	-	**31.10**
cdo127 (LG5)	ab (Tt)	28.64	22.14	16.12	**22.50**
	bb (TT)	33.94	19.03	5.66	**16.36**
	Total	**30.42**	**21.01**	**9.26**	**21.21**

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DISCUSSION

QTL analysis has revealed a number of genomic locations that have significant bearing on seed setting ability in outbreeding *L. perenne*. The QTL on LG4 indicates the presence of one or more recessive genes that severely limit seed setting ability. It would appear to be an example of ryegrass' high genetic load, expressed through aberrant gametophyte development, exposed by inbreeding that is even present in a commercially available cultivar. Another QTL with considerable influence on reducing seed set was revealed on LG7, again controlled by one or more recessive genes. The underlying process is unclear. We cannot separate the QTL for seed set with the heading date QTL so cannot dismiss the possibility that *hd1* and *hd3* underlying this QTL are directly responsible. It is unlikely that differences in seed set are simply a consequence of late flowering genotypes being exposed to a smaller pollen cloud as the difference in heading date caused by this QTL in the ILGI family is only a matter of four days. We cannot dismiss the possibility that the difference results from differential resource allocation from flag leaves especially in the light of Fang *et al.*'s (2004) finding of a genetic correlation between seed yield and flag leaf size. Also, of considerable interest is the putative location of an orthologue of the *S5* wide compatibility gene of rice on chromosome 6 (Qiu *et al.*, 2005; Ji *et al.*, 2005) that lies halfway between the *hd1* and *hd3* gene that results in halving seed set in rice.

We did not expect to find QTL for seed set associated with the incompatibility loci in the F2 population. The QTL cannot be accounted for on self-compatibility data alone. Firstly, it would not be expected that a self with 50% compatible pollen-tube growth would limit final seed set compared to a 100% compatible self. Secondly, in the case of the *T* locus, the 50% compatible selfings actually set more seed than the fully compatible selfings (Table 4). We can only conclude that the SI reaction in the multi-locus grass system involves more than just control of the initial stigma-pollen interaction. Heslop-Harrison (1982) observed variation for pollen-tube growth on grass stigma recognising a three-step process of recognition, rejection and pollen-tube growth rate. Our results suggest that the final step governing pollen-tube growth rates may play a part in influencing final seed set and that the incompatibility genes have a direct role. This may be through gene interaction between incompatibility genes or even within the individual super-gene complexes that the incompatibility loci are thought to consist of. Up until recently, the SI genes have been thought to have little influence on the seed setting ability of usually highly heterogeneous ryegrass breeding material because the large extent of estimated SI polymorphism (Fearon *et al.*, 1994). This allelic classification was based on the initial rejection reaction, not taking into account further incongruities that might occur through the stigma transmitting tract. Our findings may require a re-examination of this assumption.

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Relation between seed yield potential and turf quality in *Poa pratensis* L.

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ABSTRACT

Twenty seven entries (10 cultivars and 17 breeding strains) of smooth-stalked meadowgrass (*Poa pratensis* L.) were tested during 2002 – 2004 for seed yield and turf quality. Seed yield potential was estimated on the basis of: seed yield per plot (SY), seed yield per panicle (SPP), seed heads per area unit (SH), panicle length (PL), 1000 seed weight (TSW), plant height (PH), leaf width (LW) and heading time (HT). Turf quality was estimated on the basis of: visual merit (VM), shoot density (SD), leaf fineness (LF) and color (C). Tested entries were significantly different for all traits measured. No significant correlations were calculated for SY and turf quality traits. None of top quality turf varieties (BARCELONA, LIMUSINE and CONNI) yielded as high as the highest yielding entries (BALIN, BARON etc.). The best marker trait for seed yield was SPP and for turf quality – late HT, short panicle (PL) and low SPP. Efforts should be made to improve seed yield components but only minor chances are to combine excellent turf quality with very high SY in one variety.

Key words: Kentucky bluegrass, smooth-stalked meadowgrass, seed productivity, lawn quality

INTRODUCTION

From many cool season turf grasses, smooth-stalked meadowgrass (*Poa pratensis* L.) is one of mostly popular species, due to combination of softness, medium to fine-leaf texture, high shoot density, dark green color and persistency (Wedin and Huff, 1996). It is variable species, with cultivars that differ in color, texture, density, vigor, disease and drought resistance and tolerance to close mowing (Johnston *et al.*, 1997; Prończuk and Prończuk, 2003; Martyniak, 2003 a).

Experience has shown that however outstanding a variety may be in terms of excellence of turf, it is little hope of making an impact on the agricultural market without a satisfactory seed production (Griffiths *et al.*, 1980). In many breeding programs turf quality receives more attention, which has to do with strong VCU testing system in most EU countries. Only cultivars with excellent turf performance, prominently appearing in cultivars lists, are interesting enough to commercialize, without enough attention paid on seed productivity (Wijk, 1996). Many cultivars still continue to be sold in large quantities even though they have lower turfgrass quality of any commercial cultivar (Watkins and Meyer, 2004).

The aim of our study was to compare seed production of smooth-stalked meadowgrass with turf quality traits in order to select best turf quality strains with high seed yield.

MATERIALS AND METHODS

Materials used were: 10 common European turf cultivars (BALIN, BARON, BARONIE, BARCELONA, CONNI & LIMUSINE), 4 Polish turf cultivars (ALICJA, ANI, BILA & NANDU) and 17 Polish breeding strains of smooth-stalked meadowgrass (*Poa pratensis* L.). Seed was sown in April of 2001 in two experiments: (1) for seed yield determination - on plots of 2 m^2 in 4 rows 0.25m apart, 5 g of seed per $1m^2$, (2) for turf quality determination - on plots of $1m^2$, 10 g per plot.

Both experiments were arranged in three replicate design on silt sandy soil (pH 6.7) in central Poland (Radzików, 52°12'N, 20°37'E). Seed yield experiment was fertilized two times with mineral fertilizer starting from sowing year (June & August) and during further vegetation (March & August). During each fertilizer application mineral compounds were applied with following doses (in kg*ha^{-1}): N – 60, P – 26 and K – 50. Herbicide treatment (fluroxypyr) against broad-leaf weeds was applied twice in sowing year and once in 2002. Post-harvest residues were cut with tractor mower and hand raked. For turf quality assessment, plots after sowing were covered with propylene non-woven cover and watered until seedlings emerged. During full maintenance (2002-2004) turf plots were cut with rotary mower (clippings collected) 22 – 25 times per year at 3 cm height and fertilized with mineral fertilizer (in kg*ha^{-1}): 180 N (5 doses per year), 75 K (2 doses) and 26 P (1 dose). No special management practices (i.e. aeration, rolling, top dressing etc.) were applied during evaluation period. During prolonged periods of drought turf plots were watered with sprinklers.

For each experiment three replicate design was used and following observations were taken during three years: (1) seed yield traits: heading time (HT), plant height (PH) and panicle length (PL) were measured according to OECD rules (1971); number of seed heads per area unit (SH) – total number of fertile seed heads inside frame 20 x 20 cm counted directly on filed and results calculated for $1m^2$; for seed yield per panicle (SPP) - ten panicles per one entry on replication were collected, seed was threshed, cleaned and weight; for seed yield per unit area (SY) all panicles from plot were collected, seed was treated as mentioned above and results were calculated for 1 m^2. Leaf width (LW) was evaluated on 1-9 scale basis (Prończuk, 1993). (2) Turf quality traits: at the middle of June, August and October shoot density (SD), visual merit (VM), turf color (C) and leaf fineness (LF) were determined according to Prończuk (1993). SD and VM were evaluated on 1-9 scale basis: 9 being outstanding, ideal turf or of maximum density and 1 being poorest or dead. For C - 1 means no turf and 9 – dark green. For LF 9 is very narrow leaf and 1 – very wide. A rating of 6 is generally considered as the least acceptable turf (Prończuk, 1993).

All statistical analysis were performed with SAS statistical package (SAS, 2000). Means were separated with Fisher's protected LSD (P=0.05). Multiple regression analysis was performed on standardized data basis due to different units used in particular traits.

RESULTS

Significant differences with probabilities higher than 99.9% were found among all tested entries for all examined traits (Table 1). Seed yield traits were generally more variable than turf quality traits and the most variable traits were SH, SY and PH.

The group of the highest seed yielding entries (SY from 98 to 109 g*m^{-1}) consists of four breeding strains and two varieties: BALIN and BARON. Multiple regression analysis indicated that from all traits related to seed yield: SPP, SH and TSW accounted for 79% of total variation of SY (Table 2). VM of tested entries ranged from values close to 7.0 (CONNI, BARCELONA, LIMUSINE & BA-1036) to quite unacceptable turf (less than 6.0 – for varieties ALICJA, ANI, BARONIE, BALIN and five breeding strains). SD and C accounted for 94% of total VM variation (Table 2).

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Table 1. Results of evaluation of smooth-stalked meadowgrass (entries ordered according to descending VM values).

Name of variety or breeding line	Turf quality traits				Seed yield traits								
	VM	SD	C	LF	HT	PH	PL	LW	SH	SPP	TSW	SY	
	scale				days	cm	Cm	scale	nb.per m^2	gram			
Conni	7.5 A	8.0	7.8	6.7	47.8	35.8	6.0	6.3	2806	0.69	0.338	75.55	DEF
Barcelona	7.1 AB	7.8	8.3	6.9	47.5	48.3	7.8	6.0	2999	0.79	0.302	72.75	DEFG
BA-1036	7.0 ABC	8.1	7.9	7.6	48.3	46.7	5.7	6.0	4864	0.54	0.218	80.00	DE
Limousine	7.0 ABC	8.0	7.6	7.4	47.5	40.0	6.0	7.0	4166	0.57	0.247	74.45	DEF
Chałupy	6.9 BCD	7.4	7.7	7.4	47.2	50.0	6.5	7.0	2777	0.67	0.331	82.90	CDE
Dresa	6.8 BCDE	7.0	7.8	6.3	47.2	51.7	7.1	5.0	3061	0.93	0.318	105.80	A
BA-2090	6.8 BCDE	7.9	7.5	7.4	47.3	45.0	5.9	5.7	5454	0.54	0.258	97.95	ABC
BA-2028	6.5 CDEF	6.9	8.6	6.5	44.8	60.0	8.2	5.7	2187	0.82	0.334	57.30	GHIJ
BA-915	6.3 EFGH	7.0	7.8	5.9	47.8	60.0	7.2	4.7	2086	0.83	0.273	63.70	FGHI
Bila	6.3 DEFG	6.5	8.8	5.8	46.0	53.5	7.8	5.0	2319	0.99	0.362	71.15	DEFG
BA-2196	6.2 FGHI	6.6	8.2	6.0	48.2	47.5	7.5	4.7	1908	0.74	0.240	42.90	K
6/85	6.2 FHGI	6.4	8.0	6.2	47.8	48.3	7.2	5.0	1879	1.36	0.278	99.80	AB
BA-2169	6.2 FGHI	5.8	7.6	5.4	46.2	50.0	7.5	4.3	1712	0.95	0.324	52.40	IJK
Nandu	6.2 EFGH	6.8	8.6	5.5	48.2	47.0	9.6	5.7	1552	1.07	0.331	63.85	FGHI
Contra	6.1 FGHIJ	6.4	8.0	5.9	47.7	47.5	6.7	5.0	1886	1.18	0.284	83.65	CD
BA-2012	6.1 FGHIJ	6.9	7.5	6.4	45.7	65.0	6.7	5.7	4048	0.62	0.238	60.05	FGHI

Baron	6.0	FGHIJ	6.8	8.4	5.9	48.3	42.5	6.5	4.7	3026	0.98	0.349	105.55	A
RA-1571	5.9	FGHIJK	6.7	7.6	5.9	41.5	71.5	8.0	5.0	1725	1.40	0.322	81.80	DE
Alicja	5.8	GHIJKL	6.8	7.6	5.7	47.5	42.5	7.0	5.0	2423	0.86	0.324	71.80	DEFG
Ani	5.8	GHIJK	6.5	7.6	5.6	47.7	47.0	7.5	4.3	1941	0.95	0.322	74.40	DEF
Baronie	5.7	IJKLM	6.7	7.7	5.9	45.0	69.2	7.5	6.0	2107	0.72	0.323	61.90	FGHI
43/83	5.7	HIJKLM	6.4	7.8	6.2	48.0	45.0	7.2	4.7	2022	1.43	0.269	102.80	A
Przełęcz S	5.4	KLM	6.0	7.8	5.8	41.3	73.8	9.5	4.3	1664	1.36	0.262	85.70	BCD
NIB-176	5.3	LM	5.9	7.6	6.3	45.3	46.7	7.5	6.0	1123	1.06	0.384	67.30	EFGH
NIB-398	5.2	M	6.0	7.9	6.0	44.7	47.5	7.4	5.7	1689	1.09	0.300	71.70	DEFG
BA-2260	4.4	N	5.0	7.7	5.4	46.8	44.2	7.8	5.3	2171	0.83	0.315	36.75	K
Balin	3.6	O	4.1	7.4	6.8	34.0	88.3	11.2	5.0	1525	1.65	0.322	109.05	A
LSD (P<95%)	0.56		0.71	0.31	0.46	1.5	5.4	0.9	0.7	25.8	0.38	2.2E-09	17.55	
CV (%)	12.4		13.4	4.7	10.3	6.6	22.8	15.9	13.9	42.5	31.1	13.6	24.8	

Entries of high visual merit values (CONNI, BARCELONA, LIMUSINE & BA-1036) yielded only slightly above mean value (76 g*m^{-1}). No correlation was found between seed yield and visual merit, shoot density or color. Visual merit and shoot density were positively correlated with late heading, narrow leaf and high number of seed heads per unit area. Negative correlations were calculated between mentioned turf traits and plant height, panicle length and seed yield per panicle. Turf color was not correlated with any from seed yield traits.

Table 2. Results of multiple regression analysis for seed yield and turf quality traits.

Seed yield (SY):

$R^2 = 0.79$, $F(7,19) = 10.59$, $p < 0.00002$

Trait	coeff.of regression	standard error	t - statistic values	significance
SPP	1.42	0.19	7.30	0.00
SH	1.10	0.19	5.80	0.00
TSW	0.33	0.13	2.50	0.02
HT	0.30	0.26	1.13	0.27
PH	0.22	0.23	0.97	0.34
LW	0.11	0.14	0.83	0.42
PL	-0.23	0.20	-1.14	0.27

Visual merit (VM):

$R^2 = 0.94$ $F(3,23) = 121.56$, $p < 0.00000$

Trait	coeff.of regression	standard error	t - statistic values	significance
SD	0.95	0.06	14.71	0.00
C	0.12	0.05	2.24	0.03
LF	-0.02	0.06	-0.34	0.74

Table 3. Correlation coefficients for all traits examined.

Traits	SD	C	LF	HT	PH	PL	LW	SH	SPP	TSW	SY
VM	0.96 ***	0.27	0.49 **	0.66 ***	- 0.50 **	- 0.67 ***	0.42 *	0.60 **	- 0.64 ***	- 0.24	0.03
SD		0.16	0.56 **	0.63 ***	- 0.49 **	- 0.72 ***	0.51 **	0.70 ***	- 0.69 ***	- 0.31	0.06
C			- 0.18	0.29	- 0.19	0.12	- 0.07	- 0.15	- 0.01	0.23	- 0.13
LF				0.01	- 0.08	- 0.39 *	0.70 ***	0.71 ***	- 0.41 *	- 0.36	0.35
HT					- 0.86 ***	- 0.74 ***	0.14	0.34	- 0.59 **	- 0.16	- 0.22
PH						0.68 ***	- 0.24	- 0.28	0.47 *	- 0.01	0.13
PL							- 0.36	- 0.64 ***	0.67 ***	0.28	0.00
LW								0.43 *	- 0.54 **	0.01	- 0.10
SH									- 0.69 ***	- 0.51 **	0.19
SPP										0.23	0.45 **
TSW											- 0.02

*. **. *** indicate significance of correlation coefficient at 0.05. 0.01 and 0.001 levels of probability, respectively

DISCUSSION

The basic factors contributing to seed yield in grasses are the number of inflorescences produced per plant, the number of florets produced per inflorescence (or head size), the proportion of florets which set seed (or seed setting) and individual seed weight (Griffiths *et al.*, 1980; Martyniak, 2003 b). As it was shown in our experiment in case of BALIN variety, early plant heading and many long panicles are among the best components to select for high seed yield (Ensign *et al.*, 1989).

We have noticed that seed yield of smooth-stalked meadowgrass had the most positive correlation with seed yield per panicle but not with number of seed heads per unit area as contrary to Canode and Law (1975). Insignificant effect of number of seed heads on seed yield was also described by Ensign *et al.* (1983) however other authors suggested positive and significant relation between mentioned traits (Canode and Law, 1975; Ensign *et al.*, 1989; Johnson *et al.*, 2003).

Results similar to ours, concerning correlation of seed yield per unit area with seed yield per panicle were also noted for timothy, tall and meadow fescue (Griffiths *et al.*, 1980). Our results confirmed previous findings about significant and negative correlation between seed yield per panicle and number of panicles per unit area (Canode and Law, 1975). According to Canode and Law (1975) the major components of seed yield variation in cool season grasses were: seed yield per panicle, number of panicles per unit area and 1000 seed weight. Similar conclusions appeared from multiple regression analysis in our experiment.

Numerous experiments have shown that there was no positive correlation between seed yield and turf quality (Johnston *et al.*, 1997; Żyłka, 2001; Johnson *et al.*, 2003). The first turf-type smooth-stalked meadowgrass varieties released, including MERION, had low to moderate seed-yielding capability. Later introductions of other varieties, including BARON, with high seed yield and good turf quality has made seed yield an important criterion in turfgrass breeding (Meyer and Funk, 1989).

Traits, which are usually the components of seed yield are of generative nature, as contrary to turf quality traits, representing the vegetative phase of plant development. For example low growth - trait desirable for turfgrass, will result in seed heads close to or even below leaf canopy, and finally difficult seed harvest and cleaning (Johnston *et al.*,1997).

The combination of excellent turf quality with high seed productivity was not found among tested entries. However, combinations at little lower quality of turf and little lower quantity of seed were present in few entries.

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Variation in seed yield related and other morphological traits in a collection of Irish *Lolium perenne* ecotypes and bred varieties

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ABSTRACT

In general, grasses show a huge diversity in gross morphology. Several reproductive and vegetative traits are used as taxonomic descriptors for the description of species and for distinctiveness, uniformity and stability (DUS) variety testing. Variability in both types of traits, reproductive and vegetative, is a prerequisite for improving these traits by breeding. In 2005 we used 2,481 spaced plants derived from 50 *Lolium perenne* ecotypes and bred varieties to assess variation in reproductive and vegetative morphological traits. One measurement per plant was taken for all qualitative traits and four taken for quantitative traits. In a principal coordinate analysis 42.24% of the total morphological variation could be explained in the first two dimensions. We found surprisingly little variation in vegetative traits among the 50 *Lolium* forage accessions, but a high degree of variation for seed yield components among populations. Average values for the seed yield related traits, rachis length, spikelet per spike, florets per spikelet and glume length, were mostly highest in diploid and tetraploid bred varieties, except for the number of florets per spikelet. Rachis length was significantly correlated with glume length, number of spikelets per spike and florets per spike. Highly significant regression models could be built for seed yield related traits. Future work will include an association mapping study using molecular markers for the regions including a major QTL for the seed yield related traits rachis length and spikelets per spike.

Key words: perennial ryegrass, spikelets per spike, florets per spikelet, glume length, rachis length, heading date

INTRODUCTION

Apart from some outlying groups within the grass family all grasses are characterised by a standard spikelet containing one or more florets with specialised structures known as glumes, lemmas, paleas, and lodicules (Hubbard, 1984). Morphological traits were the earliest markers to be used in the management of germplasm. They provide an indirect method of analysing genetic diversity but at the same time assess genotypic performance. Within *L. perenne*, morphological traits have been used to assess genetic diversity in several studies (Loos, 1993; Kolliker *et al.*, 1999; Gilliland *et al.*, 2000; Roldan-Ruiz *et al.*, 2001; Van Treuren *et al.*, 2005; Hazard *et al.*, 2006) and the most commonly used morphological traits in genetic diversity studies are a mixture of vegetative traits (plant height at ear emergence and 30 days afterwards, growth habit, length and width of the flag leaf at ear emergence) and reproductive traits (date of ear emergence, ear length, spikelets per spike, length of spikelet,

and glume length). Morphological traits are also used by the International Union for Protection of Varieties (UPOV) in DUS (distinctness, uniformity and stability) testing of new varieties (UPOV, 2002). As well as using morphological characters to investigate taxonomic relationships, morphological characters have also been used to investigate genetic variability within the genus *Lolium* (Loos, 1993; Loos, 1994; Fernando *et al.*, 1997; Kolliker *et al.*, 1999; Gilliland *et al.*, 2000; Roldan-Ruiz *et al.*, 2001; Van Treuren *et al.*, 2005). Manipulating inflorescence/reproductive traits (such as spikelets per spike, and florets per spikelet) during cultivar development in *L. perenne* is important. New cultivars of *L. perenne*, need improved quality traits, but also require increased numbers of seeds in order to make breeding of the new cultivar a viable option.

The objectives of this research were to (1) describe morphological diversity in a collection of Irish *Lolium perenne* ecotypes, along with European *L. perenne* ecotypes and bred varieties, (2) determine if accessions or geographic groups of populations can be differentiated using morphological measures and traits, and (3) determine if morphological traits are dependent on each other by means of correlation and regression analysis.

MATERIALS AND METHODS

A total of 2,481 individuals from a selection of 50 *L. perenne* accessions were used to investigate morphological diversity. Between 46 and 50 individuals per population were analysed. Seeds were grown, and plants transferred to the field in Oak Park, Carlow in 2003. Plants were laid out in the field as spaced plants in 2m x 4.5m blocks with 5 plants in each row, 0.5m apart. Blocks were spaced 1m apart from each other in rows of 17 blocks. Each plant was scored for the following morphological traits in 2005: Spring growth (on a scale of 1 excellent to 9 very poor), late summer growth (*ditto*) and date of ear emergence (measured in days from April 1st 2005). Measurements were taken with a tape measure or Vernier callipers for the following traits: height at ear emergence measured from the base of the first tiller to the tip of the spike (cm), length of the flag leaf at ear emergence measured from the ligule to the tip of the blade (cm), width of flag leaf at ear emergence measured at the maximum width of the flag leaf (cm), height 30 days after ear emergence measured as height of the plant from the base of the first tiller to the tip of the spike (cm), rachis length measured as length of the rachis from the base of the first spikelet to the base of the terminal spikelet (cm), and glume length measured as length of the lower glume from the base at the spike to the tip of the glume (mm). Counting was done for the number of spikelets per spike and number of florets per spikelet. For all quantitative traits, four measurements per single plant were taken, and the mean of the measurements used. For qualitative traits, a single record per plant was taken. All data analysis for basic statistics, data transformations, correlations and regression analyses were performed using Minitab® Version 15 Statistical Software (Minitab Incorporated, 2000). For quantitative data, means and standard deviations were calculated for each plant, for each population and for four population groups: Irish ecotypes, European ecotypes, diploid cultivated varieties and tetraploid cultivated varieties. Two-sided t-tests were used to determine if the means of each group were significantly different from each other. Normality tests were performed using the Kolmogorov-Smirnov test. On non-normally distributed data transformation was performed. Pearson correlation coefficients were calculated for each pair of traits. For pairs of traits with strong significant correlations stepwise linear regression analysis was carried out. Principal components analysis (PCA) was performed on the population means data using NTSYSpc V2.2 software (Rohlf, 2005). In order to determine which traits influenced the separation of accessions in each dimension, a canonical variates (CVA) analysis was performed. One-way analysis of variance (ANOVA) tests were performed to determine the variation between different groups of accessions (between accessions, between bred varieties and ecotypes, between Irish and European

ecotypes, and between diploid and tetraploid bred varieties). The percentage of variation accounted for between groups and its significance was determined. The difference between pairs of populations and pairs of groups was determined using the Scheffé test since the group sizes differed in each case.

RESULTS

The traits rachis length, length of flag leaf, width of flag leaf, height at ear emergence and height at 30 days after ear emergence were normally distributed. The other traits investigated followed a normal distribution after data transformation.

Different levels of variation were seen across the different traits both within and among accessions. Generally variation among all investigated populations (ecotypes for Ireland and Europe, diploid and tetraploid breeding material) was highest in the growth related traits: spring growth (40.86%, p <0.001) and summer growth (49.42%, p <0.001), and in the reproductive trait date of ear emergence (41.36%, p <0.001). Variation among populations was lowest and often non significant (ns) in the vegetative traits: height at ear emergence (1.97%, p = not significant (ns)), height 30 days after ear emergence (6.90%, p <0.0001), and length of flag leaf (2.12%, p = ns) and width of the flag leaf (2.51, p = ns). Other morphological traits had intermediate variation: rachis length (30.19%, p <0.001), spikelets per spike (29.12%, p <0.001), florets per spikelet (32.27%, p <0.001) and glume length (24.55%, p <0.001). However, within population variation for certain groups was different from the among population variation across all groups. Some vegetative characters were useful to assess for within population variation.

For comparisons between cultivars and ecotypes, most variation was found in spring growth (23.08%), summer growth (27.34%), date of ear emergence (10.17%), rachis length (6.13%) and spikelets per spike (5.33%). Low variation was observed between cultivars and ecotypes for the characters florets per spikelet (0.48%), glume length (0.74%), and height 30 days after ear emergence (0.19%). The remaining characters did not show significant variation between cultivars and ecotypes. Most variation in comparisons between diploid and tetraploid cultivars was seen for date of ear emergence (12.14%), spring growth (5.33%), glume length (3.12) and florets per spikelet (2.79%). Between Irish and European ecotypes, most variation was found for the character glume length (8.65%).

For calculated Pearson's correlation coefficients three correlations had significant positive coefficients with values more than 0.4 (rachis length versus spikelets per spike, rachis length versus florets per spikelet, and rachis length versus glume length). There were also weaker significant positive correlations (spikelets per spike versus florets per spikelet, spikelets per spike versus date of ear emergence, florets per spikelet versus glume length and spring growth versus summer growth) and weaker significant negative correlations (rachis length versus spring growth, rachis length versus summer growth, and florets per spikelet versus date of ear emergence). Linear stepwise regression analysis was performed for the three pairs of characters: rachis length versus spikelets per spike, rachis length versus florets per spikelet, and rachis length versus glume length. When rachis length increased, the number of spikelets per spike increased. This finding was significant at p<0.001. 31.1% of the variation in spikelets per spike was accounted for by the relationship with rachis length. When rachis length increased, the number of florets per spikelet increased. Coefficient values in the regression equation were significant at p<0.001. 16.2% of the variation in florets per spikelet was accounted for by the relationship with rachis length. When rachis length increased, the glume length increased. Coefficient values in the regression model equation were significant at p<0.001. 19.7% of the variation in glume length was accounted for by the relationship with rachis length. In a PCA the first three dimensions explained more than 50% of the variation in the dataset (first dimension 27.29%, second dimension 14.96%, and third dimension 11.87%).

A good separation was found between the cultivars, which were mostly in the two right hand quadrants of the diagram, and the ecotypic material (in the left two quadrants). The scores for each character in the CVA indicated that the characters rachis length, spikelets per spike, spring growth, summer growth and date of ear emergence were the main characters which caused the split between ecotypes and cultivars.

DISCUSSION

While low levels of morphological variation would be expected for the cultivated material, because consistency of these characters have been selected for during breeding programmes, higher levels of variation should be expected in accessions of the ecotypic material. Often reproductive characters showed higher levels of variation among populations. For example, for the character florets per spikelet, among population variation accounted for 32.27% of the total variation. Moderate levels of within population variation were also seen for other reproductive characters. For example, within populations variation for glume length ranged from 12.80% (an ecotype from the Nordic Gen Bank) to 20.89% (in Irish ecotype IRL-OP-02250). Similar results were seen for both ecotypes and cultivars in the study of Dutch accessions by Van Treuren *et al.* (2004), as well as in studies of morphological variations in cultivars alone (Gilliland *et al.,* 2000; Roldan-Ruiz *et al.,* 2001). High levels of among population variation were seen for date of ear emergence, e.g. for the character date of ear emergence, 41.36%. However, very low levels of within population variation were seen for date of ear emergence. High levels of among population variation in date of ear emergence were also seen in other studies (Gilliland *et al.,* 2000; Roldan-Ruiz *et al.,* 2001; Van Treuren *et al.,* 2004). The low level of within population variation combined with the high among population variation in date of ear emergence for both ecotypes and cultivars could be as a result of adaptation to environmental factors such as day length, temperature and precipitation that may influence fitness. The high among and within population variation seen in spring and summer growth may be considered quite surprising, given that the cultivars should all be selected for good spring and summer growth, however, a lot of the variation in spring and summer growth was seen in the ecotypes, and these would be adapted to local environmental conditions and so have varied growth in spring and summer.

More variation was seen between ecotypes and cultivars than either between diploid and tetraploid cultivars, or between Irish and European ecotypes. Ecotypes and cultivars were separated from each other by PCA. Similar separations of ecotypes and cultivars were seen in studies of Dutch ecotypes (Loos, 1994; Van Treuren, 2005). The CVA scores of our study were similar to findings of Loos (1994) and Van Treuren (2005). This could reflect the breeding history of cultivars, which would normally be selected for later heading date, and good growth in the growing season. Based on these results, cultivars which would eventually be selected for commercial breeding would be expected to have more spikelets per spike and increased rachis lengths.

Positive relationships between rachis length and reproductive characters (spikelets per spike, florets per spikelet, and glume length) were found in our study in both correlation and regression analyses. While it would seem intuitive that with increased rachis length, the number of spikelets increase (because there is simply more space available for spikelets), this would not explain the positive relationship between rachis length and florets per spikelet, and between rachis length and glume length. Numbers of spikelets and numbers of florets are directly related to inflorescence branching processes. The more branching within a rachis the more spikelets will be produced; the more branching within a spikelet the more florets will be produced (unless reproductive structures fail to develop from these branches). Quantitative trait loci (QTL) studies in sorghum (Brown *et al.,* 2006), have found a low correlation between the number of primary and secondary inflorescence branches. QTL studies in rice (Li

et al., 2006) found moderate correlation between primary and secondary branch number and also between number of branches at both orders of branching and numbers of spikelets. This suggests that in rice, regulation of branching is related at all orders of branching. Similar correlations were seen in this study, i.e. the moderately significant correlations between rachis length, spikelets per spike, and florets per spikelet. This could indicate that regulation of branching in *L. perenne* could be controlled in a similar way to rice. Brown *et al.* (2006) suggested that allelic variation in genes controlling branch length causes morphological variation in inflorescence branch length within a species. Such allelic variation in *L. perenne* could account for the high levels of variation seen between the different populations and groups of populations of *L. perenne* in this study. A full study of these genes involved in branching in *Lolium* is required to investigate the contribution of these genes to *Lolium* inflorescence morphology. However, the results of this morphological study have helped determine basic patterns of morphological diversity and correlations on which these developmental genetic studies can be based.

As rachis length and spikelets per spike are characters which would be convenient to measure in the field, the prediction model could be used easily by breeders as a selection method for reproductive characters in breeding programmes. Rachis length is already used as a character in DUS testing under UPOV guidelines. While such a model has not been proposed for *L. perenne*, panicle elongation was seen as the best estimate of seed number in sorghum (Gerik *et al.*, 2004). While high numbers of spikelets per spike do not necessarily equate with higher seed yields, studies have shown that high seed yield is derived from plants with larger heads (Brown, 1980). Also, seed number per unit area was found to be closely associated with the number of floret sites per unit area in tall fescue by Young *et al.* (1998).

The results of this study will be highly valuable to botanists and breeders who need to understand and manipulate vegetative and reproductive traits in *Lolium*. Future studies should examine seed set and the genes involved in controlling inflorescence architecture and attempt an association mapping study in natural populations for seed yield component related traits.

ACKNOWLEDGEMENTS

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Genetic characterisation of seed yield and its components in perennial ryegrass (*Lolium perenne* L.)

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ABSTRACT

Seed yield is of major interest for the key grassland species *Lolium perenne* L. since the ability to produce a reasonable seed yield is essential to ensure the commercial success of a variety. This is particularly true for Denmark, where large amounts of forage seeds are produced. Although seed yield is a complex trait and affected by agricultural practices and environment, several studies revealed reasonable genetic variation for seed yield and seed yield components, which promises good prospects for improvement by selection. In this study, an F_2 mapping population of perennial ryegrass (VrnA) was assessed in the glasshouse for genetic variation of seed yield and its components based on lattice designs with four replications. The traits heading date (GDD), plant height (PH), number of panicles (NPa), length of panicles (LPa), seed yield per panicle (SYPa) and total seed yield per plant (SYP) revealed repeatability values ranging from 41 to 65%, supporting a considerable amount of genetic variation in this population. The adjusted entry means of the lattice analysis and a genetic linkage map consisting of 31 SSR, 50 AFLP and 17 CAPS markers were used for QTL analysis. Two QTL on linkage group (LG) 1 and LG 2 each explaining 25% of observed phenotypic variation (Vp) for the trait SYP were of particular interest, because they co-located with QTL found for SYPa. This indicates that seed is important for the target trait total seed yield per plant. Here we report on the identification of (candidate gene based) genetic markers closely linked to QTL for seed yield traits in perennial ryegrass and discuss the interference of gametophytic self incompatibility on seed production in the investigated F_2 mapping family.

Key words: *Lolium perenne* L., QTL analysis, seed yield, seed yield components, self incompatibility

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is one of the most important forage grass species of temperate grassland. For both forage and turf varieties, a reasonable seed yield capacity is of outmost importance for both forage and turf varieties since basic seed multiplication has to be profitable regarding the necessity of novel grass cultivars to compete in the commercial market. Although being affected by agricultural practices and environment, several studies in forage grasses revealed reasonable genetic variation for seed yield and its underlying components (Bugge, 1987; Elgersma, 1990; Elgersma *et al.*, 1994), enabling genetic improvement by targeted selection (Bugge, 1987; Marshall and Wilkins, 2003). However, breeding progress for seed yield may be a trade-off to forage quality and thus requires detailed characterisation of its underlying genetic components. Genetic linkage mapping and QTL analysis have the potential to unravel such complex traits, but requires characteristic

population designs based on biparental crosses. Therefore, this study aims (1) to assess the genetic variation for seed yield and seed yield components within a F_2 perennial ryegrass mapping population and (2) to identify genetic markers closely linked to QTL affecting seed yield.

MATERIALS AND METHODS

The VrnA perennial ryegrass mapping population recently characterised for vernalisation response (VrnA; Jensen *et al.*, 2005) was assessed in the glasshouse for genetic variation of seed yield and its components. The 184 F_2 individual were assessed in the glasshouse on single spaced plants based on a 12 x 8 lattice design with four clonal replications for the traits GDD, PH, NPa, LPa, SYPa and SYP. For SYPa, three selected panicles representative for the respective genotype were separately harvested and threshed. Their seeds were separated and cleaned from glumes and weighed (g/panicle). The same cleaning procedure was applied to assess the trait SYP (g/plant). Lattice analysis using the PLABSTAT software (version 2 P; Utz 2000) was performed in order to estimate quantitative genetic parameters. Repeatability was calculated by dividing the genotypic variance component σ^2_g with the sum of σ^2_g and the effective mean square of the error. For QTL analysis, the VrnA linkage map consisting of 31 SSR, 50 AFLP and 17 CAPS markers and the MapQTL software (Van Ooijen and Maliepaard, 1996) were used. QTL analysis was based on a multiple QTL model (MQM) and automatic cofactor selection (backward elimination, $P < 0.02$) was used for the detection of significantly associated markers as cofactors. LOD significance threshold levels were determined using 200 permutations.

RESULTS

The traits showed considerable variation within the VrnA mapping population. Lattice analysis revealed highly significant ($P < 0.01$) genotypic variance components for all the investigated traits. Repeatability values ranging from 0.41 to 0.65 supported that the observed Vp in this population was caused by genetic effects (Table 1). Phenotypic data of the traits SYPa and SYP were highly correlated (0.73, $P < 0.01$; data not shown).

QTL for GDD revealed large effects of the observed Vp on LG 3, 4 and 7. The genetic markers derived form putative *VRN1* and *VRN2* orthologues from *Triticum monococcum* mapped on the top of the QTL on LG 4 and LG 7. For PH, QTL effects were found on LG 3 and 7, explaining 11 and 27% Vp, respectively. Although significant QTL have been identified for the traits NPa and LPa, no major effect (i.e. exceeding 20% explained Vp) was observed. Of particular interest were two QTL for SYPa and SYP on LG 1 and LG 2 (Table 2). QTL for both traits co-located at position 20 centiMorgan (cM) on LG 1 and 28 cM on LG 2, respectively. For SYPa, a high proportion (29%) of observed Vp was explained by the QTL on LG 1, revealing the highest LOD value at the map position of the SSR marker PR37. The QTL on LG 2 explained 14% of Vp and was closely linked to the CAPS marker LpRGA7 with a distance of 2.8 cM. Similar results were found for SYP QTL with 25% explained Vp on both LGs.

Table 1. Key values of the phenotypic characterisation of heading date (GDD), plant height (PH), number of panicles (NPa), panicle length (LPa), seed yield per panicle (SYPa) and total seed yield per plant (SYP) of 184 F_2 genotypes from the VrnA mapping population. Traits were assessed in a glasshouse experiment based on a lattice design with four replicates per genotype.

Trait	GDD[a]	PH (cm)	NPa (counts)	LPa (cm)	SYPa (g/panicle)	SYP (g/plant)
Gen variance[b]	$8993^{**}(190)$	$45.1^{**}(190)$	$229^{**}(190)$	$3.99^{**}(190)$	$1.56^{**}(189)$	$1.54^{**}(190)$
Repeatability	0.65	0.44	0.41	0.50	0.65	0.63
Mean value	775.38	92.66	56.95	17.92	0.052	1.68
Maximum	1031	110.56	101.07	23.97	0.196	6.54
LSD[c]	96.25	10.56	25.07	2.79	0.041	1.87

$^{**}P < 0.01$

[a]GDD was determined using the formula GDD=$(T_{max}+T_{min}/2)-T_{base}$, where T_{max} is the daily maximum temperature, T_{min} is the daily minimum temperature and T_{base} is the basal temperature set at 3°C, defined as the temperature below which development of perennial ryegrass ceases
[b]Genotypic variance components, degree of freedom is given in parenthesis
[c]Least significant difference at $P < 0.05$

Table 2. Detailed description of QTL for seed yield per panicle (SYPa) and total seed yield per plant (SYP) observed in the F_2 mapping population VrnA. Results are based on multiple QTL model (MQM) mapping using MapQTL and a genetic linkage map based on 31 SSR, 50 AFLP and 17 CAPS markers.

Trait	LG	Cofactor	Position	Lod	% expl Vp	Closest marker
SYPa	1	PR37 (20.1)	20.1	21.02	29	PR37
	2	LpRGA7 (31.0)	28.2	3.46	14	LpRGA7 (2.8)
SYP	1	PR37 (20.1)	20.1	11.8	25	PR37
	2	LpRGA7 (31.0)	28.2	5.17	25	LpRGA7 (2.8)

DISCUSSION

Phenotypic variation for the target trait SYP in the VrnA mapping population was caused by different seed setting abilities and was largely explained by two QTL on LG 1 and LG 2. Their co-location with the QTL for SYPa as well as highly correlated phenotypic data supports that seed set is an important component affecting SYP. Similar results were found in the closely related grass species *Festuca pratensis* Huds., where path coefficient analysis identified panicle fertility to be a major trait contributing to SYP (Fang *et al.*, 2004). However, the magnitude of the explained Vp for both QTL was surprisingly high, especially since SYP is known to be a complex trait influenced by a number of other traits. Moreover, marker segregation around the QTL region on LG 1 and LG 2 was highly distorted ($P < 0.01$). Considering the markers most closely linked to the respective QTL, some allele combinations were strongly under- (or even not) represented in the investigated F_2 population. Such significant segregation distortion ratios are indicative for gametophytic incompatibility (SI).

SI is mediated by two loci in ryegrass (Cornish *et al.*, 1979) and have been used to map alleles involved in SI of rye (Fuong *et al.*, 1993). The fact, that QTL for SYPa and SYP map on similar regions on LG 1 and LG 2 like the SI loci S and Z (Thorogood *et al.*, 2002) confirms the SI system to be involved in different seed setting abilities. Furthermore, the large proportions of explained Vp of the described QTL for SYPa and SYP are caused by negative effects of F_2 individuals heterozygous for their most closely linked markers. Such individuals may share the maximal number of different SI alleles and, therefore, showed a significantly ($P < 0.01$) reduced seed set, even if the whole population was used as pollinator. This study shows the effect of SI on SYPa and SYP in an F_2 population and the necessity of the selection of favourable combinations of incompatibility alleles when designing populations. Even if synthetics are used for the production of commercial forage grass cultivars, selection for traits that are linked to S and Z may seriously affect the ability to produce a reasonable seed set.

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New developments in the International Union for the Protection of New Varieties of Plants (UPOV)

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ABSTRACT

UPOV, which continues to be the only internationally harmonized, effective *sui generis* system of plant variety protection, is continuing to expand. In December 2005, UPOV published a report on the impact of plant variety protection according to the UPOV Convention, some of the findings of which are summarized in this paper. With the expansion of UPOV in both geographical terms and in terms of the number of genera and species for which protection is sought, there are increasing demands for general information on the UPOV Convention. This paper explains some of the initiatives taken by UPOV in recent years to meet those needs, including the launch of a distance learning course and the development of new guidance documents on the examination of distinctness, uniformity and stability (DUS). One aspect of the UPOV Convention explored in this paper is the provision for essentially derived varieties. The relationship between initial varieties and essentially derived varieties is explained and the role of the authorities in matters concerning essentially derived varieties is considered. An overview of the current situation with regard to the possible use of molecular techniques in the DUS examination is also presented by reference to proposals considered within UPOV.

Key words: Distinctness, Uniformity and Stability ("DUS"), Essentially Derived Varieties, Molecular Techniques, Plant Variety Protection, UPOV Convention

DEVELOPMENT OF THE UPOV SYSTEM

The UPOV Convention was adopted in 1961 and was amended in 1972, 1978 and 1991. As of July 31, 2007, UPOV had 64 members of which 36 were bound by the 1991 Act of the Convention. (For the latest status, please refer to the UPOV website: http://www.upov.int/en/about/members/pdf/pub423.pdf). UPOV, which continues to be the only internationally harmonized, effective *sui generis* system of plant variety protection, is continuing to expand. As of July 31, 2007, 18 States and one international organization had initiated with the Council of UPOV the procedure for becoming UPOV members (http://www.upov.int/en/about/pdf/pub437.pdf) and another 46 States had been in contact with the Office of the Union for assistance in the development of legislation on plant variety protection.

Figures 1 and 2 illustrate how UPOV has expanded since 1990 to cover the most important agricultural producers and many countries from the developing world.

Fig. 1. Members of UPOV (shown in green): 1990.
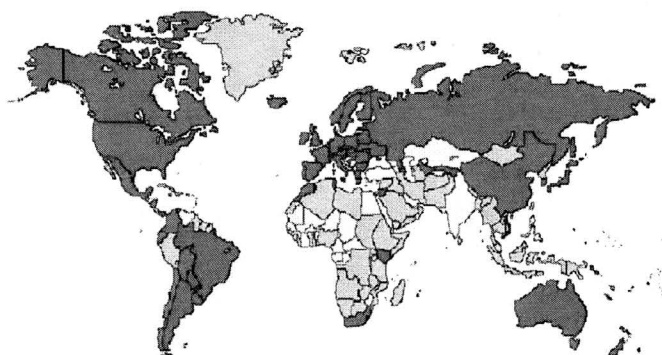
Fig. 2. Members of UPOV (shown in green) and initiating States and organizations (shown in yellow): July 2007.

In December 2005, UPOV published a report on the impact of plant variety protection according to the UPOV Convention (see Literature). The report, the first of its kind since the adoption of the UPOV Convention in 1961, includes a study on the effects of plant variety protection in five countries, namely, Argentina, China, Kenya, Poland and the Republic of Korea. The report also includes an overview of the evolution of the UPOV system.

An illustration of the overall impact of the UPOV system is provided by the number of titles of protection in force within the UPOV system. Figure 3 shows the number of titles in force with members of the Union and the Community Plant Variety Office (CPVO) for the period 1968 to 2003.

With the expansion of UPOV, the importance of PVP has grown in different regions, as illustrated in Figure 4. The growth in the UPOV membership of countries from Asia, Latin America and countries in transition to a market economy between 1983 and 2003 is reflected in their growing use of the PVP system.
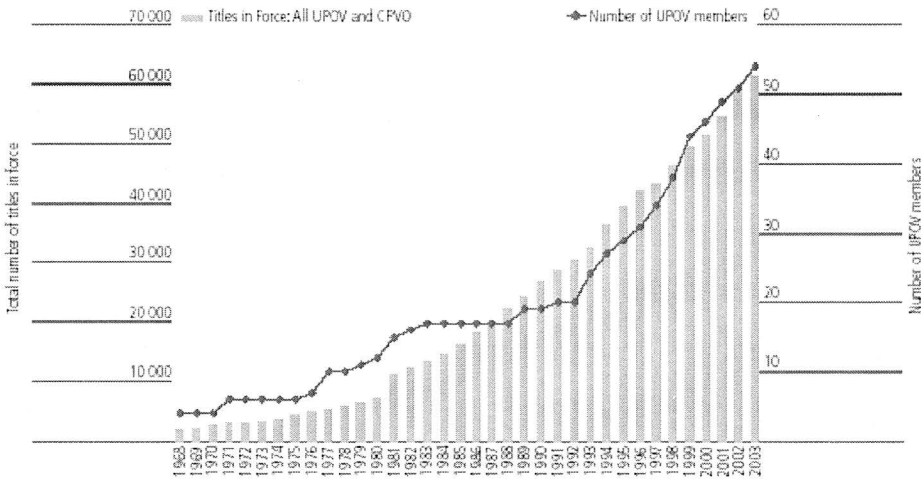
Fig. 3. Titles in force; all UPOV and CPVO.
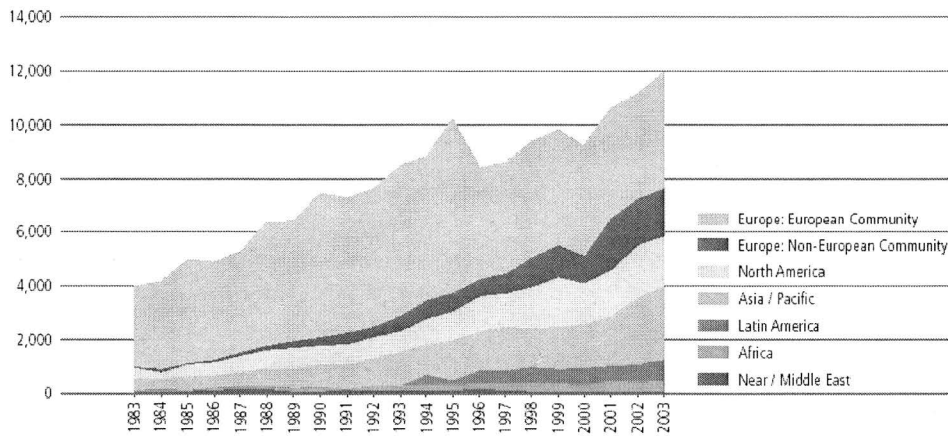
Fig. 4. Applications: all UPOV and CPVO: by region.

In addition to the geographical expansion of UPOV, Article 3 of the 1991 Act of the UPOV Convention, made provision for protection to be offered to all plant genera and species, which has extended the coverage of the UPOV system and contributed to the growth in the number of titles granted. Even before the 1991 Act of the UPOV Convention came into force in 1998, members of the Union had responded to demands for protection for an ever-increasing number of genera and species. In 1975, protection had been granted to varieties of approximately 500 plant genera or species, growing to around 900 by 1985 and over 1,300 by 1995. It is estimated that protection had been granted to varieties of around 2,300 genera or species by 2005.

The expansion of UPOV in both geographical terms and in terms of the number of genera and species for which protection is sought means that there is a growing need for information, guidance and cooperation for plant breeders' rights authorities and breeders. In response to those needs, UPOV has undertaken a number of initiatives in recent years.

RECENT DEVELOPMENTS: INFORMATION ON THE UPOV CONVENTION (ESSENTIALLY DERIVED VARIETIES)

There are increasing demands for general information on the UPOV Convention and, in 2005, UPOV launched a distance learning course to provide a comprehensive introduction to the UPOV system of plant variety protection under the International Convention for the Protection of New Varieties of Plants. The feedback from that course allows UPOV to identify aspects of the UPOV Convention where there is a particular need for clarification. One such aspect is the provision for essentially derived varieties, which was introduced in the 1991 Act of the UPOV Convention. The following summary has been developed to address the aspects where clarification is most often sought.

ESSENTIALLY DERIVED VARIETIES

Article 14(5) of the 1991 Act means that the scope of the breeder's right applies to essentially derived varieties and certain other varieties (see Table 1). One of the main purposes of the provision on essentially derived varieties is to ensure that the Convention encourages sustainable plant breeding development by providing effective protection for classical plant breeding techniques as well as newer techniques, such as genetic engineering.

Issues which commonly arise with regard to essentially derived varieties are: firstly, the determination of what an essentially derived variety is; secondly, the relationship between the initial variety and varieties which are essentially derived from an initial variety; and thirdly, the rights of the breeder of an essentially derived variety. These matters are summarized below:

The UPOV Convention Article 14(5)(b) contains the following definition of an essentially derived variety (EDV):

"(b) [...] a variety shall be deemed to be essentially derived from another variety ("the initial variety") when

- it is predominantly derived from the initial variety, or from a variety that is itself predominantly derived from the initial variety, while retaining the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety,
- it is clearly distinguishable from the initial variety and
- except for the differences which result from the act of derivation, it conforms to the initial variety in the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety."

and indicates in Article 14(5)(c)) some of the ways in which an essentially derived variety *may* be obtained as follows: "Essentially derived varieties may be obtained for example by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, backcrossing, or transformation by genetic engineering.".

Table 1. 1991 Act of the UPOV Convention: Article 14; Scope of the Breeder's Right.

(a) Subject to Articles 15 and 16, the following acts in respect of the propagating material of the protected variety shall require the authorization of the breeder:

   (1)   [*Acts in respect of the propagating material*]

      (i)   production or reproduction (multiplication),
     (ii)   conditioning for the purpose of propagation,
    (iii)   offering for sale,
     (iv)   selling or other marketing,
      (v)   exporting,
     (vi)   importing,
    (vii)   stocking for any of the purposes mentioned in (i) to (vi), above.

(b) The breeder may make his authorization subject to conditions and limitations.

   (2)   [*Acts in respect of the harvested material*] Subject to Articles 15 and 16, the acts referred to in items (i) to (vii) of paragraph (1)*(a)* in respect of harvested material, including entire plants and parts of plants, obtained through the unauthorized use of propagating material of the protected variety shall require the authorization of the breeder, unless the breeder has had reasonable opportunity to exercise his right in relation to the said propagating material.

   (3)   [*Acts in respect of certain products*] Each Contracting Party may provide that, subject to Articles 15 and 16, the acts referred to in items (i) to (vii) of paragraph (1)*(a)* in respect of products made directly from harvested material of the protected variety falling within the provisions of paragraph (2) through the unauthorized use of the said harvested material shall require the authorization of the breeder, unless the breeder has had reasonable opportunity to exercise his right in relation to the said harvested material.

   (4)   [*Possible additional acts*] Each Contracting Party may provide that, subject to Articles 15 and 16, acts other than those referred to in items (i) to (vii) of paragraph (1)*(a)* shall also require the authorization of the breeder.

   (5)   [*Essentially derived and certain other varieties*] *(a)* The provisions of paragraphs (1) to (4) shall also apply in relation to

      (i)   varieties which are essentially derived from the protected variety, where the protected variety is not itself an essentially derived variety,
     (ii)   varieties which are not clearly distinguishable in accordance with Article 7 from the protected variety and
    (iii)   varieties whose production requires the repeated use of the protected variety.

(b) For the purposes of subparagraph *(a)*(i), a variety shall be deemed to be essentially derived from another variety ("the initial variety") when

      (i)   it is predominantly derived from the initial variety, or from a variety that is itself predominantly derived from the initial variety, while retaining the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety,
     (ii)   it is clearly distinguishable from the initial variety and
    (iii)   except for the differences which result from the act of derivation, it conforms to the initial variety in the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety.

(c) Essentially derived varieties may be obtained for example by the selection of a natural or induced mutant, or of a somaclonal variant, the selection of a variant individual from plants of the initial variety, backcrossing, or transformation by genetic engineering.

With regard to the relationship between the initial variety and varieties which are essentially derived from the initial variety, Figures 5 and 6 provide a summary of the situation. In those figures the term "commercialization" encompasses the acts concerning a protected variety which require the authorization of the breeder. It is important to note that the scope of the plant breeders' rights is only extended to essentially derived varieties in respect of an initial variety. In that regard, a variety which is essentially derived from another variety cannot be an initial variety. Thus, in Figure 5, the rights of Breeder 1 extend to EDV 'B' and EDV 'C'. However, although EDV 'C' is predominantly derived from EDV 'B', Breeder 2 has no rights as far as EDV 'C' is concerned. Another important aspect of the provision on essential derivation is that no rights extend to essentially derived varieties if the initial variety is not protected. Thus, in Figure 6, if Variety 'X' was not protected or if the protection of variety 'X' had ceased (e.g. because of expiration of the period of protection, or cancellation or nullification of the plant breeders' rights), Breeder II and Breeder III would be able to commercialize varieties 'Y' and 'Z', respectively, without the authorization of Breeder I.
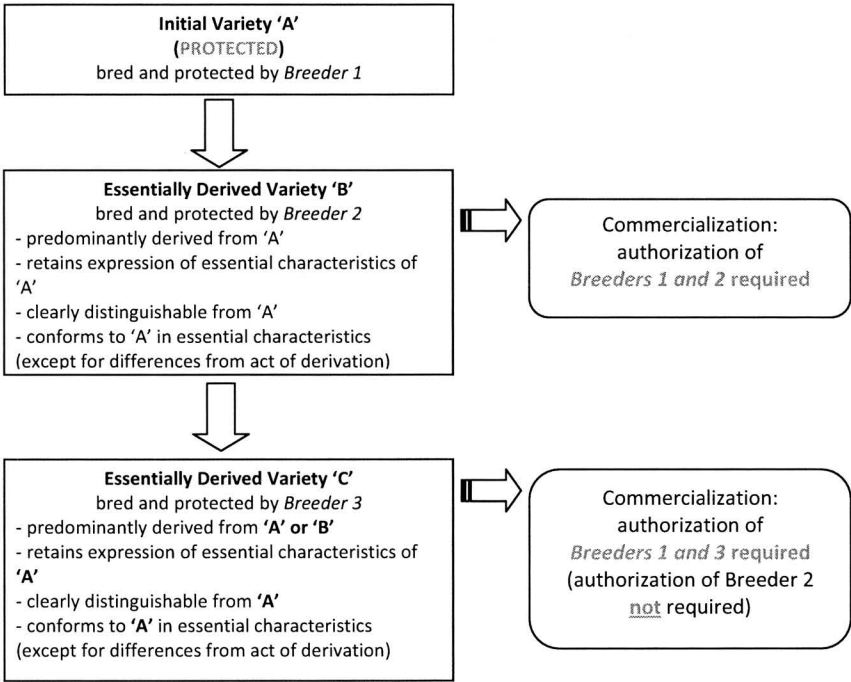
Fig. 5. Initial variety protected.

It is important to recall that a decision on whether to grant protection to a variety does not take into account whether the variety is essentially derived or not: provided the conditions for protection as set out in Article 5 of the UPOV Convention are fulfilled (novelty, distinctness, uniformity, stability, variety denomination, compliance with formalities and payment of fees) the variety will be granted protection. If it is subsequently concluded that the variety is an essentially derived variety, the breeder of that essentially derived variety still has all the rights conferred by the UPOV Convention. However, the breeder of the initial variety will *also* have rights in that variety. Thus, in the case of an essentially derived variety, the authorization of both the breeder of the essentially derived variety and the breeder of the

initial variety is required for its commercialization. With regard to establishing whether a variety is an essentially derived variety, a common view expressed by members of the UPOV is that the existence of a relationship of essential derivation between protected varieties is a matter for the holders of plant breeders' rights in the varieties concerned.
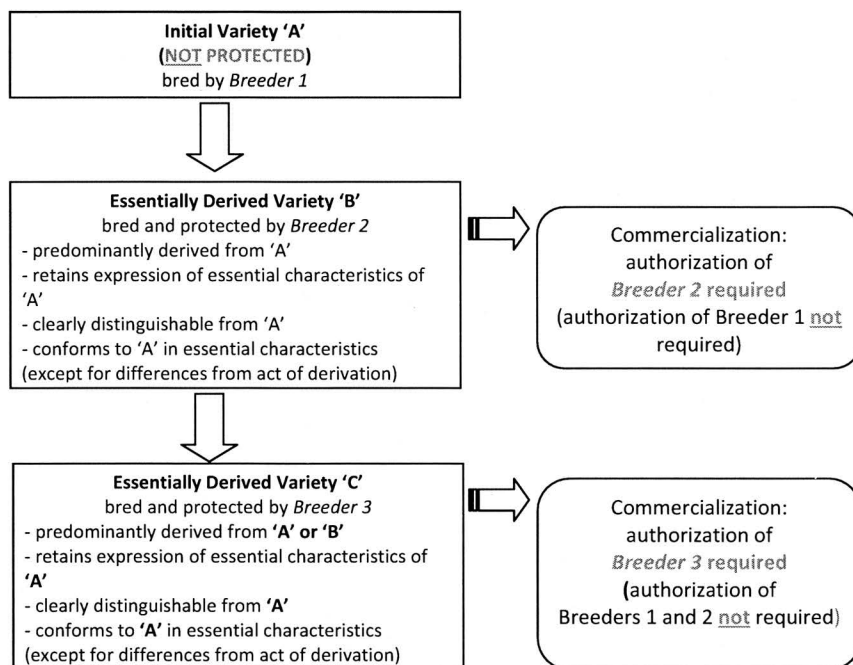
Fig. 6. *Initial variety not protected.*

UPOV has established a section on its website (see Literature) where case law relevant to plant breeders' rights, including case law concerning essentially derived varieties, is published.

RECENT DEVELOPMENTS: INFORMATION ON THE EXAMINATION OF DUS AND MOLECULAR TECHNIQUES

In 2002, UPOV revised its guidance document on the examination of distinctness, uniformity and stability (DUS) "General Introduction to the Examination of Distinctness, Uniformity and Stability and the Development of Harmonized Descriptions of New Varieties of Plants" (document TG/1/3) (see Literature). That revision resulted in the establishment of a series of associated documents (TGP documents) (see Literature) which are being developed to provide more detailed guidance on the application of the principles set out in the General Introduction. This extends to information on arrangements for DUS testing and the possibilities for cooperation between authorities and with breeders in DUS testing. In addition, UPOV continues to develop "Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability," or "Test Guidelines", for many individual species or other variety groupings (see Literature). The purpose of those Test Guidelines, of which there are now more than 230, is to elaborate certain of the principles contained in the General Introduction and the associated TGP documents into detailed practical guidance for the harmonized

examination of DUS and, in particular, to identify appropriate characteristics for the examination of DUS and production of harmonized variety descriptions. One aspect which is not covered in those documents at present is the possible use of molecular techniques in the DUS examination. This paper provides an overview of the current situation.

Possible use of molecular markers in the DUS examination

In 2000, UPOV established an *ad hoc* subgroup of technical and legal experts on biochemical and molecular techniques (BMT Review Group) to assess the possible use of biochemical and molecular techniques in relation to the examination of DUS. The purpose of the BMT Review Group is to assess possible application models proposed by the Technical Committee, on the basis of the work of the BMT[1] and Crop Subgroups[2], for the utilization of biochemical and molecular techniques in the examination of Distinctness, Uniformity and Stability in relation to:

(a) conformity with the UPOV Convention, and

(b) potential impact on the strength of protection compared to that provided by current examination methods and advise if this could undermine the effectiveness of protection offered under the UPOV system.

The BMT Review Group reports its assessment to the Administrative and Legal Committee, but its assessment is not binding for the position of the Administrative and Legal Committee.

Table 2. Working Group on Biochemical and Molecular Techniques, and DNA-Profiling in Particular (BMT) – the terms of reference of the BMT.

The BMT is a group open to DUS experts, biochemical and molecular specialists and plant breeders, whose role is to:
(i) Review general developments in biochemical and molecular techniques;
(ii) Maintain an awareness of relevant applications of biochemical and molecular techniques in plant breeding;
(iii) Consider the possible application of biochemical and molecular techniques in DUS testing and report its considerations to the TC;
(iv) If appropriate, establish guidelines for biochemical and molecular methodologies and their harmonization and, in particular, contribute to the preparation of document TGP/15, "New Types of Characteristics." These guidelines to be developed in conjunction with the Technical Working Parties;
(v) Consider initiatives from TWPs, for the establishment of crop specific subgroups, taking into account available information and the need for biochemical and molecular methods;
(vi) Develop guidelines regarding the management and harmonization of databases of biochemical and molecular information, in conjunction with the TWC;
(vii) Receive reports from Crop Subgroups and the BMT Review Group;
(viii) Provide a forum for discussion on the use of biochemical and molecular techniques in the consideration of essential derivation and variety identification.

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[1] Working Group on Biochemical and Molecular Techniques, and DNA-Profiling in Particular (BMT) – the terms of reference of the BMT are provided in Table 2.

[2] Crop subgroups are groups of DUS experts and experts in molecular techniques established to discuss questions concerning the possible application of molecular techniques for the assessment of distinctness, uniformity and stability in relation to specific crops: UPOV has established Crop Subgroups for Maize, Oilseed Rape, Potato, Rose, Ryegrass, Soybean, Sugarcane, Tomato, Wheat and Barley and a Crop Subgroup covering vegetatively propagated crops

Models and proposals considered by the BMT Review Group
The following models have been developed by the Crop Subgroups:

Option 1: Molecular characteristics as a predictor of traditional characteristics
      (a)   Use of molecular characteristics which are directly linked to traditional characteristics (gene specific markers)
The Crop Subgroups noted that molecular markers which are directly linked to traditional characteristics might be useful for the examination of traditional characteristics that cannot be consistently or easily observed in the field, or require additional special arrangements (e.g. disease resistance characteristics).
      (b)   Use of a set of molecular characteristics which can be used reliably to estimate traditional characteristics; e.g. quantitative trait loci
The Crop Subgroups considered a proposal to predict the difference in traditional characteristics by a linear function of a set of molecular characteristics.
The BMT considered that a proposal based on this approach should not be presented at this time, but it was emphasized that work on this approach was ongoing.

Option 2: Calibration of threshold levels for molecular characteristics against the minimum distance in traditional characteristics
The Crop Subgroups developed this option with the aim to ensure that there would be no significant shift in the typical minimum distances as measured by traditional characteristics. However, they noted that the lack of a clear relationship between molecular marker distances and differences in traditional characteristics would lead to the need to consider how to handle potentially different decisions on distinctness.

Option 3: Development of a new system
The Crop Subgroups considered that this approach would mean that clearly distinguishable differences in molecular characteristics would be considered as threshold levels for judging distinctness. It noted that it would be necessary that the impact of the new system, compared to the existing system, should be analyzed, e.g. by a review of possible differences in decisions.

Boxes 1 and 2 present proposals developed by experts from members of the Union under Option 1(a) and Option 2, respectively, which were considered by the BMT Review Group in 2002. The assessment by the BMT Review Group of the proposals under Option 1(a) and Option 2, and the conclusions of the Technical Committee and the Administrative and Legal Committee concerning those proposals, are also presented in the boxes. Proposals under Option 3 were also put forward to the BMT Review Group in 2002. However, for the proposals under Option 3, there was no consensus in the BMT Review Group on the acceptability of those proposals within the terms of the UPOV Convention and no consensus on whether they would undermine the effectiveness of protection offered under the UPOV system. The proposals under Option 3 are not presented here. As explained above, the BMT considered that a proposal based on Option 1(b) should not be presented at that time, but it was emphasized that work on this approach was ongoing.

     The work of the BMT and the Crop Subgroups is continuing and where further proposals are developed they will be put forward for assessment by the BMT Review Group.

Box 1

> MODEL
>
> <u>Option 1</u>: Molecular characteristics as a predictor of traditional characteristics
>
> > (a) Use of molecular characteristics which are directly linked to traditional characteristics (gene specific markers)

PROPOSAL FOR A MARKER LINKED TO HERBICIDE TOLERANCE

1. A variety is genetically modified by the insertion of a gene for tolerance to herbicide "Formula X." Varieties containing this gene are not harmed when sprayed with Formula X; however, varieties without this gene are always killed if sprayed with this particular herbicide. Tolerance of Formula X, examined in field trials by spraying of plots, is an accepted DUS characteristic, and it can be used to establish distinctness between varieties.

2. It is proposed that, rather than spraying varieties in the field (this is difficult to organize in the standard DUS trial), the characteristic "tolerance of Formula X" is examined by conducting a test for the presence of a molecular marker *linked* to the gene. This marker is located on a part of the gene "construct." The gene "construct" comprises all the elements which are inserted into the plant during the genetic modification and, in addition to the gene itself, contains additional elements for regulating the gene when in the plant. The marker may be located within the gene, partly on the gene or outside the gene itself.

ASSUMPTIONS TO BE MADE IN THE PROPOSAL

3. The following assumptions are made:

(a) The DUS Examination

It is assumed that the test for the marker would be conducted to the same extent as for the field test, i.e. the same number of individual plants, over the same number of years and with the same criteria for distinctness, uniformity and stability.

(b) Reliability of the Linkage

It is assumed that the link between the marker and the gene would be checked to ensure that the marker is a reliable predictor of tolerance to Formula X. This check would be necessary to ensure, for example, that the marker does not become separated from the gene and that the presence of the gene is still resulting in tolerance to Formula X.

(c) Development of Different Molecular Markers for the Same Gene

It would be possible to develop different gene constructs containing Formula X tolerance and to identify separate molecular markers for these individual gene constructs, all of which would be linked to exactly the same gene for Formula X tolerance. If all the different markers for the same gene were accepted as different methods for examining the *same existing phenotypic characteristic*, the consideration of the approach would be the same. Under Option 1, "Molecular characteristics as a predictor of traditional characteristics," it is necessary to work on the basis that the markers correspond to a traditional, i.e. existing, approved characteristic. Therefore, it is assumed that different markers for the same gene would be treated as different methods for examining the same characteristic, i.e. tolerance to Formula X.

(d) Different Genes Producing Tolerance to the Same Herbicide

It might be possible to develop different genes which confer tolerance to Formula X. In the simplest case, this could be considered in the same way as different markers for the same gene, i.e. the different genes, with their respective markers, would be considered as different methods for examining the same characteristic, i.e. tolerance to Formula X. However, the different genes are likely to have a different chemical mechanism to produce the tolerance to Formula X. Thus, the chemicals produced from the different genes will be different and, these different chemicals might be a basis for establishing distinctness in some circumstances. Nevertheless, under Option 1, it would first be necessary to approve the chemical components as UPOV characteristics, before accepting molecular markers linked to these potential characteristics. This in turn would be a separate proposal. Therefore, it is assumed that different genes would be treated as different methods for examining the same characteristic, i.e. tolerance to Formula X.

(e)   Different Gene Constructs Producing the Same Herbicide Tolerance but With Different Control of the Expression

It is also possible that different gene constructs could be developed which contain the same gene for tolerance to Formula X, but which had different regulatory control. For example, the regulatory elements may result in the Formula X tolerance only being switched on at certain stages of development. For simplicity, in considering this proposal, it is assumed that the different markers linked to different regulatory elements for the same gene would all be treated as different methods for examining the same characteristic of tolerance to Formula X. However, it is also assumed that further consideration would be given to this matter at a later stage.

POTENTIAL IMPACT

4.   In the basic proposal and on the basis of the assumptions made in [paragraphs 3] (a) to (e), it would appear that the potential impact on the strength of protection compared to that provided by the "current" examination method (i.e. the field test for tolerance to Formula X) should be nil, because the results of the DUS examination would always be the same regardless of whether the field test or test for the marker was used.

CONCLUSIONS OF THE BMT REVIEW GROUP, THE TECHNICAL COMMITTEE AND THE ADMINISTRATIVE AND LEGAL COMMITTEE

The BMT Review Group concluded that the proposal above was, on the basis of the assumptions in the proposal, acceptable within the terms of the UPOV Convention and would not undermine the effectiveness of protection offered under the UPOV system. The Technical Committee and the Administrative and Legal Committee agreed with the conclusions of the BMT Review Group.

Box 2

MODEL

Option 2: Calibration of threshold levels for molecular characteristics against the minimum distance in traditional characteristics

PROPOSAL FOR OILSEED RAPE

1.   Option 2 is based on a calibration of threshold levels for molecular characteristics against threshold levels in traditional characteristics, principally based on information obtained in France on Maize, Oilseed Rape and Rose. In this particular proposal, the threshold levels in the traditional characteristics are based on an overall distance assessment, rather than a characteristic by-characteristic approach and the application of the proposal is in the "management of reference collections." In this context, the term "management of reference collections" encompasses, in particular, the selection of varieties of common knowledge that can be excluded from the growing trial used for examination of distinctness, on the basis of comparing harmonized descriptions. A key feature of the process of eliminating varieties of common knowledge prior to the growing trial is that the threshold for deciding which varieties can be safely excluded (i.e. are distinct on the basis of descriptions), can be set with a suitable margin of safety, because those varieties which are not eliminated, but which are actually distinct, will be discovered in the growing trial. This threshold, with a safety margin, is termed the "Distinctness plus" threshold in this paper. In this proposal, the aim is to develop a Distinctness plus threshold for molecular characteristics.

Measuring distance in traditional characteristics

2.   The first step is to consider how to measure the distance between varieties using traditional characteristics. This proposal is based on the use of an approach, using the GAÏA computer software, developed by France. This approach works by estimating the phenotypical difference

between two varieties, based on the addition of the differences observed for the different characteristics. Each difference observed is weighted by the crop expert according to the value of the difference and to the reliability of each characteristic.

Measuring differences in molecular characteristics

3. The difference between varieties on the basis of information from molecular markers is calculated, in this option, by the use of Rogers' distances.

Calibrating threshold levels for molecular characteristics against the minimum distance in traditional characteristics

4. The calibration of threshold levels for differences in molecular characteristics against differences in traditional characteristics would be straightforward if there was a strong correlation between these two ways of measuring the differences between varieties. In such a situation, a graph of the different methods would look like figure 7. The threshold for Distinctness plus in molecular markers could be extrapolated from the Distinctness plus threshold in traditional characteristics in such a way that the same decisions would be made, regardless of which method of assessing variety differences was used.

5. However, in the case of Oilseed Rape, the correlation is less good, as illustrated in figure 8. It can be seen that, wherever the Distinctness plus threshold is set for the molecular markers, there would be some varieties with different decisions according to the method used for calculating the differences. The implications of this situation are explored in the section "Potential Impact."

ASSUMPTIONS TO BE MADE IN THE PROPOSAL

6. The following assumptions are made:
(a) Uniformity and Stability

The uniformity and stability requirements for the molecular markers have not been developed in this proposal. However, the available information suggests that variability for molecular characteristics within varieties seems to be higher than that observed in traditional characteristics. It is assumed that the differences calculated between varieties on the basis of molecular markers fully take into account the variation within varieties.

Furthermore, it is assumed that suitable uniformity standards could be developed for molecular markers without requiring varieties, in general, to be more uniform. This assumption is on the basis that molecular markers would be used for the establishment of a "Distinctness plus" threshold, based on genetic distance, in the management of reference collections and not for the judgment of distinctness on a characteristic by characteristic approach.

(b) Application of the Proposal

As explained in the Introduction, this proposal is made on the basis that it would only be used for the establishment of a "Distinctness plus" threshold in the management of reference collections.

(c) Reliability of the techniques

It is assumed that the techniques would meet all the normal requirements for any characteristic to be used in the DUS examination and, in particular, would be checked to ensure they are sufficiently consistent and repeatable.

POTENTIAL IMPACT

7. The graph provided in figure 8 highlights the possible ways in which this proposal could have an impact on the strength of protection. In summary, the situation can be represented as follows:

	Distinctness plus (Traditional characteristics)	Distinctness plus (Molecular characteristics)
Type 1	Yes	Yes
Type 2	No	No
Type 3	Yes	No
Type 4	No	Yes

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8. Types 1 and 2 outcomes would have no impact on the strength of protection because the result is the same for both methods used.

9. Type 3 outcomes would also have no impact on the strength of protection because the varieties would be discovered to be distinct using traditional characteristics in the growing trial.

10. Type 4 outcomes could have an impact on the strength of protection because they could result in varieties being considered to be distinct which would not have previously been considered to be distinct. Determining whether type 4 outcomes could undermine the effectiveness of protection offered under the UPOV system would require an analysis of such cases.

11. At present, type 4 cases are known in oilseed rape (examples can be provided). However, these cases only relate to pairs of varieties which were found to be distinct in a growing trial. The situation in which different decisions on distinctness would result can only be investigated where varieties are rejected for distinctness in the growing trial. This would require analysis of pairs of varieties rejected for distinctness in the past or, if such material is unavailable, a system of "parallel running" of the two systems in real time on candidate varieties. It would then be possible to discover if any such cases would occur and if these would undermine the effectiveness of protection. If it was considered that these cases would undermine the effectiveness of protection it could then be decided if a sufficiently high threshold could be set to eliminate these cases without losing the benefit of the approach for the management of reference collections.

12. It should be recognized that the case studies, envisaged in paragraphs 10 and 11, may not provide a complete assessment of the potential impact, since breeders would be operating under the existing system of DUS examination. Consideration should also be given, for example, to whether it would be easier under the proposed new system, if accepted, for new varieties to be selected from entirely within existing protected varieties. If this was the case, it could encourage "breeders" to try to select new varieties in this way, whereas, under the existing system there would be no incentive to do so because the varieties would not be considered distinct. This situation might be more likely to occur if the uniformity criteria for molecular markers was lower than for traditional characteristics.

CONCLUSIONS OF THE BMT REVIEW GROUP, THE TECHNICAL COMMITTEE AND THE ADMINISTRATIVE AND LEGAL COMMITTEE

The BMT Review Group concluded that the proposal above for Oilseed Rape and similar proposals for Maize and Rose, where used for the management of reference collections were, on the basis of the assumptions in the proposals, acceptable within the terms of the UPOV Convention and would not undermine the effectiveness of protection offered under the UPOV system. The Technical Committee and the Administrative and Legal Committee agreed with those conclusions, namely that those proposals could be pursued on the basis of the assumptions, whilst recognizing the need for further work to examine those assumptions and to improve the relationship between morphological and molecular distances.
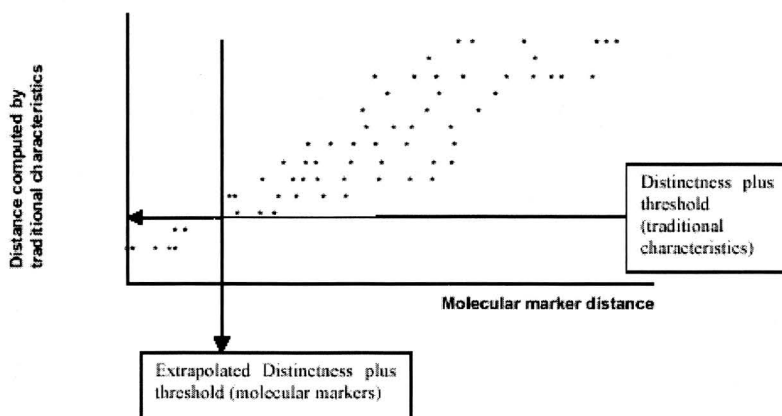
Fig. 7. Correlation between differences in molecular characteristics and differences in traditional characteristics.

GAÏA Distances = f(Rogers' Distances) for 28 varieties in the reference collection
Tests 1997/1998
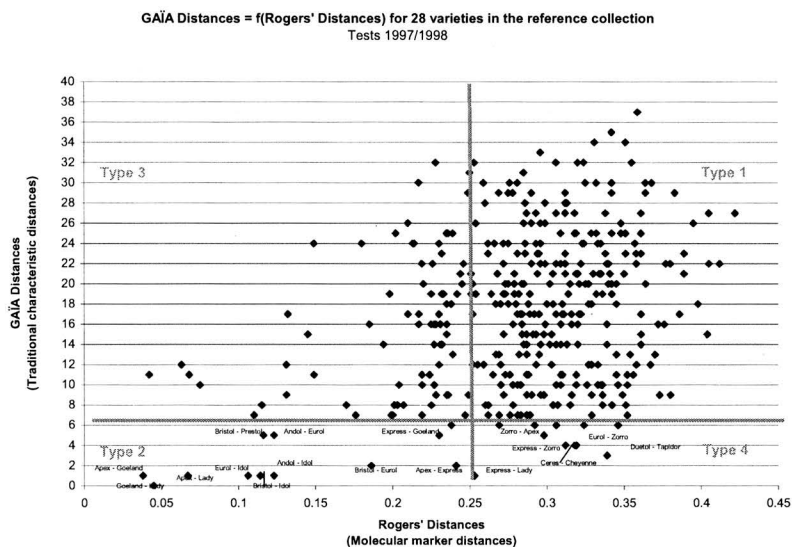
Fig. 8. Correlation in the case of Oilseed Rape.

LITERATURE

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Development of allele-specific markers for sustainable grassland improvement (GRASP)

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ABSTRACT

GRASP was a EU framework V project involving eight public institutions and one commercial partner. The major topic of GRASP was the development of "functional" allele-specific markers associated with relevant traits in *L. perenne* such as forage quality, nitrogen use efficiency, disease resistance, and abiotic stress tolerance. More than 100 candidate genes for these traits were isolated and the respective alleles of 20 genotypes (*Lolium* test set = LTS) were sequenced in order to derive allele-specific DNA markers. In parallel, the LTS was used to develop synthetic populations to be propagated under divergent selection over two generations for the different traits of interest. The frequency shift of DNA marker alleles over generations was monitored and used to validate candidate gene – trait associations. In addition to markers and populations, genomic tools were developed including a BAC library for *L. perenne*, subtracted cDNA libraries, microarrays for expression profiling, and genetic reference maps. Information generated in GRASP will be made available through the existing forage grass database in the UKCropnet (http://ukcrop.net/grass.html). GRASP is expected to be a platform for modern forage grass breeding but also for international follow-up projects.

Key words: GRASP, ryegrass, *Lolium perenne*, allele specific markers

INTRODUCTION

In the years to come European livestock production will face a number of challenges to remain competitive at the international market. At the same time European agricultural policies emphasise the need for improved quality, environmentally sustainable production methods, traceability of the feed and food supply, and diversity.

On-farm forage is vital in underpinning European livestock production of the future. To fulfil this strategy improved production of forage grasses is essential. In contrast to high performing cereals and other "cash crops", forage grasses allow for economic and consumer-friendly feed production under low input conditions (fertilizers, pesticides). Traditionally, forage grass breeding objectives have concentrated on improving sward yield and persistency (Johnson and Beyer, 1974). However, in the future breeding programs must pay more attention to specific nutritional needs of grazing ruminants and the impact of livestock farming on the environment (Spangenberg *et al.*, 2001). In the past few years the amount of basic biological information has increased dramatically on the function of hundreds of plant genes, largely through studies on the molecular genetics of the model plant Arabidopsis thaliana. Current research in the field of plant genomics has focused on analyses of mutant alleles, created by insertional mutagenesis, and sequencing the entire genomes of selected plant species. This has provided extensive knowledge on gene functions. Genes controlling important agronomic traits have been identified and characterised.

Genomics projects acts at the level of genes, i.e., gene function is determined by comparison of "wildtype" with "loss of function" alleles. However, plant breeding primarily acts at the level of minor allelic variation among wildtype alleles of a given gene. Thus, allele and SNP identification is of crucial importance to effective and competitive plant breeding but very little information has been generated on the naturally occurring allelic sequence variation in crop plants (Thornsberry *et al.*, 2001). It constitutes a complementary approach to map based cloning and insertional mutagenesis and with the technologies currently available for most of our crop plants it may be the most realistic and readily applicable strategy for improvement of forage grasses to determine allele functions for relevant genes.

Targets for improvement using allele-specific markers developed in this project comprised traits relevant to environmental and nutritive value needs, efficient control of developmental pathways (e.g., flowering, leaf, and root formation), as well as tolerance to biotic and abiotic stresses are important components of environmental adaptation. Moreover, animal nutritionists urge plant breeders to consider the balance between energy and protein supply at various stages of ruminant digestion in addition to assessing overall digestibility. Up to 80% of the protein in fresh forage may be wasted to the environment. This results from the inability of rumen microbes to capture the nitrogen released during the breakdown of plant proteins due to limitations in the supply of readily available energy.

The genus Lolium contains the most important forage grass species in Europe, *L. perenne* and *L. multiflorum*. Together the two species cover 23% of the grassland area (52 million ha) in Europe with the perennial *L. perenne* being the most prevalent grass species. About 45.000 tonnes of ryegrass seed is used each year at a cost of about 160 million EUROs with Europe being a net exporter of Lolium seeds. Progress in conventional Lolium breeding is hampered by the application of simple but inefficient population breeding schemes and by the slow phenotypic evaluation process. Taking into account the high degree of diversity of the breeding materials used by ryegrass breeders, molecular tools for early selection would dramatically accelerate progress in breeding especially of perennials. This project established molecular markers based on relevant genes. Specifically, Single Nucleotide Polymorphism markers (SNPs) were developed.

Outline of GRASP

A set of 20 Lolium genotypes highly differing for the traits of interest have been assembled by the nine partner institutions. The genetic distance among them was determined using molecular markers (WP1). Candidate genes for forage quality and for traits improving environmental sustainability in Lolium were isolated (WP2) by partners with trait-specific expertise. We identified a set of promising candidate genes and supplemented this portfolio by genes isolated via differential gene expression in relevant tissues. In parallel, a BAC library was produced (WP2) for isolation of full-length genes and promoters. In the second year, a standard protocol was employed to characterize the 100 candidate genes isolated within this project including genetic mapping (WP2). Gene-specific sequence variation was studied on the set of 20 Lolium genotypes (WP3), established in WP1. All molecular (sequence, map) data will be made available through a database of the partnership (WP4). Trait specific selections were carried out over at least two generations using the set of 20 genotypes as starting materials (WP5). Changes in allele frequencies associated with divergent selections were used for validation of candidate genes. WP6 was dedicated to technology transfer including (1) development of SNP marker tool kits for allele discrimination in grass breeding, (2) available BAC and cDNA / ESTs libraries, (3) breeding populations selected for specific traits and evaluated at the level of several relevant genes, and (4) development of new breeding strategies implementing allele specific SNP markers developed in this project.

WORKPACKAGES

Workpackage 1
A collection of 20 diverse diploid *Lolium perenne* genotypes (Lolium test set: LTS) was been established. Most of the LTS genotypes have been well characterized by individual partners, such as parents of QTL mapping populations. DNA from all 20 LTS genotypes has been distributed to all partners. Fingerprinting of the 20 LTS genotypes was conducted with AFLPs, SSRs, RAPDs, and ISSRs (Posselt *et al.*, 2007).

Workpackage 2
More than 100 candidate genes for agronomically relevant traits were isolated either based on sequence homology to characterized genes (see Figure 2), or based on differential expression. The respective clones / PCR fragments were assembled to generate project microarrays for expression profiling experiments (e.g., Van Daele *et al.* 2007). Full-length sequences for the genes of interested were isolated from a BAC library generated within GRASP (Farrar *et al.*, 2007).

Workpackage 3
For about 100 genes, allele sequences for all 20 diploid and heterozygous LTS genotypes were generated. Allele sequencing revealed generally high levels of nucleotide diversity and low levels of linkage disequilibrium, as expected for an outcrossing species, with substantial variation between individual genes (e.g., Xing *et al.*, 2007). SNP and INDEL polymorphisms have been converted into markers assays, using a broad range of technologies by the different partners such as the Sequenome approach (MassARRAY), EcoTILLING, or primer extension using Taqman.

Workpackage 4
A project homepage (http://www.grasp-euv.dk) was established for communication between partners. Protocols, plans, data, reports, etc relating to individual workpackages were uploaded under the respective workpackage sections. Parts of the information from this

homepage is made publicly accessible. Project data were collected at IGER curating the FoggDB (http://www.igergru.bbsrc.ac.uk/dev/Welcome/IGER/foggdb/foggdb.htm). Upon agreement by GRASP partners, project data will be released to the public domain.

Workpackage 5
Synthetic populations have been developed for selection experiments for all traits of interest (Figure 1). The respective partners have traced allele frequencies for alleles of their candidate genes as well as SSRs covering all seven chromosomes. Significant differences between divergent selections were taken as first indication for an involvement of the respective genes or genome regions in the trait under investigation
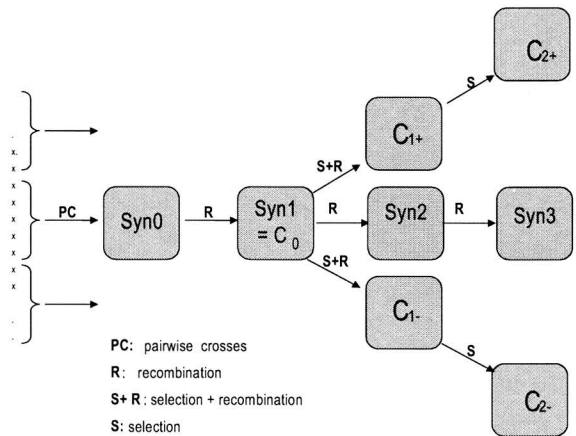
Fig. 1. Outline of a selection experiment, starting from a subset of genotypes of the Lolium test set (LTS).

Workpackage 6
Technology transfer was addressed by (1) developing SNP marker tool kits for allele discrimination, (2) making available BAC and cDNA / ESTs libraries, as well as breeding populations selected for specific traits and evaluated at the level of several relevant genes, and (3) developing new breeding strategies implementing allele specific SNP markers developed in this project.

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Identification of promising candidate genes for abiotic stresses and forage quality in perennial ryegrass

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ABSTRACT

An essential topic of the EU-project GRASP was the identification of candidate genes in perennial ryegrass (*Lolium perenne* L.) for traits that are relevant in relation to variation in response to abiotic stresses and forage quality. To this end, various trait-specific cDNA libraries were created and used for the production of cDNA microarrays. The arrays were subsequently used for various expression profiling studies. The results are exemplified for the trait nitrogen-use efficiency.

Key words: cDNA microarray, nitrogen-use efficiency, expression profiling

INTRODUCTION

The primary aim of the EU-project GRASP (Lübberstedt *et al.*, 2003) was to study the influence of selection in a few synthetic populations on the frequencies of alleles from genes involved in the trait of interest. To this end, the project partners had to choose genes being functionally related to the subject of their study. Traits under study were flowering and vernalization (DLF), carbohydrate allocation and cell-wall composition (IGER), nitrogen-use efficiency (PRI), self- incompatibility and fertility (ILVO), cold tolerance (LIA, UMB) and leaf area expansion in response to temperature and light (INRA). One obvious route to come to a set of promising candidate genes is expression profiling for each of the traits under study using a dedicated cDNA microarray. Various sets of cDNAs contributed by the GRASP partnership were used to generate the arrays. They were subsequently used for a variety of expression studies, one of them concerning expression profiling of genes involved in nitrogen-use efficiency (NUE). This trait is of utmost importance for grassland husbandry. A genetic study in the EU-project NIMGRASS showed that a small number of QTLs explained a major part of the genetic variation (Loo *et al.*, 2003). Dolstra *et al.*, (2007) showed that the trait was amenable to marker selection. Application of differential mass selection in one of the GRASP synthetic populations, using the same selection procedures, also showed a large genetic response.

MATERIALS AND METHODS

NUE-related cDNA libraries. Shoots and roots of about 20 4-weeks old plants of genotype LTS01 grown on a hydroponics system (Loo *et al.*, 1992) set to produce plants with an N-content of about 4.5% (high N) were harvested, pooled, and used to extract RNA. In parallel and in the same way the system was used to generate RNA from shoots and roots aiming at 2.7% N (low N). The contrasting root and shoot samples were then used to produce specific root and shoot cDNA libraries by means of suppression subtractive hybridization (SSH) using

the PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, USA). The clones were partially sequenced.

cDNA microarray production. A GRASP cDNA microarray with about 4200 clones printed in duplicate was generated, using the equipment and procedures as described by Aharoni and Vorst (2002). This set of clones comprises nine collections of cDNA clones contributed by the GRASP partnership and originates from different types of tissues collected from plants exposed to various conditions (Table 1). All clones were obtained from perennial ryegrass, except those from UMB which originated from meadow fescue. Most collections were enriched for clones of genes affecting specific plant responses, either through SSH or cDNA-AFLP. In all, 49 arrays were generated and used for expression studies.

Table 1. Summary of clones printed on GRASP cDNA microarray.

Partner	Material	Trait of interest	Number of clones	Enrichment way
PRI	shoots	nitrogen-use efficiency	321	SSH
PRI	roots	nitrogen-use efficiency	351	SSH
DLF	leaves	flowering, vernalization	738	SSH
IGER	leaves	carbohydrate metabolism	672	
ILVO	pistils	self-incompatibility	195	cDNA-AFLP
ILVO	shoots	forage quality	263	
LIA	meristematic tissue	rhizome formation	576	SSH
INRA	leaves	light quality, temperature	384	SSH
UMB	crown tissue (Fp)	cold tolerance	576	SSH
total			4076	

Expression profiling. Comparative swap-dye experiments with various pairs of differently labelled total RNAs were performed to study the influence of environmental and genetic factors on gene expression in various plant tissues. To this end, 40 µg of total RNA was used for samples to be labelled with Cy3, and 20 µg of total RNA for samples to be labelled with Cy5. After indirect labelling both samples were mixed in 50 µl hybridization buffer (Slidehyb#1, Ambion, Austin, USA) and applied on to a microarray slide. Hybridization was performed in a hybridization station (Hybarray 12, Perkin–Elmer Applied Biosystems, Foster City, USA) overnight at 50°C. After hybridization, the array was washed in several SSC-SDS solutions to remove unbound material, dried, and scanned for fluorescence emission using ScanArray Express HT (Perkin–Elmer Applied Biosystems, Foster City, USA). Using the Scan Array software (Perkin–Elmer Applied Biosystems, Foster City, USA), fluorescence signals obtained upon scanning for a specific dye are converted into a digital image. A grid consisting of defined circles fitting the size of each DNA spot was superimposed over the image so that the median pixel intensities for each DNA spot were calculated for both dyes. Each contrast was repeated in reverse labelling (swapping of dyes). Aharoni and coworkers

(2000, 2002) presented more detailed information on the technical aspects of such experiments.

Criteria for clones to be selected as being 'differentially expressed' were a mean of the log2 ratios of the fluorescence signals > 0.72 or <-0.72 with a standard deviation less than half the mean value.

RESULTS AND DISCUSSION

Expression profiling for NUE. A summary of the swap-dye experiments to study differential expression of NUE-related genes in shoots and roots is shown in Figure 1. Only clones showing a significant difference in expression between high and low N in roots or shoots were depicted by single markers. The profiling with the root contrast resulted in the identification of 175 clones with overexpression at high N and only 71 with overexpression at low N. The corresponding figures for the shoot contrast were 34 and 124. In all, 366 clones out of 4076 were implicated in NUE of which 38 showed a significant differential expression in the root as well as in the shoot contrast. Figure 1 also shows remarkable differences between collections of clones spotted on the arrays with respect to the proportion of clones showing differential expression. The collection of INRA yielded quite a large number of clones showing overexpression at low N in shoots. In roots the two PRI collections were the most rewarding with respect to the discovery of clones showing differential expression between high and low N. It was somewhat surprising that most of these clones revealed overexpression of genes at high N. The clone collections of UMB, ILVO and LIA yielded relatively few candidate genes for NUE. Rhizome formation and self-incompatibility are apparently traits having little in common with NUE. The low yield of candidate genes from the UMB collection is more likely to be caused by a lower degree of similarity between the expressed genes from ryegrass and the corresponding clones from meadow fescue. Quite a large proportion of INRA clones implicated in NUE showed a high degree with overexpression in shoots and roots under N stress.

Expression profiling for other characteristics. In addition to the two profiling studies on NUE the PRI collection of clones was screened in four other studies (UBM, DLF, IGER, and INRA). It turned out that a large part of clones from PRI spotted on the arrays revealed no differential expression in the six expression studies performed. This held true for 330 out of 672 clones. The rest of the clones revealed differential expression: 94 (one study), 104 (two studies), 31 (three studies), 12 (four studies), and 1 (five studies). The clones showing differential expression in more than 2 studies were in most cases involved in the synthesis/functioning of aquaporins and germins.

Promising candidate genes for NUE. The most rewarding cDNA library for NUE was not surprisingly the root collection of PRI. Out of 110 clones 24 gave no hits in neither of the BlastX or BlastN searches performed. The rest of searches pointed to putative functions which mostly can be associated to responses to abiotic stress, N metabolism and/or the functioning of other nutrients. This is illustrated in Table 2. Some clones were associated to the same function (Table 2). The remaining clones were associated to different functions. Most of them were up-regulated at low N, except for the ones affecting nitrate transport. All other clones identified were linked to different gene functions. It is striking that in root tissue gene expression for most groups of genes was up-regulated at high N or perhaps more likely down-regulated at low N. The group of clones concerning nitrate transport is one of the few striking exceptions to the general tendency to down-regulate gene expression at low N. The functions found for the three other clones showing up-regulation at low N in root tissue are

putatively an ACC oxidase, acid pathogenesis-related protein, and a plasma membrane ATPase, respectively. In shoot tissue only a few clones showed overexpression at low N.
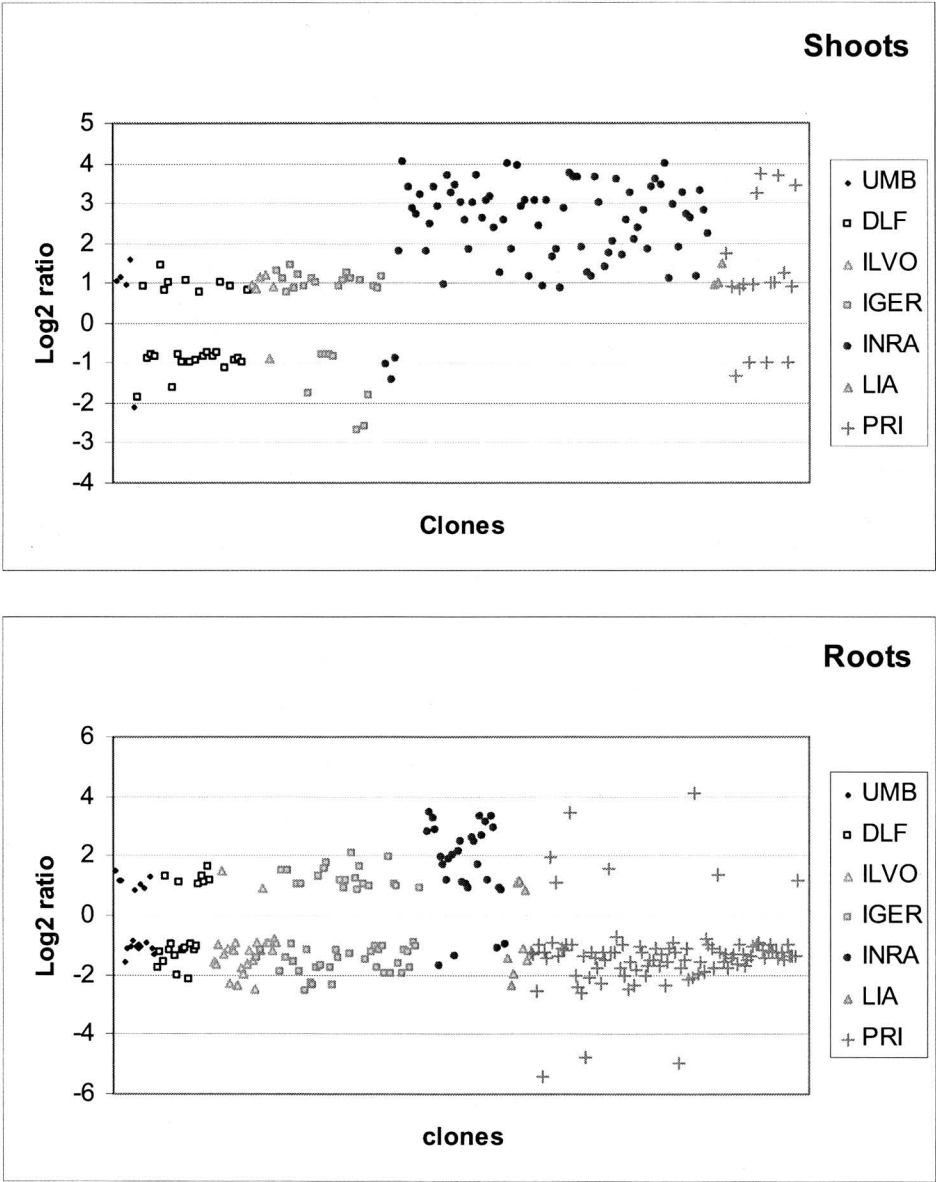
Fig. 1. Overview of cDNAs showing differential expression for NUE in shoots and roots, respectively. The cDNAs were grouped by partner. Values >0 indicate overexpression at low N and those having a value <0 overexpression at high N.

Table 2. Clones from the PRI cDNA libraries showing differential expression for NUE in root (R) and/or shoot (S) tissue from LTS01 grouped by putative function. The tissue in the two swap-dye experiments in which the expression was up-regulated is indicated.

Putative function	Number of clones (R/S)	Root NUE-contrast	Shoot NUE-contrast
aquaporin	8/0	high ↑	-
pathogenesis-related protein	5/1	high ↑	low ↑
nicotianamine synthase	5/0	high ↑	high ↑
cysteine proteinase	4/1	high ↑	high ↑
senescence-associated protein	3/0	high ↑	-
plasma membrane ATPase	2/1	low ↑, high ↑	low ↑
high affinity nitrate transporter	3/0	low N	-
putative thiamine biosynthesis protein	1/1	high ↑	high ↑
heat shock protein	2/0	high ↑	-
germin-like protein	2/1	high ↑	low ↑
elongation factor 1-alpha	2/0	high ↑	-
ankyrin-like protein	2	high ↑	-
amino acid transporter	2	high ↑	-
alpha-tubulin	2	high ↑	-
no hit	24		

The profiling studies for NUE as all the other profiling studies yielded numerous candidate genes for further studies. The study of the allelic diversity of such numbers of genes was impossible within the GRASP framework. Therefore there was a need to reduce the number to the most promising ones. One strategy was the mapping of the candidates prior to starting the analysis of the allelic diversity. The ones localized in the vicinity of QTLs for NUE are considered to be the most promising for further study. This approach turned out to be too time-consuming and gave limited success because of a lack of polymorphisms between the parents of the mapping. Therefore it is necessary to get full-length gene sequences first, which can be done at the moment in a straightforward manner using the BAC-landing approach developed in GRASP (Farrar *et al.*, 2007). So pre-screening of clones using their map position is now better feasible.

CONCLUSION

In conclusion, the expression studies were time-consuming and also laborious. In fact, they have delayed the start of the searches for allelic diversity. On the other hand, the joint activities needed to realize this task certainly strengthened the cohesion of the GRASP

consortium. The expression profiling approach gave a lot of possible candidates, for sure a promising result of the project. However, the number was far beyond our capacity for the analysis of allelic diversity. In all, a homology-based strategy is often a more rewarding method to get good trait-specific candidate genes for genetic studies in perennial ryegrass.

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Development of ryegrass allele-specific markers for sustainable grassland improvement (GRASP): SNP discovery

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ABSTRACT

GRASP was an EU framework V project involving eight public institutions and one commercial partner. The major target of GRASP was the development of "functional" gene-derived and allele-specific single nucleotide polymorphism markers associated with relevant traits in *Lolium perenne* such as forage quality, nitrogen use efficiency, disease resistance, and abiotic stress tolerance. For each of the candidate genes, allele sequences (minimum length 1000 bp per allele) of 20 diverse genotypes (*Lolium* test set = LTS) were sequenced in order to derive allele-specific SNP markers. For a total of 104 genes, allele sequencing was initiated. Allele sequencing was completed for 91 genes and have been used to derive informative SNP markers for selection experiments. At University of Århus, 11 expressed resistance candidate genes were analysed in detail, including 6 nucleotide binding site and leucine rich repeat (NBS-LRR) like genes and 5 non-NBS-LRR resistance gene analogues. The number of haplotypes per gene ranged from 9 to 27. On average one single nucleotide polymorphism (SNP) was present per 33 bp between two randomly sampled sequences for the 11 genes. NBS-LRR like gene fragments showed higher degree of nucleotide diversity, with one SNP every 22 bp between two sampled sequences. NBS-LRR like gene fragments showed very high nonsynonymous mutation rates, leading to altered amino acid sequences. Particularly LRR regions showed very high diversity with one SNP every 10 bp between two sequences on average. In contrast, non-NBS LRR resistance gene candidates showed lower

degree of nucleotide diversity, with one SNP every 112 bp. 78% of haplotypes occurred at low frequency (<5%) within the collection of 20 genotypes. Low intragenic LD was detected for most R genes, and rapid LD decay within 500 bp was detected. Comparative analyses with regard to SNP density, heterozygosity, linkage disequilibrium, etc. across different gene families will be presented.

Key words: ryegrass, GRASP, crown rust, molecular markers, SNPs

INTRODUCTION

Plant breeding primarily acts at the level of minor allelic variation among wildtype alleles of a given gene. Thus, allele and SNP identification is of crucial importance to effective and competitive plant breeding but very little information has been generated on the naturally occurring allelic sequence variation in crop plants (Thornsberry *et al.*, 2001). It constitutes a complementary approach to map based cloning and insertional mutagenesis and with the technologies currently available for most of our crop plants it may be the most realistic and readily applicable strategy for improvement of forage grasses to determine allele functions for relevant genes. Development of gene-derived and allele-specific markers associated with relevant characters is the goal in the EU project GRASP (http://www-grasp-euv.dk; Lübberstedt *et al.*, 2003). GRASP is an EU framework V project involving eight public institutions and one commercial partner. The major target of GRASP is the development of "functional" gene-derived and allele-specific single nucleotide polymorphism markers associated with relevant traits in *Lolium perenne* such as forage quality, nitrogen use efficiency, disease resistance, and abiotic stress tolerance. Results from seven expressed resistance gene analogues will be described in more detail. We sequenced about 1kb regions of 11 expressed disease resistance candidate genes and 3 laccases from 20 genotypes (Lolium Test Set, LTS) employed in the EU project GRASP (Lübberstedt *et al.*, 2003). The objectives were to (1) compare the nucleotide diversity within and between different gene classes, (2) study effects of natural selection on gene mutations, (3) determine the amount and structure of LD within these genes, and (4) to discuss the prospects of candidate-gene based association mapping in ryegrass.

MATERIALS AND METHODS

A total of 20 genotypes of perennial ryegrass (*Lolium perenne* L.) originating from various sources were included in this study (= Lolium Test Set, LTS). These genotypes represent a wide range of genetic diversity within ryegrass (Posselt *et al.*, submitted). Three laccase genes and 11 potential disease resistance genes were selected from the annotation of EST sequences generated within the project DAFGRI (http://www.dafgri.dk), which included homologues of nucleotide binding site and leucine rich repeat (NBS-LRR) like, pathogenesis related (PR), Mitogen-activated protein kinase (MAPK), enhanced disease resistance (EDR), and plastid pyruvate kinase A (PKpA) protein coding genes (Table 2). On the basis of candidate mRNA sequences, 14 pairs of primers were designed to amplify about 1 kb genomic fragments from the 20 genotypes for each of the 14 genes (Table 3). A touch down PCR program was used beginning with 5 min at $94^{o}C$, followed by 12 cycles of 30 s at $94^{o}C$, 60 s at annealing temperature $67^{o}C$, 60 s at $72^{o}C$ with the annealing temperature decreasing by $1^{o}C$ per cycle, followed by 29 cycles of 30 s at $94^{o}C$, 60 s at $55^{o}C$, 60 s at $72^{o}C$ and 10 min at $72^{o}C$. All 14 primer pairs ran with the same PCR program on a MJ Research thermocycler (Applied Biosystems, Califonia) in 25 µl reaction mixtures containing 20 ng DNA, 0.2 µM primer, 0.2 mM dNTPs, 0.4 u BD Advantage 2 polymerase, and 2.5 µl 10×BD advantage 2 PCR buffer.

The PCR products were purified from agarose gel using QiaQuick spin columns (Qiagen, Valencia, USA) according to manufacturer instructions. Purified fragments were cloned into vector pCR®2.1-TOPO (TOPO TA cloning kit, Invitrogen, Califonia). Five clones per gene for each genotype were randomly picked for plasmid DNA extraction. Purified plasmid DNA was used for allele sequencing on the MegaBACE1000 (Amersham Bioscience, Califonia). Sequence chromatogram files from the same genotype were assembled into contigs by using SEQMAN (DNA star, Madison, WI), and consensus sequences were edited manually to resolve discrepancies. Consensus sequences for all the 20 genotypes were aligned by using CLUSTAL alignment. Polymorphisms which appeared only in one genotype were rechecked in chromatogram files.

When calculating the number of haplotypes, all polypmorphic sites including Indels and segregating sites with 2 and more variants were taken into consideration. Direct comparison of mRNA sequence and its corresponding genomic DNA sequences was used to determine exon and intron regions. Alignment data for each candidate gene were used for nucleotide diversity and linkage disequilibrium (LD) analysis. DnaSP version 4 (Rozas et al., 2003) was used for the following analyses. All calculations were based on 40 alleles from the 20 heterozygous diploid genotypes. If one genotype was homozygous in the sequenced region, its allele sequence was presented twice in the alignment in order to determine the allele frequency for the 20 genotypes.

LD was estimated by using standardized disequilibrium coefficients (D') (Hedrick, 1987) and squared allele-frequency correlations (r^2) (Weir, 1996) for pairs of SNP loci. Sites with alignment gaps or polymorphic sites segregating for three or four nucleotides were completely excluded from the analysis. Fisher's exact test (1935) was used to determine the statistical significance of LD. Decay of LD with distance in base pairs (bp) between sites within the same gene was evaluated by nonlinear regression in Statistica (Hill & Lewicki, 2006).

RESULTS

About 1kb long sequences have been obtained from all 14 genes and 20 heterozygous LTS genotypes investigated. The 14 genes will be subdivided in the following into NBS-LRR resistance candidate genes (NBS genes: six different NBS genes), Non-NBS-LRR resistance candidate genes (NNL genes: five different NNL genes), and Laccases (LAC genes).

The average number of haplotype-alleles within the collection of LTS genotypes was 20.4, 11.4, and 14.0 for NBS, NNL, and LAC genes, respectively. For the SSRs, the everage number of different alleles was 10.7. The number of haplotypes varied between 12-27, 9-15, and 7-21 for NBS, NNL, and LAC genes, respectively. The majority of alleles was present at low frequency (<5%): 16.6, 8.0, and 10.6 for NBS, NNL, and LAC genes, respectively. In contrast, only few haplotype alleles were detected at frequencies above 20% with average absolute frequencies of 0.6, 1.2, and 1.4 for NBS, NNL, and LAC genes, respectively.

The level of homozygosity found in the 20 LTS genotypes as determined by the haplotype and SSR alleles was much higher as expected under an assumption of random mating. Homozygosities ranged from 20-60% (expected: 4-19%), 35-75% (expected: 17-33%), 50-69% (expected: 10-32%), and 10-93% (expected: 6-44%), for NBS, NNL, LAC, and SSR alleles, respectively.

Generally, Indel and SNP polymorphisms were more frequent in non-coding as compared to coding regions, and synonymous SNP mutations were more frequent than non-synonymous SNP mutations in coding regions. However, for two NBS genes higher SNP frequencies were found in non-coding as compared to non-coding regions. Moreover, in most NBS genes more non-synonymous than synonymous SNP polymorphisms were found. The SNP density was highly variable between genes and ranged from 16 SNPs in 1030 bp to up to 277 SNPs in 1036 bp within the LTS.

The intragenic LD between pairs of SNP polymorphisms as expressed by r^2 ranged from 0.07-0.19, 021-0.54, and 0.17-0.63 for individual NBS, NNL, and LAC genes, respectively. Percentages for significant LD between SNP pairs ranged from 8-33%, 31-58%, and 22-79%. When plotting r^2 values against the distance between SNP pairs, r^2 values fell below 0.2 within distances of 15-220 bp, 300-1600 bp, and 250-195000 bp for individual NBS, NNL, and LAC genes, respectively.

The minimum number of SNPs required to discriminate all haplotypes within the LTS was below 12 for each of the 14 genes investigated. For the majority of these genes, as few as 3-6 SNP polymorphisms were sufficient for complete differentiation of all haplotypes found within the collection of LTS genotypes

CONCLUSIONS

Substantial sequence variation for three classes of expressed resistance candidate genes was found in a collection of 20 unrelated diploid perennial ryegrass genotypes, employed in the EU project GRASP. For most genes, both high SNP densities and low intragenic LD were found. The on average highest SNP densities and and lowest LD were found for NBS-LRR resistance gene candidates. This is in agreement with the biological role of these genes, which are key players in pathogen recognition. Often multiple alleles at respective resistance loci are involved in gene-by-gene interactions with pathogen genes.

For two laccases, LD was extending over larger regions, supporting earlier findings from other species that the degree of LD is strongly genome region-specific. However, the otherwise generally low LD substantially decaying within genes is in agreement with good prospects for candidate-gene based association studies in ryegrass, which should allow detection of short sequences or even sequence motifs associated with the traits of interest, comparable to the study of Thornsberry *et al.* (2001). In contrast, genome-wide association studies based on natural populations would require a very high density of markers with at least a few markers / 1 kb.

Alternatively, artificial populations with high or moderate levels of LD can be employed in association studies, such as the synthetic populations studied within GRASP (Lübberstedt *et al.*, 2003). For tracing and discrimination of allele sequences in this kind of experiments only 3-6 SNP markers per gene are sufficient. This information can be implemented into SNP marker assays allowing a high degree of multiplexing.

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Association studies using synthetic varieties: Case study of GAI gene and leaf length in perennial ryegrass

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ABSTRACT

Identification of genome regions controlling quantitative trait variations is generally obtained from QTL analyses on mapping populations exhibiting a low allelic diversity. An alternative approach would be association studies using collections of individuals. Any structure of these collections would hinder detection of associations. An ideal plant material for association studies would be multi-allelic and unstructured populations.

The objective of this study was to test whether synthetic varieties could be used for association studies.

In a first experiment, three perennial ryegrass varieties with contrasting numbers of parental plants were studied for their structuration, variability and linkage disequilibrium (LD) decline. This decline defines which association study approach has to be envisaged: "candidate gene" when LD rapidly decreases or "genome scan" when LD slowly decreases. The varieties were not structured. LD decreased more rapidly when the number of parents increased. LD is low beyond 150 bp for Herbie, a variety built from 336 parents whose molecular and morphological diversity was similar to a core collection.

In a second experiment, an association study was performed between the Gibberelic Acid Insensitive gene and leaf length in the variety Herbie. One specific SNP explained 4% of leaf length variation. Differences between genotypic classes of this SNP reach 30 mm for a mean value of 303 mm.

Key words: candidate gene, forage, gibberelic acid insensitive, grasses, linkage disequilibrium, *Lolium perenne* L., morphogenesis

INTRODUCTION

The pattern of linkage disequilibrium (LD) decline determines whether a genome scan or a candidate gene approach can be used in an association study between genotype and phenotype (Rafalski, 2002). A synthetic variety is obtained after three to four panmictic bulking generations starting from a given number of parents. Such varieties should be valuable for association studies since they should be unstructured, variable and present a LD between two loci only due to a physical link. Moreover, they are common in the breeding scheme of most outcrossing species such as perennial ryegrass.

The objective of this study was to test whether synthetic varieties could be used for association studies using perennial ryegrass. In a first step, we evaluated the effect of the number of parents in the original polycross of a synthetic variety on the pattern of LD decay and we then performed an association study between the Gibberelic Acid Insensitive gene (GAI) and leaf length in a highly variable variety. Leaf length appears to be an important agronomic trait for grassland, especially when grazed (Barre *et al.*, 2006). GAI was shown to

play an important role in plant growth in different species by acting on the gibberelin signal (Ogawa *et al*., 2000).

MATERIALS AND METHODS

First experiment: LD decline (details in Auzanneau *et al*., 2007)
Three synthetics varieties were chosen for their contrasting number of parents in the initial polycrosses: Aberavon (Abe) with six related plants, Brest (Bre) with ten unrelated plants and Herbie (Her) with 336 plants. For each variety 47 plants were studied. In addition, 47 plants of a core collection (Cc) were used. To estimate the LD decline around GAI on LG4, each plant has been genotyped with 3 SSRs and 4 developed STSs including a part of GAI. Direct sequencing of PCR products were applied on the STSs leading to SNP data. Genotypic LD between markers, including both not rare SNPs and SSRs, were tested using Fisher exact test in GENEPOP software. Moreover, 2 STS of 1322 bp and 874 bp were cloned and sequenced to calculate gametic LD using DNAsp software. Finally, 3 SSRs in different linkage groups have been used as unlinked markers.

Second experiment: Association study between leaf length and GAI
Three replicates of 186 genotypes of Herbie were phenotyped for their leaf growth: leaf elongation rate (LER), leaf elongation duration (LED) and leaf length at maturity (Llength) on one leaf per plant, in a glasshouse in fall 2004 at Lusignan (France). One hundred plants of the previous core collection (Cc) were included. Each genotype of Her was genotyped by direct sequencing of PCR products of the same fragment (232 bp) of GAI studied previously. The effect of GAI's SNPs on leaf length was tested using a stepwise multiple linear regression analysis.

RESULTS AND DISCUSSION

First experiment: LD decline
Results on SSRs (Table 1) and STSs (not shown) revealed a gradient of polymorphism among varieties similar to the one obtained from the number of parents used to build the varieties. For all SSR markers, the three varieties were at panmictic equilibrium and did not present any substructure within populations (not shown).

Table 1. Number of alleles (A), expected heterozygosity (H) and fixation index Fis averaged on the 6 SSRs studied.

Populations										
SSRs markers	Aberavon			Brest			Herbie			Core collection	
	A	H	Fis	A	H	Fis	A	H	Fis	A	H
Total	3.3	0.42	0.14 NS	7.5	0.63	0.05 NS	10.3	0.60	0.06 NS	16.3	0.76

NS : Not Significant at P < 0.05 after Bonferroni correction, deficit or excess of heterozygous relative to HW expectations.

Results on gametic LD at short distance showed a gradient of LD values with Abe having the highest values followed by Bre and then by Her and Cc (Figure 1). Furthermore, considering all populations, pattern of LD decline was dependent on the distance for STS5 but not for

STS4 which had a higher level of LD (not shown). Data on genotypic LD showed that at a distance of 174-175 kb, the proportions of significant tests were 50%, 10%, 4% and 4% for Abe, Bre, Her and Cc, respectively and at a distance of 1.6-1.8 Mb, the proportions of significant tests were 17%, 16%, 6% and 3% for Abe, Bre, Her and Cc, respectively. These results indicated that for long distances Abe had the strongest LD followed by Bre. No LD was detected beyond 14 Mb (about 4 cM) for all the populations.
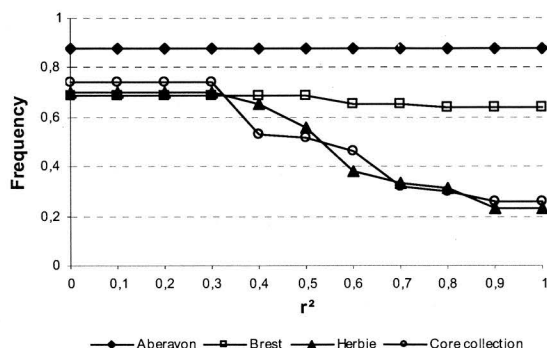
Fig. 1. Frequency of significant tests (after bonferroni correction) taking into account 2 STSs in relationship with values of r² for studied perennial ryegrass populations.

Second experiment: Association study between leaf length and GAI

Leaf length variation in Herbie (mean: 301 mm; standard deviation: 65) was as wide as in a core collection (mean: 270 mm; standard deviation: 69). It was explained primarily by leaf elongation rate (correlation of 0.93) and to a lesser by leaf elongation duration (correlation of 0.48). LD in GAI was low beyond 150 bp with r² value lower than 0.2. The association study between leaf length and SNPs polymorphism revealed that 11% of variations in leaf length were explained by the polymorphism of 4 SNPs. One of these SNPs explained 4% of leaf length variation and differences between genotypic classes reached 30 mm for a mean of 303 mm.

Thus, this study revealed that synthetic varieties are valuable material for association studies. Synthetic varieties with a wide genetic basis are adapted for association studies with a gene candidate approach such as the one presented on leaf length and GAI. Varieties with a narrow genetic basis can be used for association studies with a genome scan approach. In all cases, LD should be estimated in the working population.

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Changes in SSR allele frequencies during phenotypic selection for forage Water-Soluble Carbohydrate (WSC) in an experimental population of perennial ryegrass

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ABSTRACT

Carbohydrate content is an important component of forage quality with direct effects on nutritive value. Increasing carbohydrate content increases the utilisation of protein in the rumen, which in turn increases milk and meat production whilst concomitantly reducing nitrogen losses to the environment. C0 (Syn2) seed of 3 experimental populations was produced from an initial set of 20 genotypes of perennial ryegrass (the most important forage grass for temperate livestock production) as part of the EU GRASP project. One of these populations was used to phenotypically-select for high and low WSC over two generations, producing C2+ and C2- populations. A C2= (Syn4) reference population was also produced with no conscious selection. The C2+ and C2- populations had significantly different WSC content in spring 2007, with the C2= population being intermediate. Allele frequencies of SSR markers were compared between the various populations. Shifts in allele frequency between the C2+ and C2- populations that could not be explained by genetic drift (assessed by comparing the original C0 population and the C2=) indicated effects of selection. The map location of the markers with the greatest shift in allelic frequency has been compared with known WSC QTL positions.

Key words: association studies, fructan, *Lolium perenne*, population genetics, quantitative trait loci (QTL)

INTRODUCTION

Temperate grasslands support most of the world's milk and meat production. Presently around 75% of feed requirements are obtained from grass and forage, although this varies from 60% of the feed for some dairy cows up to 90% for sheep (Wilkins & Humphreys, 2003). In the UK the most commonly grown forage grass is perennial ryegrass (*Lolium perenne* L.). This shows considerable natural variation for water soluble carbohydrate (WSC) and this variation has been employed in the breeding of high-sugar ryegrasses. These have been demonstrated to provide improved nutritional value for ruminants, by supplying an easily fermentable energy source in the rumen which leads to improved protein utilisation, and so boosts milk and meat production whilst reducing nitrogen losses in waste products (Miller *et al.*, 2001). The major component of the total WSC pool in perennial ryegrass forage is fructan. The genetic control of fructan and total WSC variation in perennial ryegrass has been analysed by quantitative trait locus (QTL) mapping in an F2 family (Turner *et al.*, 2006). QTL were identified on four linkage groups with two regions of the genome conferring particular control. Major QTL for tiller base WSC were found on linkage group 1 and for leaf WSC on linkage group 6.

As part of the EU GRASP project (http://www.grasp-euv.dk/) trait specific selections were carried out over two generations using populations derived from an initial set of 20 test genotypes which included members of the F2 mapping family in which WSC QTL were characterised. Changes in allele frequencies associated with divergent selection were used for the validation of SNP markers in candidate genes during the GRASP project. The object of the work described here was to use SSR markers spread across the genome to compare changes in allele frequency in the C2 generations with the known QTL locations.

MATERIALS AND METHODS

Plant material of the C2+, C2- and C2= (unselected, Syn4) populations was produced from C0 seed according to the schedule in Figure 1. C0 populations were subjected to divergent phenotypic selection based on WSC content over two generations (to produce C2+ and C2- populations) with a selection intensity of 10% (30 plants taken for each round of recombination from a population of 300). The control population (C2=) was obtained without conscious selection with the same number of plants retained at each generation.
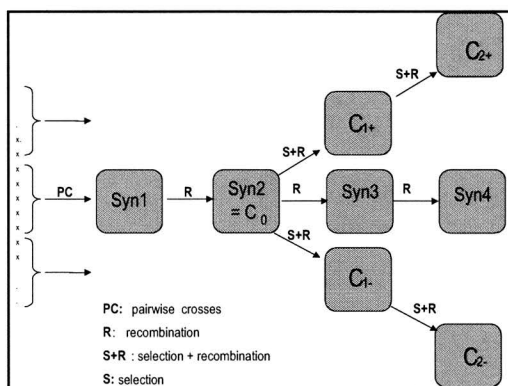
Fig. 1. Selection protocol for production of C2+, C2- and C2= plants.

Seed was sown in August on each occasion and the resulting plants maintained in 9cm pots of Humax John Innes No3 with wetting agent in a frost-free, unlit glasshouse throughout the year. WSC content was analysed the following spring to identify plants for the next round of recombination. Crossing was carried out as polycrosses in pollen-proof glasshouses. Plants were retained and renewed each year from a small sub-set of tillers. WSC analyses were carried out on the full C0, C1 and C2 selection populations of 300 plants, but only on the 30 plants randomly selected to produce the next generation of the unselected population.

WSC was analysed in total herbage from a cut at about 4cm stubble height in early March. The material was oven-dried at $80^{\circ}C$ and WSC extracted following Turner *et al.* (2002). WSC was analysed directly by the anthrone colourimetric assay (Yemm & Willis, 1954) modified for a microtiter plate reader.

DNA was extracted from the plant material with the QIAGEN DNEasy Plant Mini Kit and SSR markers run on an ABI 3100 sequencer as described by Turner *et al.* (2006). Markers with the prefix 'rv' were produced by ViaLactia Biosciences and used under licence.

Association studies were carried out with two software packages, FSTAT (Goudet, 2001) and GENEPOP (Raymond & Rousset, 1995). Analyses included deviation from Hardy-Weinberg equilibrium, linkage disequilibrium and population, allele and genotype differentiation between the various populations.

RESULTS

The material analysed for WSC in this study was predominantly leaf, but did contain some sheath/tiller base material. The parents from the initial set of *Lolium* test genotypes (LTS) used for the C0 population covered the majority of the variation for WSC content within the full set of 20 LTS plants (Table 1). There was significant divergence between the high and low selections after the first round of recombination. Further divergence occurred after the second round of recombination. The non-selected population was intermediate, but more similar to the low selection than the high selection.

Table 1. WSC content of forage from the various generations during phenotypic selection. Significant differences were analysed by ANOVA in GENSTAT.

Population	n	Minimum	Maximum	Mean
LTS Genotypes	20	7.3	20.8	12.7 ± 0.9
LTS Pop. 3 parents	5	8.2	20.8	13.6 ± 1.8
C0	300	2.6	32.9	10.0 ± 0.3
C0 Low Selection parents	30	2.6	4.5	3.6 ± 0.1
C0 High Selection parents	30	15.9	32.9	19.6 ± 0.7
C1- Low	300	1.4	28.7	11.8 ± 0.3
C1+ High	300	2.0	35.7	13.5 ± 0.3 ***
C1- Low Low Selection parents	30	1.4	6.1	4.7 ± 0.2
C1+ High High Selection parents	30	20.0	35.7	24.0 ± 0.8
C2- Low	300	0.3	19.7	6.1 ± 0.4
C2+ High	300	0.3	33.0	14.1 ± 0.4 ***
C2- Low Low Low Selection parents	30	0.3	2.9	2.0 ± 0.1
C2= Rand Rand Rand Selection parents	30	3.2	19.7	4.3 ± 0.8
C2+ High High High Selection parents	30	21.5	33.0	25.0 ± 0.6

Marker studies were carried out on the selected parental populations of 30 plants in all cases. Significant population differentiation that could not be explained by genetic drift occurred in both the high and low selections (Table 2). The C2= unselected population was not significantly different from the C0 or the LTS parents. The C2+ and C2- populations were different from each other and both were also different from the C0 and C2= populations.

Table 2. Population differentiation analysed in FStat.

	LTS	C0	C2=	C2-	C2+
LTS	0	-0.068	-0.043	-0.035	0.049
C0		0	0.005	**0.055	**0.071
C2=			0	*0.048	**0.112
C2-				0	**0.185
C2+					0

Allelic differentiation between the C2+ and C2- populations occurred in markers spread across the whole genome (Table 3).

Table 3. Significant effects in allelic differentiation between the C2+ and C2- populations analysed in Genepop.

Marker	linkage group	P value		Marker	linkage group	P value
rv1391	1	0.00000		rv0190	4	0.00000
rv0659	1	0.00005		rv0250	5	NS
rv0327	1	0.00000		rv0757	5	0.00000
M4-136	2	0.00000		rv0260	5	NS
rv0116	2	0.00000		rv1112	5	0.00063
rv0706	2	0.00000		rv1423	6	0.00000
B1C9	3	0.00000		rv0641	6	0.00000
14ga1	3	0.00000		rv0739	6	0.00000
25ca1	3	0.00023		rv0440	7	NS
rv0454	4	0.00012		rv0264	7	0.00000
LpSSR023	4	0.00000		LpSSR020	7	NS

The six markers with the largest differences in allele frequency between the C2+ and C2-populations were identified and compared to the locations of WSC QTL (Figure 2). Three markers coincided with known major QTL regions and a further marker was on a linkage group with WSC QTL. However two were on linkage groups without known WSC QTL. At all these marker loci the allele which accumulated in the C2+ population was present in the LTS parents which came from the WSC F2 mapping family.
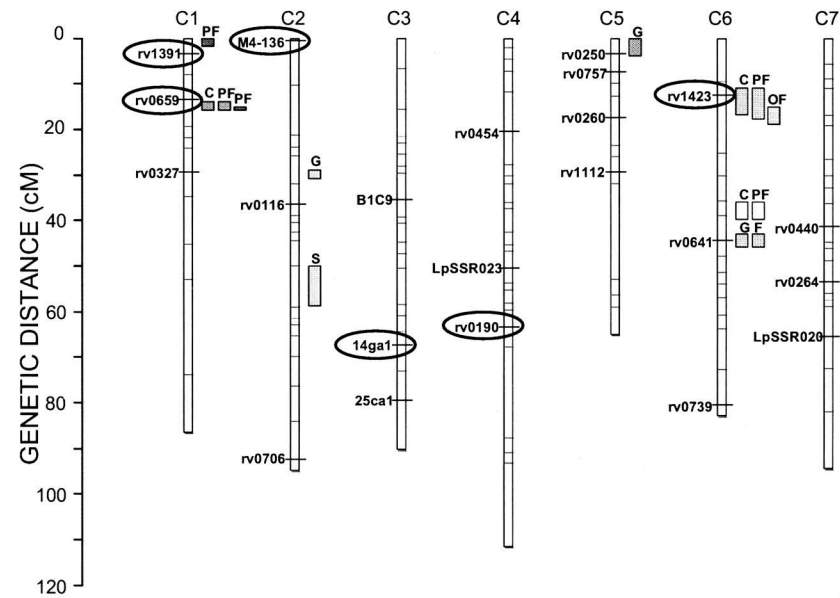
Fig. 2. Location of QTL and the six marker loci (circled) with the greatest differentiation in allele frequency. Bars represent the 2-LOD interval from MQM mapping for leaf tissue in the spring (white background) and autumn (light grey background) and tiller bases in the spring (mid grey background) and autumn (dark grey background). Total WSC, C; polymeric fructan, PF; oligomeric fructan DP>3, OF; sucrose, S; glucose, G; fructose, F.

DISCUSSION

The phenotypic selection carried out in these experiments was successful in creating significant divergence in forage WSC content after three rounds of selection and two rounds of recombination. This was undoubtedly due in part to the wide range of variation available in the initial material and the presence of known high WSC alleles.

The extent of allele differentiation across the whole genome was unexpected but does perhaps illustrate the truly quantitative nature of the WSC trait. Nevertheless the most extensive accumulation of high WSC alleles (from the WSC F2 family) did locate the known major QTL regions. However such widespread changes in allele frequency across the whole genome following a relatively short period of phenotypic selection, if proved to relate to the WSC trait, does have implications for the strategies which should be used during marker selection for the WSC trait. Selection based on single markers may be unlikely to have a major effect on phenotype in the short term. Selection indices based on a number of markers at relevant loci across the genome may be more effective.

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Genetic characterisation of persistence in red clover (*Trifolium pratense* L.)

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ABSTRACT

Red clover cultivars improved for persistence may help maintaining optimal legume proportions in temperate grasslands and thus may substantially contribute to high forage yield and quality over several years. However, persistence is a complex trait, which is difficult to improve. Phenotypic or genetic markers closely linked to genes controlling complex traits may offer additional tools for breeding to complement traditional approaches. The objectives of this study were to optimise the phenotypic evaluation of persistence, to identify QTLs for this important trait and to investigate the association of persistence with other important traits. Plant vigour and various morpho-physiological characters were evaluated during four growing periods in a mapping population consisting of 280 F_1 individuals and QTL analysis was performed based on a genetic linkage map consisting of 42 SSR and 216 AFLP markers. A weighted average of vigour scores assessed over two winters and three growing seasons was identified as the optimal method to phenotype persistence. For this index, one QTL explaining 12.2% of the total phenotypic variation was identified. No negative correlation between persistence and seed yield was observed, but persistence was positively correlated to length of stem, which in turn was positively correlated to seed yield. The phenotypic and genetic markers identified in this study have the potential to assist future breeding efforts for the improvement of persistence in red clover.

Key words: AFLP, marker assisted selection, phenotypic characterisation, plant vigour, QTL, SSR

INTRODUCTION

Red clover (*Trifolium pratense* L.) is an insect pollinated, outcrossing species, particularly valued for its high forage quality, its high yield potential and its ability to fix atmospheric nitrogen (Taylor and Quesenberry, 1996). It is an important component of multi-annual and multi-species swards where persistence is important in order to maintain an adequate legume proportion over time. Targeted utilisation and selection of red clover germplasm has resulted in cultivars considerably improved for persistence, constantly high yielding across several growing seasons (Suter *et al.*, 2006). These cultivars are often based on Swiss Mattenklee germplasm which traces back to Swiss landraces and is characterised by better persistence when compared to foreign field clover cultivars (Kölliker *et al.*, 2003). Despite the considerable success through phenotypic selection, persistence remains a trait which is difficult to improve. In addition to the laborious long-term field experiments required for phenotypic selection, the trait may also be negatively correlated to other important traits such as seed yield (Deneufbourg, 2004). A detailed characterisation of the genetic control and the

development of molecular genetic markers linked to persistence may help to improve this complex trait and to complement traditional breeding approaches through marker assisted selection. Therefore, the aim of this study was to provide a basis for the development of alternative breeding strategies for persistence in red clover by (i) identification of quantitative trait loci (QTL), (ii) clarification of the relationship of persistence and seed yield and (iii) identification of traits associated with persistence which could be used for indirect selection.

MATERIALS AND METODS

An F_1 mapping population segregating for persistence was established based on a reciprocal cross between one plant each of the field clover cultivar Violetta (ILVO-Plant Unit, Melle, Belgium), characterised by poor persistence and high seed yield, and the Mattenklee cultivar Corvus (Agroscope Reckenholz-Tänikon ART, Zurich, Switzerland), characterised by excellent persistence but rather low seed yield. Plant vigour was continuously evaluated during four growing periods (2003 - 2006) in a replicated field experiment consisting of 280 F_1 progeny and four clonal replicates per genotype. Vigour was scored at 16 time points using a scale from 1 (poor vigour) to 9 (very vigorous). Persistence was calculated based on weighted vigour scores (WV, Formula 1, Table 1) where V_n is the average vigour score at time point n and t_n is the number of days of the growing season up to time point n.

Formula 1.
$$WV = \frac{(V_1 t_1 + V_2 t_2 + V_3 t_3 + ...)}{(t_1 + t_2 + t_3 + ...)}$$

In addition, persistence was also calculated by counting the number of days of the growing season until the vigour of a plant dropped to a score of 3 and failed to recover (time of survival, TSf05, Table 1). Analyses of variance (ANOVA) were performed using the general linear model (GLM) of the SAS statistical package (version 8.1, SAS Institute, Cary, N.C., USA) considering the factor genotype as random. Heritability was calculated using Formula 2, where $\sigma^2_{g(mp)}$ represents the variance component of the genotype nested within the maternal plant, σ^2_e represents the variance component of the error term and r represents the number of replicates.

Formula 2.
$$h^2 = \sigma^2_{g(mp)}/(\sigma^2_{g(mp)} + \sigma^2_e/r)$$

Correlation of persistence to seed yield and length of stem was calculated based on results from a previous study (Herrmann *et al.*, 2006) using Pearson's product moment correlation coefficient. QTL analysis was performed as described by Herrmann *et al.* (2006) based on a genetic linkage map consisting of 42 SSR and 216 AFLP markers.

RESULTS

Analyses of variance revealed highly significant variation among the 280 F1 genotypes for all five persistence indices (Table 1). No significant effect of the maternal plant was observed, i.e. there was no difference whether seed was harvested from one or the other maternal plant (data not shown). Heritability ranged from 0.28 for the time of survival (TSfa05) to 0.64 for the weighted vigour scores WVfa04 (Table 1). Product moment correlation coefficients revealed significant positive correlations among the different persistence indices, with coefficients ranging from r = 0.698 for the WVsu05/TSfa05 comparison to r = 0.925 for the comparison of WVsu05 and WVsp06 (Figure 1). There was also as a significant positive correlation of persistence indices to the non-weighted vigour scores (Herrmann *et al.*, 2008).

In addition, a slightly positive, significant correlation between persistence indices and seed yield per plant was observed (Figure 1).

Table 1. Description, F-values, significance of genotype effects and heritability for five persistence indices evaluated in a replicated field experiment using 280 red clover F_1 genotypes.

Index	Description	F-value Genotype	h^2
WVfa04	Weighted vigour until fall 04	2.7^{***}	0.64
WVsp05	Weighted vigour until spring 05	1.9^{***}	0.48
WVsu05	Weighted vigour until summer 05	1.7^{***}	0.44
WVsp06	Weighted vigour until spring 06	1.9^{***}	0.49
TSfa05	Time of survival until fall 05	1.4^{***}	0.28

*** $P<0.001$

A total of 9 QTLs were identified for the five different persistence indices with a LOD score higher than the LOD threshold (Table 2). These QTLs explained 6.1% to 12.2% of the phenotypic variation observed. While all QTLs were observed for at least two different indices, one QTL on LG3 and one on LG7 were only observed for WVfa04.

Table 2. Position and description of QTLs for five persistence indices identified using multiple QTL mapping and least square means of four replications per genotype.

Persistence Index	LG	Position (cM)	Max. LOD	explained variance	(total)
WVfa04	3	9.2	5.35	7.7%	
	3	60.8	4.43	7.2%	
	4	61.4	4.57	6.8%	
	7	7.6	4.65	6.5%	(28.2%)
WVsp05	3	9.2	6.89	11.3%	
	4	61.3	3.82	6.1%	(17.4%)
WVsu05	3	9.9	6.63	12.2%	(12.2%)
WVsp06	3	9.9	4.88	8.9%	(8.9%)
TSfa05	3	9.9	5.19	9.4%	(9.4%)

The QTL on LG 3 was consistently observed for all persistence indices and explained up to 12.2% of the phenotypic variation for persistence.
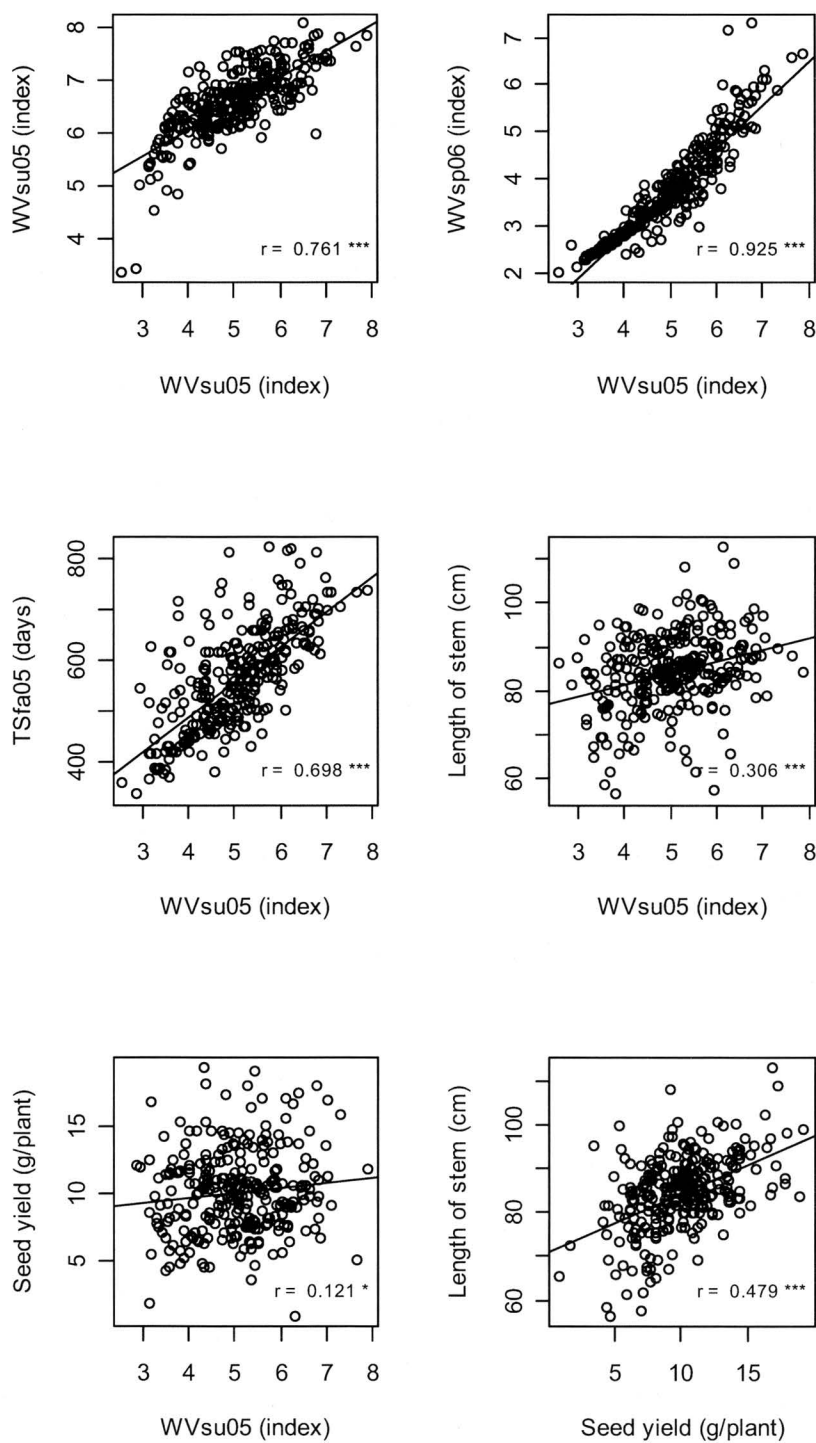
Fig. 1. Product moment correlation coefficients for persistence indices, seed yield per plant and length of stem of a red clover population consisting of 280 F₁ genotypes based on least square means obtained from four replicates per genotype.

DISCUSSION

The five persistence indices developed in this study allowed for a reliable assessment of persistence in red clover and yielded heritabilities comparable to those found for winter hardiness in ryegrass (Yamada *et al.*, 2004). Although the five measures showed significant positive correlations, the number, location and magnitude of QTLs differed for each individual trait (Table 1). In general, the number of QTLs detected decreased with increasing duration of the phenotypic evaluation. This could be partly due to factors not directly related to persistence such as plant establishment and tolerance to clonal propagation which could strongly influence plant vigour during the first growing season. Based on the fact that the number of QTLs detected remained stable after the vigour ratings of summer 2005, the index taking into account 12 vigour scores of three growing seasons (WVsu05) was considered optimal for characterising persistence in red clover.

The slightly positive, significant correlation between persistence and seed yield per plant observed in this study is in clear disagreement with previous hypotheses of a negative correlation between seed yield and persistence (Deneufbourg, 2004) and supports the findings of Taylor *et al.* (1962) who could not detect any correlation between seed yield and persistence based on phenotypic investigations. Our findings based on phenotypic observations were supported by molecular genetic analyses which revealed QTLs for seed yield and length of stem to be located close to the QTLs for persistence identified on LG 3 and 4 (Herrmann *et al.*, 2006; Herrmann *et al.*, 2008). Since a positive correlation between seed yield and length of stem was previously observed in Mattenklee (Deneufbourg, 2004) and soybean (Mansur *et al.*, 1996), this character could serve as an indirect trait primarily for the selection of seed yield, but may also be useful for the improvement of persistence due to the positive correlation observed in this study.

In conclusion, the identified genome region rich in QTLs for persistence represents a candidate region for further characterisation and for the development of tools for marker assisted improvement of persistence. In addition, our results indicate that improvement of persistence may be possible without adverse effects on seed yield.

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Mapping of *LmPc*, a major dominant gene from *Lolium multiflorum* conferring resistance to crown rust

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ABSTRACT

Crown rust caused by the biotrophic fungus *Puccinia coronata* f. sp. *lolii* is known as one of the most serious foliar diseases in Italian ryegrass. We were able to genetically identify a single dominant resistance gene for crown rust originating from the Italian ryegrass variety 'Fastyl' using a detached-leaf test. In total, 274 BC1 individuals could be defined with respect to their genetic constitution at the resistance gene locus. We have named this locus *LmPc* for *Lolium multiflorum* resistance towards *Puccinia coronata*. Bulked segregant analysis allowed us to identify linkage between *LmPc* and the microsatellite marker *M4136*, which has previously been mapped on LG2 in *Lolium*. A comparative-genomic approach based on DNA-sequence information of publicly available *Lolium*- and *Festuca*-ESTs and the rice genome data resulted in a set of novel STS-markers for *Lolium*. The probability of recombination between two flanking markers, which cover a genetic distance of 14.7 cM, and *LmPc* is only 0.0047, if both markers are taken together as a single selection criterion. Results obtained so far reveal a degree of co-linearity between the *LmPc* genomic region on LG2 and rice chromosome R4 at the genetic map level, which will allow to narrow *LmPc* systematically down with molecular markers.

Key words: comparative mapping, Italian ryegrass, *Puccinia coronata*, STS marker

INTRODUCTION

Crown rust, caused by *Puccinia coronata* f. sp. *lolii* is one of the most serious foliar fungal diseases in ryegrass (*Lolium* spp.) species. This disease results in decreased herbage yield and loss of palatability for grass-feeding domestic animals. Thus, the improvement of ryegrass varieties with both quantitatively (Hayward, 1977; Reheul & Ghesquiere, 1996) and qualitatively (McVeigh, 1975; Lellbach, 1999) inherited crown rust resistance (R-) genes is a major breeding aim. With respect to an efficient improvement of varieties with R-genes, molecular genetic markers represent indispensable breeding tools, both through the ability to perform selection at the seedling stage, as well as a tool which enables pyramidization of multiple resistance genes (Witcombe & Hash, 2000). In recent years, progress has been achieved in mapping crown rust resistance genes in perennial (Thorogood *et al.*, 2001; Dumsday *et al.*, 2003; Muylle *et al.*, 2005; Schejbel *et al.*, 2007) as well as Italian ryegrass (Fujimori *et al.*, 2003; Sim *et al.*, 2007; Studer *et al.*, 2007). All but one of these yet described crown rust R-genes in *Lolium* are quantitatively inherited, and QTLs explaining a significant part of the phenotypic variation have been mapped on LG1, 2, 3, 4, 5 and 7, respectively. A major crown rust resistance gene originating from the breeding line 'Yamaiku 130' has been

mapped using AFLP markers (Fujimori *et al.*, 2003). However, the chromosomal localization of this gene has not been determined.

The objective of our research was (i) to exploit the genetic basis of crown rust resistance observed in the *L. multiflorum* cultivar 'Fastyl', (ii) to determine the chromosomal localization of the R-gene(s) and (iii) to develop molecular markers suitable for marker-assisted selection purposes.

MATERIALS AND METHODS

Inbred lines, kindly provided by the Deutsche Saatveredelung, Thüle, were established from the *L. multiflorum* cultivar 'Fastyl' by pseudo-compatible selfing using high temperature during anthesis (Wricke, 1978). The F1 family *BAZ-3104* was established by crossing a susceptible genotype from the *L. perenne* cultivar 'Aurora' with individual genotypes from the resistant $I_{0,1}$-line *BAZ-2962*. The segregating families *BAZ-3213, BAZ-3216, BAZ-3218 and BAZ-3219* were established by backcrossing resistant F1 progeny to the susceptible parents. To assess the resistance level among the segragating progeny a detached-leaf test was carried out as described before (Lellbach, 1994). Controlled infections were performed using a uredospore mixture of *Puccinia coronata* f. sp. *lolii* from several European origins (standard mixture from the Biologische Bundesanstalt Braunschweig) as inoculum. The leaf segments were scored on a 1-3 scale, where "1" is highly resistant without visible pustules, "2" is partially resistant with small pustules sourrounded by chlorotic tissue and "3" is highly susceptible with abundant medium to large pustules and sporulation. Each individual was tested two times with 2 leaf segments per test. A random sample of resistant and susceptible BC1 genotypes were further backcrossed to the susceptible parent and detached leaf tests perfomed as described using 21 individuals of each progeny.

Genomic DNA of each 10 resistant and 10 susceptible BC1 individuals was pooled and used for a bulk segregant analysis (Michelmore *et al.*, 1991) with genomic *Lolium* SSR markers (Kubik *et al.*, 2001; Jones *et al.*, 2005), the majority of them kindly provided by Dr. J.W. Forster, CRC for Molecular Plant Breeding, Australia. SSR were assayed on automatic sequencers as described by Hackauf and Wehling (2002). Development, application and mapping of novel *Lolium* STS markers were performed using publicly accessible *Lolium* and *Festuca* ESTs as well as the rice genome data according to a recently described approach (Hackauf and Wehling, 2005). Linkage analysis was performed using the BC1 algorithm in Joinmap 3.0 (Van Ooijen and Voorrips, 2001). The Kosambi function was used to convert recombination values to genetic distances (cM). The recombination frequency between two flanking markers and a gene under selection was calculated as described (Weber and Wricke, 1994).

RESULTS

A F1 progeny of a cross between inbreds originating from the variety 'Fastyl' and susceptible *L. perenne* genotypes proved to be homogeneously resistant to crown rust. A total of 277 plants from four BC1 families were used for genetic analysis of crown rust resistance. Test conditions allowed an almost complete differentiation into the two classes 1 (resistant) and 3 (susceptible), respectively (Table 1). Only 3 out of 277 (1.08%) tested progeny were scored as partially resistant. Thus, the observed segregation ratios correspond to a monogenically acting dominant resistance gene, which was confirmed by progeny tests of 95 BC2 families (not shown). We have named this locus *LmPc* for *Lolium multiflorum* resistance towards *Puccinia coronata*. Excluding the partial resistant individuals, 274 defined *LmPc* genotypes were used for linkage analysis with molecular markers.

Table 1. Segregation of resistant and susceptible genotypes in 4 BC1 progenies.

BC1	Score			N	$\chi^2_{1:1}$
	1	2	3		
BAZ-3213	37	3	34	74	0,48
BAZ-3216	32	0	32	64	0,00
BAZ-3218	41	0	31	72	1,39
BAZ-3219	32	0	35	67	0,16

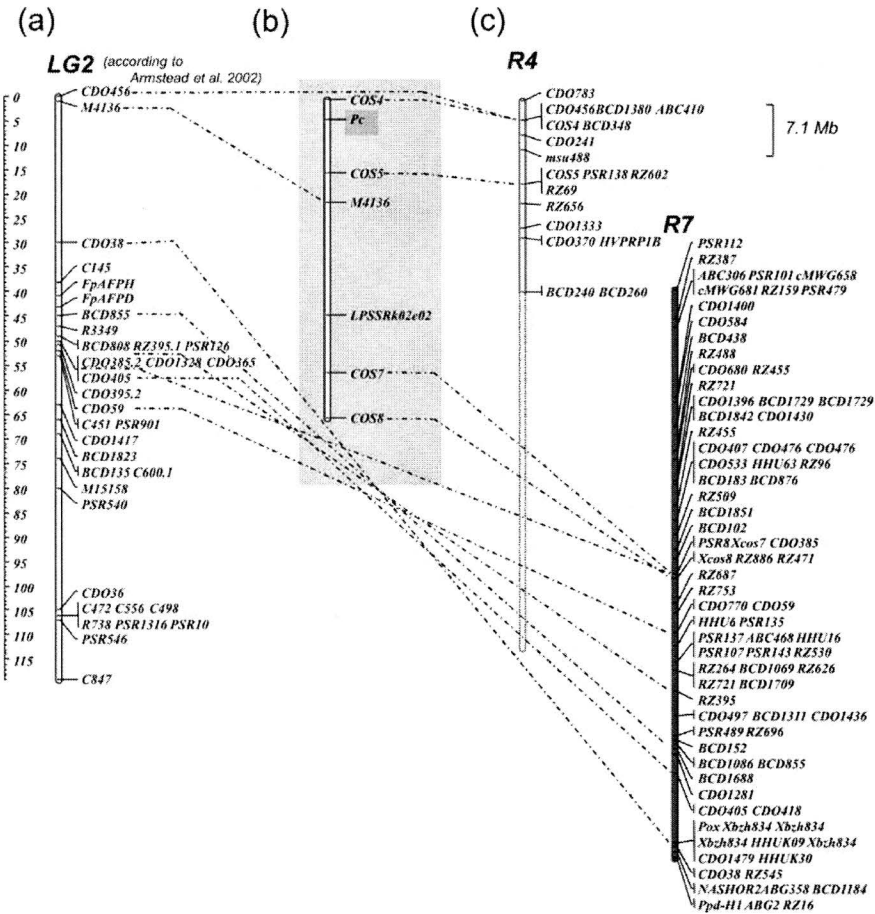Fig. 1. Comparative mapping of the crown rust resistance gene LmPc. (a) Integrated map of Lolium LG2 according to Armstead et al. (2002). (b) Linkage map of LmPc integrating the data from four mapping populations (c) In silico map of grass anchor markers on rice chromosomes R4 (clipped) and R7, respectively. The orthology of genomic regions on LG2 in Lolium and rice chromosomes R4 and R7 is indicated by dotted lines.

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Bulked segregant analysis was applied to identify SSR markers linked to *LmPc*. This approach revealed linkage of *LmPc* and the microsatellite marker *M4136* (Figure 1b), the latter of which has been mapped most distally on LG2 in *L. perenne* (Figure 1a). The SSR anchor marker *LPSSRk02e02* could be mapped proximally from *M4136* and confirms the localization of *LmPc* on LG2.

DNA sequence information of cDNA anchor probes mapping to LG2 enabled us to identify orthologous regions on rice chromosomes R4 and R7 (Figure 1c). Using the rice genome data as a blueprint, 20 *Lolium* as well as *Festuca* ESTs with similarity to rice genes on R4 and R7 have been selected for the targeted development of novel STS markers in *Lolium*. Among the 17 (85%) successfully established primer pairs, a CAPS polymorphism could be observed for 15 (88.2%) STS. These novel *Lolium* STS allowed us to narrow *LmPc* down to the distal part of LG2 (Figure 1b). The marker loci *COS4* and *COS5* could be mapped 3.6 cM distally and 11.3 cM proximally from *LmPc*. Thus, the probability of recombination between two flanking markers and *LmPc* is only 0.0047, if both markers are taken together as a single selection criterion. The rice genes used to establish *COS4* and *COS5* are located 7.1 Mb away of each other on chromosome R4 (Figure 1c).

DISCUSSION

We were able to genetically identify a single dominant resistance gene, *LmPc*, originating from the Italian ryegrass variety 'Fastyl'. A leaf segment test was applied as a rapid and reliable method for disease evaluation. QTL mappings of crown rust resistance in oat revealed a discrepancy between greenhouse and field evaluations (Zhu *et al.*, 2003a,b; Portyanko *et al.*, 2005). In contrast, resistance towards leaf rust in rye was effective in both detached-leaf tests carried out on seedlings and in field tests of adult plants (Wehling *et al.*, 2003; Roux *et al.*, 2004). A recently published study on QTLs for crown rust resistance in Italian ryegrass also reported a high correlation between disease scores obtained under controlled conditions with scores from multisite field assessment (Studer *et al.*, 2007). Thus, the leaf segment test has proven to be a suitable method to assess crown rust resistance in *L. multiflorum*.

Previously, a major crown rust resistance gene has been reported in the Italian ryegrass breeding line 'Yamaiku 130' (Fujimori *et al.*, 2003). While closely linked AFLP markers have been developed, the chromosomal position of this R-gene remains unknown. Application of genomic *Lolium* SSR markers allowed us to map *LmPc* to the distal part of LG2. Several quantitative trait loci conferring resistance towards crown rust in perennial and Italian rye grass have recently been identified on LG2 (Thorogood *et al.*, 2001, Dumsday *et al.*, 2003; Muylle *et al.*, 2005; Sim *et al.*, 2007; Studer *et al.*, 2007). The positions of these QTLs on this particular linkage group as determined by their linked markers *Xlpssrk02e02* (Dumsday *et al.*, 2003), *Xcdo385* (Muylle *et al.*, 2005) and *Xbcd1184* (Sim *et al.*, 2007) reveal, that the major resistance gene *LmPc* does not correspond to most of these QTLs. *Xlpssrk02e02* could be mapped 40.3 cM apart from *LmPc* and thus clearly allows to separate both genomic regions on LG2. This applies also for the marker *Xcdo385*, which could be mapped *in silico* on rice chromosome R7 and which served as a reference to establish the STS markers *COS7* and *COS8*, the latter of which mapping 52.2 cM and 61.6 cM proximal from *LmPc*. The map position of *Xbcd1184* on LG2 (Sim *et al.*, 2005) as well as the localization of a bcd1184-orthologous rice gene on the distal part of chromosome R7 largely excludes the QTL identified by Sims and co-workers as identical with *LmPc*. In contrast to these studies, Thorogood and colleagues (2001) identified one QTL in perennial ryegrass linked to the SSR marker *M4136*. Similarly, Studer and co-workers (2007) reported on a QTL in Italian ryegrass which explained up to 35% of the phenotypic variance and which is linked to an AFLP marker most distantly on LG2. The relative positions on LG2 suggests, that *LmPc* described

in our study corresponds to the QTLs identified by Thorogood *et al.* (2001) and Studer *et al.* (2007), respectively.

A comparative genomics approach based on functional, i.e., expressed *Lolium*- and *Festuca*-DNA sequences and the conserved genetics across the grasses allowed us to narrow the *LmPc*-genomic region on LG2 down and to establish reliable, sequence-specific and flanking markers making *LmPc1* amenable for marker assisted selection strategies. Results obtained so far reveal a degree of co-linearity between the *LmPc* genomic region on LG2 and R4 at the genetic map level, which will allow to narrow *LmPc* systematically down with additional molecular markers.

In conclusion, our results point out a reliable strategy to exploit *Lolium* germplasms with respect to R-genes conferring crown rust resistance. The genomic resources available for *Lolium* as well as the rice genome data provide valuable resources for the development of molecular markers as tools, which will efficiently allow to improve varieties with R-genes in practical ryegrass breeding.

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Genetic analysis of seed yield components

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ABSTRACT

The potential tradeoffs between vegetative and reproductive growth is a constant challenge for the forage plant breeders. Breeding for seed production has inevitably played a secondary role compared to improvements of the vegetative production. In this paper the current status regarding genetic variation, genotype x environment interactions, heritability estimates and mapping of quantitative trait loci (QTL) for seed yield and seed yield components in grasses and legumes are reviewed, with special focus on important forage grasses. Investigations of seed yield components have shown that components contributing to an increased utilization of the reproductive potential, like seed set and seed retention, seems efficient in increasing seed yield without adverse effects on the vegetative production. Mapping of QTLs, identification of markers and candidate genes associated with seed yield components, and the utilization of comparative genomics with cereal species have revealed several key components which may facilitate development of markers for marker-assisted breeding for the improvement of seed yield.

Key words: seed yield components, genetic variation, genotype x environment interaction, indirect selection, quantitative trait loci (QTL), comparative genomics

INTRODUCTION

Improvement of vegetative traits like leafiness, tillering capacity and persistency of forage species may lower their ability to produce seeds. Thus the most important characteristics of forage plants from a farmer's perspective can be a problem for the seed producer. However, outstanding cultivars will hardly be commercialized if the seed production is not satisfactory. This constraint and the potential tradeoffs between vegetative and reproductive growth is a constant challenge for the forage plant breeders, and breeding for seed production has inevitably played a secondary role. The lack of deliberate breeding for seed productivity may also be due to the opinion that seed and forage production are negatively correlated (Bugge, 1987). However, it has been quoted that this negative correlation probably can be changed by selection. Andersen (1981) reported a modest negative correlation between seed yield and dry matter yield or persistency, based on a survey of a number of commercial cultivars, mainly perennial ryegrasses (*Lolium perenne* L.). He concluded that it should be possible to combine high seed yield with high dry matter production in grasses. Griffiths (1965) pointed out that overall seed yield was not correlated with forage production in perennial ryegrass. Elgersma (1990a) concluded that high seed-yielding capacity and high dry-matter yield were not mutually exclusive. Recent studies of selection for seed yield in perennial ryegrass support these conclusions (Marshall & Wilkins, 2003).

SEED YIELD AND SEED YIELD COMPONENTS

Seed yield is a highly complex trait which is influenced both by numerous interacting genetical, physiological and environmental factors. The seed production capacity varies with species and with type of cultivar, e.g. turf and forage cultivars. In grasses, seed yield can be divided into components like the numbers of fertile tillers, spikelets per panicle and florets per spikelet, which determine the seed yield potential, and fertility and 1000-seed weight, which determine the utilization of the seed yield potential (Bean, 1972). Utilization of the yield potential is thus heavily dependent on successful pollination, fertilization and seed growth, which is influenced by physiological and genetical factors like assimilate allocation, source-sink competition, self-incompatibility and pollen production. In addition, seed yield is influenced directly or indirectly by a number of agronomic traits such as plant height, leaf area, dry-matter yield, heading date, lodging resistance and proneness to seed shattering (Griffiths, 1965).

GENETIC VARIATION AND SELECTION FOR SEED YIELD AND SEED YIELD COMPONENTS

Many studies of seed yield and its component traits have demonstrated large genetic variation and high heritability for these traits, and these estimates are often larger than for vegetative production traits. The larger genetic variation for seed production traits is usually attributed to the lower selection pressure than for vegetative production during breeding. In a study of high-latitude populations of timothy, Rognli (1987) estimated broad sense heritabilites of seed yield, date of ear emergence and plant height to 0.64, 0.86 and 0.80, respectively with corresponding genotypic coefficients of variation (GCV%) as 24.8, 13.2 and 5.8. Elgersma (1990b) found that narrow-sense heritabilites, estimated from parent-offspring regressions, were highest for earliness, flag leaf width, ear length and the number of spikelets per ear in perennial ryegrass. Fang *et al.* (2004) studied phenotypic and genotypic variation for seed yield and related traits in a full-sib family of meadow fescue (*Festuca pratensis* Huds.) grown at two locations in Norway. Their estimates of broad sense heritabilities (H_B^2) for the traits were high (0.50-0.80), and highest (0.80) for seed yield per plant. Seed yield per plant and reproductive components like seed weight per panicle and fertility exhibited the largest genotypic coefficients of variation (GCV %), being around 34%. Fang *et al.* (2004) also conducted a path coefficient analysis of seed production components in meadow fescue. The path analysis showed that fertility (measured as seed weight/panicle divided by panicle length) was the most important component trait contributing to seed yield with a direct effect of 0.60, while number of fertile tillers, plant height and flag-leaf width were also important with direct effects around 0.25. Variation in 1000-seed weight had little influence on seed yield per plant. Since panicle fertility is highly correlated with seed weight per panicle, this component trait could be used instead since it is easier to handle in selection programmes for seed production. The path analysis also demonstrated that flag-leaf width (FLW) had an important direct effect on seed yield and indirectly through panicle fertility (PF). This indicates that large flag leaves contribute to a good seed-set (panicle fertility) through assimilate reallocation via the stems to the inflorescence in the period of anthesis, and that this contributes to higher seed yields. The importance of the flag-leaf for grain yield in cereals is well-known, recently demonstrated by Quarrie *et al.* (2006) in wheat, and it is not surprising that this is the case also in grasses. In perennial grasses the competition for assimilates is probably stronger than in annual cereal crops since the seeds have to compete with other sinks, i.e. actively growing organs like roots and new vegetative tillers, for assimilates. Indeed, studies in herbage grasses have shown that the number of florets that are produced is very large, but that a high percentage is aborted probably due to lack of fertilization and/or

competition for nutrients and assimilates (Elgersma, 1990a). The path analysis also showed that plant height (PH) had an indirect positive effect on seed yield via panicle fertility, and this effect was nearly as large as the direct effect. This might be explained by the fact that taller plants will have a better chance of capturing pollen, which will increase the proportion of florets being successfully fertilized.

The major importance of fertility and seed set has been confirmed in several studies. Elgersma (1990a) found that variation in seed yield was more related to variation in seed number than to variation in seed weight in a study of nine diploid late-flowering perennial ryegrass cultivars. Elgersma *et al.* (1994) found that the number of spikelets per ear was negatively correlated with seed yield. Marshall & Wilkins (2003) conducted two generations of recurrent phenotypic selection for seed yield per plant under controlled pollination in the perennial ryegrass cultivar AberDart. Selected and unselected varieties (AberDart and AberElan, respectively) and control varieties were grown for seed in pots in a glasshouse experiment and in two field plot experiments over 5 harvest years. Selection gave significant improvements in seed yield both in green house and in field plots and the increased seed yield of AberDart was attributed to a higher seed set, greater seed number per tiller and more reproductive tillers per plant.

It can be concluded that seed component studies have demonstrated that after the establishment of a sufficient number of fertile tillers, panicle fertility is the most important determinant of seed yield. It should therefore be possible to breed for an increased efficiency of the reproductive system rather than an increased size of the reproductive system, without negative effects on the forage production.

GENOTYPE X ENVIRONMENT INTERACTIONS

A major problem in improving seed yield by breeding is environmental interactions, i.e. inconsistent correlations between estimates obtained in spaced plantings and in dense stands. Selection among spaced plants, either by a component of seed yield or seed yield directly, constitutes a form of indirect selection. A prerequisite for successful indirect selection is a high genetic correlation between yield on spaced plants and yield in plots, i.e. absence of genotype x environment interactions. Elgersma (1990c) studied seed yield and seed yield components using spaced plants and drilled plots of nine perennial ryegrass cultivars, and found that spaced-plant traits in general showed poor correlation to corresponding traits in drilled plots. Spaced-plant traits in two perennial ryegrass cultivars were assessed using clones and their open-pollinated progenies in four environments by Elgersma *et al.* (1994). The results indicated that spaced-plant data was of limited value in predicting seed production. They concluded therefore that direct selection for seed yield in drills of progenies in later stages of the breeding programme was the best method for obtaining varieties with sufficient seed production. I think this reflects the current opinion and practice among forage grass breeders.

On the other hand, in lucerne, Annicchiarico (2006) reported that indirect selection for seed yield based on spaced plants was only 19% less efficient than direct selection in dense stands and the heritability was higher on spaced plants. However, indirect selection for seed yield components was not efficient. As pointed out by Marshall & Wilkins (2003), the poor correspondence between seed set of individual spaced plants and genetically related plants grown as drilled plots observed in perennial ryegrass by Bugge (1987) and Elgersma (1990c), could well be attributed to the lack of pollination control in field spaced plants. These plants are more likely to receive pollen from genetically unrelated plants than plants grown closely together in drilled plots. Perennial ryegrass (and many other grass species) is highly self-incompatible, and the male parent is as important as the female parent in determining whether a particular floret sets a seed. Marshall & Wilkins (2003) argue that phenotypic selection for

improved seed set can be effective provided that pollination is closely controlled. In addition, lodging and insufficient pollination leading to abortion and low seed set is much more of a problem in dense stands, and this lowers the heritability and makes correlations between spaced plants and dense stands unreliable. The effect of lodging seems to differ among species. Griffith (2000) found that lodging depressed seed yield much more in perennial ryegrass than in tall fescue.

QUANTITATIVE TRAIT LOCI (QTL) MAPPING ANDCOMPARATIVE STUDIES WITH CEREALS

QTLs for seed yield and seed yield components have been mapped both in forage grasses and legumes. Fang (2003) found a total number of 34 chromosomal regions containing QTLs for seed yield and component traits in meadow fescue. QTLs for a number of related traits clustered in a few chromosomal segments, most evident on linkage groups 1F, 4F and 5F. This indicates that there must be one or a few major gene(s) in these regions that affect reproductive development with pleiotropic effects on many traits. Concurrent QTLs for panicle fertility and seed yield were detected on chromosomes 1F, 2F, 4F and 6F, and these should be interesting for the future development of molecular markers for improved seed yield. Comparisons of the QTL positions with positions of QTLs of identical or similar traits in other grass (cereal) species, using common anchor markers, identified a number of putatively orthologous QTLs. Mapping of an orthologue of the wheat vernalization gene *Vrn1* in perennial ryegrass (Jensen *et al.*, 2005) and in meadow fescue (Ergon *et al.*, 2006) and their association with vernalization and seed yield related traits demonstrate conservation across grass species and the value of comparative genomics approaches. In red clover Herrmann *et al.* (2006) identified 38 QTLs for eight seed yield components. QTLs for several traits were often detected in the same genome region. Two genome regions contained several QTLs for different seed yield components and represent candidate regions for further characterisation of QTLs. These studies have revealed several key components which may facilitate development of markers for marker-assisted breeding for the improvement of seed yield. Recent studies in rice have revealed molecular mechanisms involved in determining seed yield components like single-spikelet weight/grain filling (Obara *et al.*, 2004), grain width and weight (Song *et al.*, 2007), and seed shattering (Konisihi *et al.*, 2006). These results might be utilized by comparative genomic approaches to improve seed yield components in grasses in the future.

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SNP haplotypes at candidate gene loci associated with frost tolerance in *Lolium perenne* L.

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ABSTRACT

The Lolium Test Set (LTS) of the EU project GRASP consisting of 20 diverse *Lolium* genotypes was used to isolate allele sequences and single nucleotide polymorphisms (SNPs) for candidate genes associated with cold acclimation and frost tolerance. Two generations of divergent selections for high- and low freezing tolerance were established from a synthetic population constructed by inter-crossing five LTS genotypes. Genetic drift was monitored by comparing allele frequency shifts against shifts in a parallel random mating population. Haplotypes were identified using haplotype specific sequencing, and high throughput SNP genotyping of experimental populations performed using the MassArray platform. For one of the candidate genes, fructan 6-fructosyl transferase (*6ft*) gene, SNP genotyping defined five haplotypes (alleles) but only two parental haplotypes were observed in the divergent selections. One of the haplotype was fixed in population selected for high frost tolerance and selection may be the driving force behind the observed allele frequency shift. Results from the analyses of two candidate genes will be presented. Our results implies that the strategy used in this study, i.e. defining SNP haplotypes and studying their allele frequency changes under selection for desired agricultural traits, is a promising strategy for identifying functional markers for plant breeders.

Key words: allele-frequency, cold acclimation, fructan-6-fructosyl transferase (*6ft*), functional markers, genotyping, MassArray, population

INTRODUCTION

Growth at periods with low, non-freezing temperatures, coupled with shorter photoperiods induces an adaptive response known as cold acclimation or hardening (Crosatti *et al.*, 1999). Upon exposure to cold acclimation, plants exhibit many physiological and metabolic changes resulting primarily from changes in gene expression (Tremblay *et al.*, 2005). Many of these genes encode proteins such as enzymes involved in respiration and metabolism of carbohydrates, lipids, phenylpropanoids and antioxidants. In addition, molecular chaperones, antifreeze proteins, and others with a presumed function in tolerance to the dehydration are expressed upon freezing (Chinnusamy *et al.*, 2003). These changes in gene expression help plants to increase their freezing tolerance, thus enabling them to survive the winter. In addition to the genes that are involved in metabolic changes and general protection of the cells during cold stress, genes encoding protein factors that regulate signal transduction pathways and gene expression in response to cold stress are induced as well.

Current evidence suggests that cold-acclimation is mediated by several parallel signal transduction pathways, whose activation leads to induction of low temperature specific gene

expression. These pathways are mainly divided into two groups, depending on the presence or absence of the phytohormone abscisic acid (ABA), and are divided into "ABA-dependent" or "ABA-independent" pathways (Thomashow, 1999). Recent evidence, however, has shown interaction and cross links between the pathways (Yamaguchi-Shinozaki and Shinozaki, 2006).

The candidate genes investigated in this project (GRASP) were selected from two sources, from differently expressed ESTs (from *Festuca pratensis* Huds.), acquired through suppression subtractive hybridisation (SSH), and from genes known to be involved in cold acclimation and/or freezing tolerance in other grass species by sequence homology (NCBI BLAST). Actual candidate genes were subsequently picked on the basis of differential expression detected by cDNA microarray analysis (Opseth, 2004; Rudi *et al.* manuscript in prep).

SNP (Single Nucleotide Polymorphism) is a polymorphism that involves one single base change in a given DNA sequence defining alleles. SNPs located in coding regions of genes, coding SNPs (cSNPs), are of particular interest since they might alter trait variation. Many SNPs are silent point mutations and do not lead to new gene products. SNPs function also as genetic markers and offer several key advantages over conventional genetic markers. Firstly, they are highly abundant and account for more than 90% of sequence difference in most organisms. Secondly, they are also predominantly phenotypically neutral, and they exist mostly in two co-dominant variants (Törjék *et al.*, 2003). In the GRASP project a *Lolium* test set of 20 diverse genotypes was used to isolate allele sequences and SNPs for candidate genes associated with cold acclimation and frost tolerance. Two generations of divergent selection for high- and low freezing tolerance were established from a synthetic population constructed by inter-crossing of five LTS genotypes. The objective of this research was to study SNP haplotypes in candidate genes and their allele frequency changes after divergent selections of frost.

MATERIALS AND METHODS

SNP detection by PCR and sequencing
SNP detection was done in collaboration with CIGENE (www.cigene.no) using their facilities at Norwegian University of Life Sciences (UMB). All PCR reactions were set up in 96 well plates, and primers were designed to yield 1000-1500 bp amplicons of the candidate genes. After designing and selecting PCR primers that fulfilled this requirement, a PCR reaction was then performed on a set of diverse individuals, in this case the 20 different LTS genotypes. The resulting PCR products were then purified and sequenced directly in both directions to ensure maximum reliability of the results. Sequence data were collected using fluorescent dye-terminator chemistry by a fully automated ABI 3730 sequencer (Applied Biosystems, USA). The traces were compiled into contigs and transferred to the automated SNP detection software pipeline developed at CIGENE (B. Hayes, pers. comm.). This software distinguishes true polymorphisms from sequencing errors, and consists of Phred/Phrap/Consed and polybayes.

Identification of mutation/variation
The Phred base call confidence score is primarily used to identify high probability for heterozygotes. The variations are then mapped back to the original sequence and the variation together with the flanking sequence is used to design assays for the SNP genotyping pipeline.

Data cleaning
SNP data from MassArray were put through a pipeline of stringent data cleaning procedures. First all SNP loci with more than 10% of missing data were discarded from the analysis. The

second step was designed to minimize the genotyping errors in the data set, as genotyping errors inflate the number of falsely inferred haplotypes. Using the four gamete test, we identified all novel genotypes that had arisen by recombination event. Because the probability of observing an intragenic recombination event between two generations is immensely low we discarded novel recombinant genotypes as genotyping errors. The third step was to infer population haplotypes again using the ELB algorithm in Arlequin software (v3.1) and to do a second screen for probable false haplotypes. Haplotypes were scored as "false" and subsequently taken out of the dataset if: I) haplotypes had very low frequency (≤ 0.01) or II) haplotypes were not observed in homozygous state either in parental genotypes or un-selected population.

RESULTS

SNP allele frequency changes in divergent selections for frost
SNP genotyping using the MassArray approach was performed on the selected and unselected plants and allele frequency changes were determined for two candidate genes, i.e. *6ft* and *PhyC*.

C_{1+} is fixed for the *6ft* haplotype 1 (Figure 1). Exact test for population differentiation were significant for differentiation between the monomorphic C_{1+} and the polymorphic C_{1-} and Syn3, but no significant differentiation was observed between C_{1-} and Syn3 (Table 1). Test for Hardy Weinberg equilibrium showed no significant deviations from expected genotype frequencies for the two polymorphic populations.
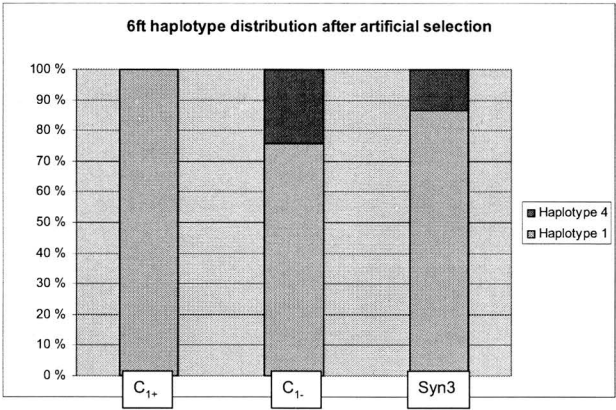
Fig. 1. Haplotype distribution in 6ft before (Syn3) and after selection (C_{1+} and C_{1-}).

Table 1. Non-differentiation P-values and 95% confidence intervals for pair-wise comparisons between C_{1+}, C_{1-} and Syn3.

	C_{1+}	C_{1-}
C_{1-}	0 (± 0.0000)	
Syn3	0.0039 (± 0.0005)	0.1146 (± 0.0019)

To asses the probability of genetic drift leading to the fixation of haplotype 1 in C_{1+} a simple two locus simulation of random drift was run for two generation (Winpop V.2.5.22). Initial allele frequency was set equal to the unselected Syn3 population (p=0.86), simulation was done by 1000 reiterations with a population size of 30. The simulation produced only 0.5% populations fixed for haplotype 1 (data not shown), indicating that the allele frequency shift is caused by selection rather than genetic drift.

For *PhyC* we observed haplotype frequency changes in C_{1-} (Fig 2). All three populations (C_{1+}, C_{1-} and Syn3) were in Hardy Weinberg equilibrium.

Exact test for population differentiation showed significant differentiation only between C_{1-} and C_{1+}/Syn3 (P=0) (Table 2).
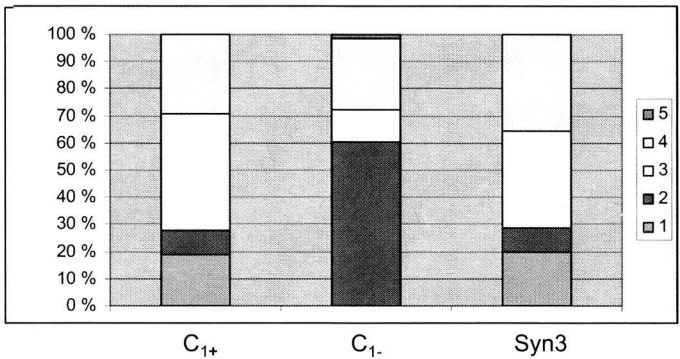
Fig. 2. Haplotype distribution in PhyC before (Syn3) and after selection (C_{1+} and C_{1-}).

Table 2. Exact test of differentiation P-values for pairwise comparisons of C_{1+}, C_{1-}, and Syn3.

	High Frost	Low Frost
High Frost	-	
Unselected	0,82	0,00
Low Frost	0,00	-

DISCUSSION

In this study we report SNP haplotype frequency change in two candidate genes after divergent selection for frost tolerance. The first gene, *6ft*, encodes the enzyme fructan 6-fructosyltransferase and has been shown to be important in frost tolerance (Gallagher *et al.*, 2004; Sprenger *et al.*, 1995). Temperatures near 0°C initiate a shift in polysaccharide formation whereby starch synthesis is suppressed and fructan synthesis is induced (Sprenger *et al.*, 1995). After selection for frost C_{1+} was fixed for the haplotype 1. Even though selection probably is the driving force behind the allele frequency shift in C_{1+}, we cannot without further investigations overrule genetic drift as an alternative explanation for the haplotype 1 fixation in C_{1+}. For the second gene, *phyC*, haplotype frequency changes in C_{1-} indicate a genotype-phenotype association with frost tolerance. Even though *phyC* is a photoperiodic response pathway gene, it is likely influencing climatic adaptation through association with the vernalization process (Beales *et al.*, 2005). *PhyC* also map to a frost tolerance QTL (Henriksen, 2005), close to the vernalization gene *VRN1* in cereals (Beales *et al.*, 2005) and in the closely related *F. pratensis* (Ergon *et al.*, 2006). Even though no significant changes in

allele frequency could be detected in C_{1+} we could see a 10% decrease in heterozygosity compared to the Syn3 populations. This may indicate a weak selection for frost.

In conclusion, this research shows that the strategy used in this study, i.e. defining SNP haplotypes and studying their allele frequency changes under selection for frost tolerance is a promising strategy for identifying functional markers for trait-selection in *Lolium* breeding programs.

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Using translational genomics to underpin germplasm improvement for complex traits in crop legumes

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ABSTRACT

The objective of this project is to create a robust physical map of the outbreeding diploid species red clover (*Trifolium pratense*) that will be anchored to the genome sequence of the legume reference species *Medicago truncatula*, and aligned to the clover genetic map. The anchored physical map will facilitate dissection of biological traits, future genetic improvement and marker assisted breeding in this important forage legume crop. The project will allow comparative analysis across legume species and create a model for translational genomics in crop legumes. Fingerprinting and end-sequencing of BAC clones from an existing red clover library will be used to obtain an estimated 2000 BAC contigs and anchor them to the *M. truncatula* genome using the closest homologue. Cytogenetics approaches will assess the level of coverage of the clover physical map and resolve issues with misaligned contigs. A total of 106 cross orthologous sequence (COS) markers have been identified with the GeMprospector program. They will be used in the physical and genetic mapping. In addition to this, 50-100 gene specific or microsatellite molecular markers, previously tested in clover, will be included for this purpose, allowing an approximate positioning in the *M. truncatula* genome to be determined. A web accessible clover information resource with alignment to *M. truncatula*, and integrated with the alfalfa resource developed in the US will be established. Integration with other databases will allow us to derive conserved orthologous sequence markers from the clover end-sequence tags that can be integrated with their counterparts in *M. truncatula* and *Lotus japonicus*.

Key words: BAC sequence; bioinformatics, cytogenetics, genetic map, *Medicago truncatula*, physical map, red clover

INTRODUCTION

Translating resources and information from model species is a key requirement if the potential of genomic approaches is to be realised in crop improvement. *M. truncatula* has been developed as a model for the study of legume biology and the gene-rich euchromatin of *M. truncatula* is currently being sequenced using an anchored, clone-by-clone strategy (Young *et al.*, 2005). *M. truncatula* is closely related to the important forage legumes white

clover (*Trifolium repens*), red clover (*T. pratense* sp.) and alfalfa (*Medicago sativa*). These species all belong to the *Trifolieae* tribe and they encompass the majority of worldwide forage legume production, underpinning temperate livestock production for the sheep, beef and dairy industries. A major challenge is to apply the wealth of genomic information from *M. truncatula* to the improvement of legume crops particularly with respect to key quantitative traits. Investigations indicate a high degree of synteny between *M. truncatula*, clovers and alfalfa (Sato *et al.*, 2005), and this provides an opportunity to utilise the high quality *M. truncatula* genome sequence to develop a robust physical map of these species anchored to *M. truncatula*. The work will create a template for translational genomics in crop legumes and a stepping stone to developing equivalent resources in crop legumes with more complex genomes.

The central objective of the proposed work is to create a robust physical map of red clover (*Trifolium pratense*), an important legume of European livestock production from grasslands, that will be anchored to the genome sequence of the closely related model legume *Medicago truncatula* and aligned to the red clover genetic map. Additional key objectives are (i) use of cytogenetic approaches to validate BAC contigs and resolve problem contigs and (ii) development of a web accessible informatics resource in which data from this work is managed, stored, curated, placed into context and integrated with other legume genomic resources.

MATERIALS AND METHODS

The size of the red clover genome has been estimated to be 420-440 Mbp (unpublished results, Sato *et al.*, 2005). A BAC library with 6X genome coverage and an average insert size of 150 Kb has been developed at IGER (Farrar and Donnison, 2007; Webb *et al.*, in preparation). We will use fingerprinting to generate contigs of the BACs. Approximately 18000 clones from this library will be fingerprinted. This will be supplemented with 9000 clones from each of two new libraries derived from *Eco*RI and *Bam*HI-cut DNA, respectively. All BACs that have been fingerprinted will also be end sequenced. These end sequences will provide anchor points in the *M. truncatula* genome (Figure 1) that will also validate the BAC contigs. The BAC end sequencing, fingerprinting and the production of the supplementary BAC libraries is being carried out at the Arizona Genomics Institute, University of Arizona, USA. From our experience with the *M. truncatula* fingerprint contig (FPC) database, we anticipate generating approximately 2,000 BAC contigs anywhere from a few hundred base pairs in length to a few Mb in length.

In order to facilitate gene isolation, map-based cloning, chromosome landing and genome organisation studies in red clover, the genetic and physical maps will be aligned. A total of 106 COS markers have been identified using the GeMprospector program (Fredslund *et al.*, 2006), and a 26356 red clover EST library (Sato *et al.*, 2005). PCR-based methods are being used to identify the BAC clones containing the markers in order to place them on the BAC contigs and physical map. Sequence polymorphisms permits us to place the markers on a genetic map based on an F_1 population derived from a cross between two genotypes from the synthetic populations Britta and Milvus. This will be supplemented with another 50-100 gene specific or microsatellite markers.
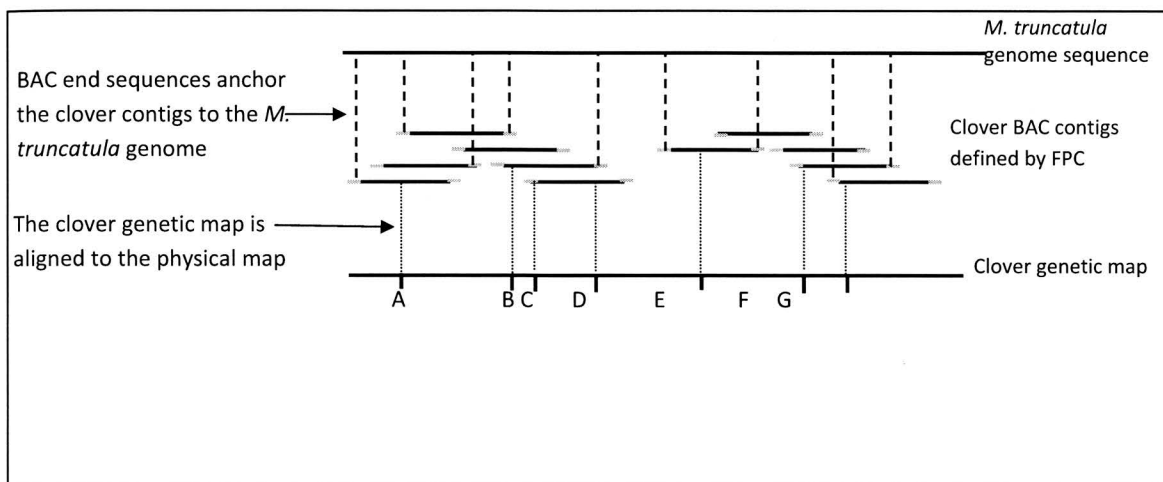
Fig. 1. A diagramatic representation of the generation of the anchored clover physical map. BAC contigs are defined through fingerprinting. Each BAC will have associated end sequences that provide anchor points to the M. truncatula genome. Clover genetic markers are positioned in the physical map to create a coherent link between M. truncatula and clover with aligned physical and genetic maps.

A molecular cytogenetic map of red clover (2n = 2x = 14) will be constructed on the basis of a pachytene DAPI karyogram. Chromosomes at this meiotic prophase stage are at least tenfold longer than at mitotic metaphase, and display a well differentiated pattern of brightly fluorescing heterochromatin segments. We will use cytogenetics to link individual chromosomes to genetic linkage groups, using BAC clones containing genetic markers as probes in FISH experiments. Furthermore, we will align red clover and *M. truncatula* (pachytene) chromosomes and map the translocations that have occurred during evolution of both species. This work provides the basis for further characterising the red clover physical map. Cytogenetics will be used to resolve problematic contigs particularly those that appear to align to separate locations on the *M. truncatula* genome. Multiple BACs on these contigs will be cytogenetically mapped to resolve whether the contigs are inappropriately aligned or represent a region of non-linearity between the two genomes. Cytogenetics also will be used to assess the level of coverage of the red clover physical map. A subset of gaps in the physical map will be sized in order to gauge the approximate amount of the genome not represented by the physical map.

We will establish a web accessible clover information resource hosting the FPC and BES data and alignments to the *M. truncatula* genome. The clover BES tags will be integrated into SIMAP (the similarity matrix of proteins) that is fundamental for the derivation of conserved orthologous sequence markers (COS markers). These COS assignments enable the clover BESs to be integrated with the orthologous counterparts in *M. truncatula* and *Lotus japonicus*. The sequence information generated during the course of the project will be subjected to a detailed analysis and embedded into a comparative plant genomics framework including *Medicago* as well as *Lotus*. FPC data and physical map information generated within the frame of the project along with the BAC end sequence tags form a powerful information basis for association of the *Trifolium* map with the model genomes of *Medicago* and *Lotus*. Detailed analysis of BAC end sequences on one hand allows derivation of detailed information about the composition and content of the clover genome. In conjunction with FPC map data these data can be integrated into a plant and legume comparative framework. The integrated BES tags along with the associated physical map information from FPC will

enable us to project and anchor individual BACs and associated FPC contigs onto the reference genome backbone of *Medicago* and *Lotus*. This will permit us to associate individual *Trifolium pratense* regions of interest, delineated by particular molecular markers and BES tags, with corresponding stretches on the genomic template of *Medicago/Lotus*. Combined comparative approaches of BES tags and FPC map data have already been demonstrated to be highly beneficial to gain insights into the structure and composition of complex plant genomes such as maize and for their potential in delineating orthologous regions among species (Messing *et al.*, 2004). The web will also be a major route of dissemination of the ongoing progress and outcomes of the work.

The anchored physical map of red clover will facilitate dissection of important physiological, morphological and agronomic traits and genetic improvement utilising marker assisted breeding in this important legume crop. In addition, the proposed work will provide information on evolutionary relationships within the legumes and also shed light on questions of generic importance both in respect of biological processes (e.g. nitrogen fixation) and as a detailed case study of evolutionary change between closely related taxa. The alignment of genetic markers with a physical map will also facilitate assessment of the level of linkage disequilibrium in red clover and thus the prospects for association mapping in this species.

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Perennial ryegrass (*Lolium perenne* L.) improvement through cisgenics®

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ABSTRACT

Perennial ryegrass (*Lolium perenne* L) is a major grass species used for forage and turf throughout the world. The yield improvements in perennial ryegrass dry matter production achieved annually in New Zealand farms between 1961 and 1999 was less than 0.5%, and it reflects a yield plateau. It is our belief that the development of cisgenic® ryegrass will advance our long-term product development plans to improve animal productivity by providing pasture cultivars with traits of interest to industry, such as drought tolerance, increased biomass, increased persistence, increased pasture quality, and increased condensed tannin content. Our functional genomics platform consists of markers based on GeneThresher® technology (Gill *et al.*, 2006), ryegrass SAGE™ (Sathish *et al.*, 2007) for transcriptome analysis and functional testing of genes in ryegrass and rice. We have developed a high throughput *Agrobacterium tumefaciens* mediated transformation method of perennial ryegrass (Bajaj *et al.*, 2006) to enable us to identify the ryegrass genes for our target traits. We have isolated the ryegrass homologue of *Atvp1*, *Lpvp1*, and produced transgenic plants driven by constitutive double strength CaMV *35S* promoter. Our preliminary experiments on T_0 plants suggest high drought tolerance in some lines. After identifying the target genes, we have replaced *Agrobacterium* T-DNA sequences with ryegrass derived 'P-DNA' sequences for *Agrobacterium*-mediated transformation. We have also produced transgenic ryegrass plants over-expressing *Lpvp1* driven by perennial ryegrass derived constitutive or inducible promoters. As part of our cisgenics® based functional genomics platform, we are now assessing *Lpvp1* and other cisgenic® genes as selectable markers.

Key words: 3'UTR, *Agrobacterium tumefaciens*, GeneThresher®, P-DNA, promoters, SAGE, selectable markers, transformation

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) is one of the most important temperate pasture grasses in the world (Jauhar, 1993). In New Zealand, it is the most widely grown pasture grass for sheep and cattle and covers more than 7 million hectares (Siegal *et al.*, 1985). Perennial ryegrass is an outcrossing, wind-pollinated and highly self-incompatible species (Thorogood *et al.*, 2002). Genetic improvement of perennial ryegrass through conventional breeding in New Zealand, like any other perennial forage crops in the world, (Tamaki *et al.*, 2007) has proven difficult and indicates a yield plateau. Biotechnology can help to overcome these problems and contribute to plant improvement for certain traits that would not have been possible with conventional breeding. Using the services of Orion Genomics, St. Louis, USA, we developed a genomic database of perennial ryegrass using the GeneThresher® technology,

which takes the advantage of differential methylation patterns by filtering genomic shotgun libraries to exclude methylated sequences and results in a gene-enriched shotgun library (Bedell *et al*., 2005; Rabinowicz *et al.,* 1999*)*. We subsequently carried out a SAGE™ (Velculescu *et al*., 1995) based transcriptome programme in perennial ryegrass sourced from livestock active paddocks (Sathish *et al.*, 2007)and laboratory raised plants. These tags were mapped to our perennial ryegrass genome database. Genes and promoters were selected based on the transcriptome data and were tested for functionality in transgenic rice, using the services of a commercial research lab – MetaHelix in Bangalore, India. Leads from the functional genomics programme are being fed into our perennial ryegrass cisgenic® program, where ryegrass will be improved using only ryegrass genetic elements, using our high-throughput *Agrobacterium tumefaciens*-mediated genetic transformation platform *(Bajaj et al.*, 2006)developed at HortResearch, Auckland, New Zealand and/or by markers assisted breeding based on SNPs and SSRs.

MATERIALS AND METODS

Perennial ryegrass GeneThresher® library was created using genomic DNA isolated from germinating perennial ryegrass seedlings and the protocol described previously (Bedell *et al.*, 2005; Rabinowicz *et al.*, 1999). SAGE™ libraries were created using the I-SAGE kit (Invitrogen, California, USA) following the manufacturer's protocol and tags were mapped to the ryegrass genome database and annotated as described before-. (Sathish *et al.*, 2007). Standard molecular biology protocols were employed to clone promoters, genes, 3'UTRs and P-DNA (Sambrook *et al.*, 1989). Perennial ryegrass was transformed using *Agrobacterium tumefaciens*-mediated genetic transformation methods (Bajaj *et al.*, 2006).

RESULTS

Ryegrass genome database
The genomic resource created using GeneThresher® technology consists of ~166 Mb of hypo-methylated sequence that assembles into 80 162 contigs and 189 697 singletons (Table 1) and is available, as tracks against the rice cv. Japonica genome sequence (TIGR Version 4), to the international research community through the Gramene database (Jaiswal *et al.*, 2006). It is estimated that the ~25 000 genes which ViaLactia has access to make up three quarters of the coding genes available in the perennial ryegrass genome.

Table 1. Perennial Ryegrass Genome Database.

Description	Number
Number of Sequences	528 539
GeneThresher® Sequences	511 987
EST Sequences	16 552
Number of Contigs	80 162
Average Contig length (bp)	964
Average number of sequences per Contig	3
Number of Singletons	189 705
Average Sequence length (bp)	507
ESTs, Contigs & Singletons with polyA features	25 126

Ryegrass transcriptome database

We used SAGE™ analysis to characterize the transcriptome from perennial ryegrass grown in active paddocks (GEO Series accession number GSE5211) and specially treated laboratory-grown plants (Sathish *et al.*, unpublished). We produced fourteen libraries (Table 2). Associated tags were mapped to our ryegrass GeneThresher® and EST sequence database. Lowly expressed transcripts, such as transcription factors, were well represented in the data set.

Table 2. Perennial Ryegrass SAGE™ Database.

Description	SAGE™ Tags Sequenced	Unique SAGE™ Tags	Unique SAGE™ Tags with Annotation
Pre-Grazed Leaves - Autumn Sample (Field)	28 798	10 402	5 964
Post-Grazed Leaves - Autumn Sample (Field)	27 748	11 086	6 301
Pre-Grazed Leaves - Winter Sample (Field)	27 764	13 567	8 082
Post-Grazed Leaves - Winter Sample (Field)	26 730	11 266	6 892
Roots - Winter Sample (Field)	16 474	9 293	5 641
Pre-Grazed Leaves - Spring Sample (Field)	18 832	8 255	4 976
Post-Grazed Leaves - Spring Sample (Field)	16 484	7 590	4 658
Immature Inflorescences (Field)	24 496	12 982	7 560
Post-Grazed Leaves - Summer Sample (Field)	19 456	9 108	5 333
Mature Leaf Tissue (Lab)	12 505	6 858	3 999
Cold-Stressed Leaf Tissue (Lab)	18 375	8 694	5 043
Hydrated Leaf Tissue (Lab)	15 746	8 094	4 664
Dehydrated Leaf Tissue (Lab)	17 068	8 629	4 768
Rehydrated Leaf Tissue (Lab)	30 416	13 478	7 676
Total	**300 892**	**67 941**	**36 643**

Perennial ryegrass improvement through Cisgenics®

Our targeted ryegrass traits include drought-tolerance, enhanced biomass, etc. Our cisgenic® approach has prompted us to identify, besides the genes that are to bring about the desired trait change, ryegrass promoters that differ in gene-expression strengths and induction, 'P-DNA's, which are ryegrass sourced equivalents of *Agrobacterium* derived T-DNA border sequences and 3'UTRs that confer different degrees of mRNA retentions. Strong constitutive promoters, not unlike double *CaMV35S* or maize ubiquitin promoters; inducible promoters, such as for drought, etc have been cloned and tested for their expression in rice and perennial ryegrass (Figure 1a - c). We have identified two right border and one left border T-DNA equivalents and evaluated their usefulness to transfer cloned genes in to ryegrass genome. Of the two combinations tested, one was found to be better than the other (Figure 1d). However, the efficiency is far below that of T-DNA, but nevertheless a workable alternative. Also, we are currently testing three different 3'UTRs from perennial ryegrass.
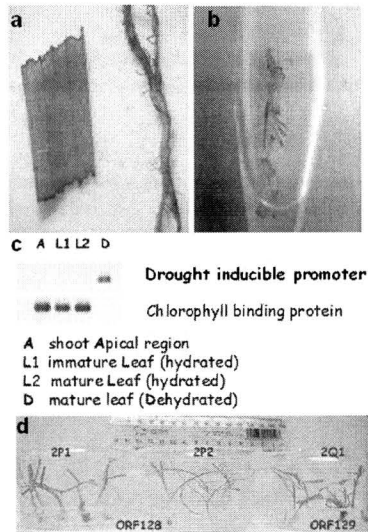
Fig. 1. Characterization of different elements involved in the creation of cisgenic® ryegrass. a) Ryegrass promoter driving bacterial uidA gene in rice; b) Ryegrass promoter driving bacterial uidA gene in ryegrass; c) drought inducible promoter activity in ryegrass characterized by RT-PCR; d) regenerated ryegrass plants transformed using 'P-DNA'.
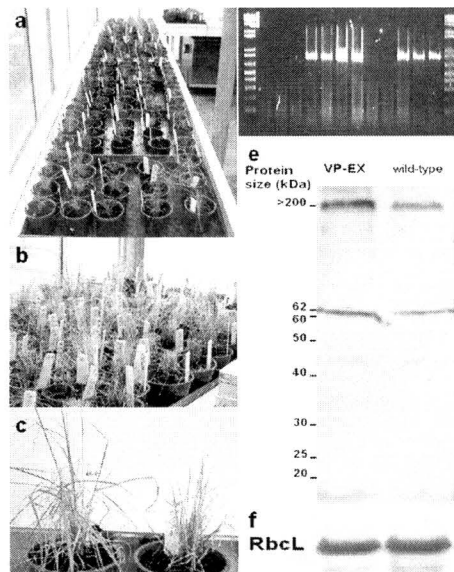
Fig. 2. Drought tolerance screen using T_0 perennial ryegrass plants transformed with Lpvp1. a) Ryegrass plants before the drought screen; b) Ryegrass plants at the end of the drought screen; c) drought tolerant T_0 perennial ryegrass plant transformed with Lpvp1 along with non-transformed control plant; d) RT-PCR analysis of select plants for cloned Lpvp1 expression; e) Western-blot analysis of drought tolerant transformed ryegrass plant and the control plant for VP expression in hydrated condition; f) coomassie blue staining of the protein gel demonstrating uniformity in protein loading.

100

Lpvp1 is one of the genes that were identified in our database as that could enhance drought-tolerance. We have transformed perennial ryegrass using *Agrobacterium*-mediated transformation procedures with *Lpvp1* expressed under either constitutive or inducible promoters derived from perennial ryegrass as well as double *CaMV35S* promoter. Screening of T_0 plants under controlled conditions produced a number of plants with increased resistance to drought (Figure 2). Currently, T_0 plants are being bulked in preparation for a drought tolerance screen involving replicate samples and multiple levels of drought.

DISCUSSION

We characterised the ryegrass genome at an unprecedented degree at three levels i) the gene, ii) the transcript and iii) the molecular genome map. With this resource, we have: successfully identified field- and lab-specific transcripts; characterized the seasonal variation for many transcripts; monitored the effects of grazing on transcript profiles; found novel transcription factors based on cluster associations; identified several natural anti-sense transcripts; discovered many SNPs; and detected alternatively spliced transcripts. We have cloned a suite of promoters to control the expression of cloned genes in ryegrass; a suite of genes to deliver the desired trait in ryegrass; and 'P-DNA' for delivering the cloned genes back in to perennial ryegrass. We are currently evaluating a suite of 3'UTRs and alternative transformation selection markers to be used towards advancing our perennial ryegrass cisgenic® programme. We believe that the general public will be less disparaging of the cisgenic® perennial ryegrass thereby paving the way for field trials in New Zealand.

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DNA marker-assisted selection for yield and nutritional traits in Italian ryegrass (*Lolium multiflorum*)

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ABSTRACT

Our project aiming for the application of DNA MAS for yield and nutritional quality traits in the breeding of Italian ryegrass has been finalised. F1-populations generated by pair-crosses between non-related elite clones were used for the identification of QTL markers. MAS was performed on genotypes issued from the self-pollination of elite F1-genotypes selected on a phenotypic basis. Although, a 'virtual' (not effectively performed) MAS of F1-genotypes led to a mean for the traits higher than the F1-population means. In the S1-populations, MAS was complicated by the presence of new allelic combinations, absent at the F1-level. The different MAS compositions, obtained after intercrossing of the selected S1-genotypes, were evaluated in yield and quality plots. Results of the MAS selections were in the same range as the standard varieties and the traditional breeding compositions of same origin. Plants out of the self-pollinated populations, not retained by MAS and intercrossed together, consistently had lower results. Significant differences were observed between the two experiment locations. Thus, the gain obtained by MAS was not as expected and no significant heterosis effect was generated. MAS allowed overcoming the negative effect of selfing, however, no real difference was observed between negative and positive selections. It can be argued if the MAS applied was more effective in selecting loci with significant genetic variation than differentiating positive and negative alleles. The possibility of MAS for complex traits such as DMY and quality traits from a whole season observations in a very heterogeneous species like ryegrass will be discussed.

Key words: grasses, markers, quality, QTL, validation

INTRODUCTION

Nearly all the genetic variation in the major agronomical traits of forages, such as yield and quality, is quantitative; very few major genes that regulate these traits have been identified. Breeders must largely rely on quantitative trait loci (QTLs) with small individual effects for genetic improvements. Even though QTL markers associated to several agronomic traits have been published in ryegrasses and related species, these investigations have not been extended beyond the step of QTL identification. The aim of the study here described was not only to identify QTL markers, but also to test their effect by marker-assisted selection (MAS) in populations derived from the segregating populations across different environments. Difficulties to apply MAS for complex quantitative traits as yield and fodder quality, in highly heterogeneous ryegrass materials were highlighted.

MATERIALS AND METHODS

Traits chosen for investigation were dry matter yield (DMY), dry matter digestibility (DMD), water-soluble carbohydrate content (WSC) and crude protein content (CP). Four mapping populations of Italian ryegrass (*Lolium multiflorum* L.) of 100 to 110 F1-progeny were generated from pair-crosses between non-related individuals of high agronomical value. This choice aimed at being closer to the real situation of breeding programmes. The four populations were phenotyped (individual plants, 5 cuts in the year after sowing) and used for linkage map construction (Joinmap 3.0) and QTL mapping (MapQTLv.4.0). AFLPs, STSs and SSRs markers were used for the linkage map construction. Several mapping methods were compared. The linkage maps obtained by direct mapping including all marker classes were used for QTL analysis. These maps ranged from 689 to 925 cM and consisted of close to seven major linkage groups (LGs). These maps were aligned to each other and to several published *Lolium* maps (Jones *et al.*, 2002; Jensen *et al.*, 2005; Muylle *et al.*, 2005; Turner *et al.*, 2005). The QTL analysis combined the Kruskal Wallis analysis (KW) test and simple interval mapping (SIM). It was performed for the total or mean value of the traits, as well as for some individual cuts depending on the trait. A specific selection scheme was elaborated in which F1 genotypes (F1s) were self-pollinated with the intent to 'fix' the QTL effects. Elite F1s with high values for the different trait were identified using the phenotypic observations.
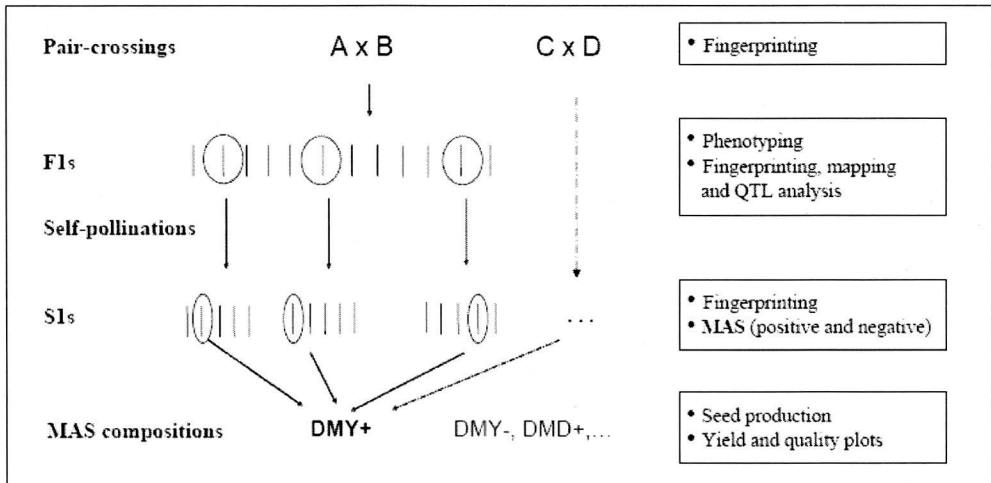
Fig. 1. Scheme of the strategy applied for the MAS.

These F1s were self-pollinated to produce S1-populations. MAS for most interesting S1-individuals (S1s) was performed using the identified QTL markers according to the different traits. These S1s were intercrossed in eight MAS (DMY+, DMY-, CP+, CP-, WSC+, WSC-, CP+ and CP-). The compositions were evaluated in yield and quality plots at two locations (Melle, Belgium and Rennes, France). Figure 1 illustrates the different steps performed prior to and after MAS. Statistical analyses were performed using SPSS 11.1.5.

RESULTS

The QTL analysis indicated several genome regions involved in each of the studied traits, and in most cases, major QTLs could be identified. The individual markers identified as associated to a trait explained from 9.7 to 40.0% of the phenotypic variation of the traits. On average for the four populations, the number of markers linked to total DMY, mean CP, mean WSC and mean DMD were 5.5, 6, 10 and 10, respectively. They explained 14.6, 17.2, 14.8 and 17.9% of the variation respectively. Some markers linked to multiple traits were identified, especially for DMD and WSC. The associated markers were in most cases different between populations. For some QTLs, similarities for their location were observed between populations. Major QTLs were identified for DMY mainly on LG4. For CP, major QTLs were present on LG4 in all populations and on LG7 and LG6 in several populations. For WSC, major QTLs were located on LG2, LG3 and LG7 in two of the populations. No consistent QTL location was revealed in the four populations for DMD (either LG2, LG3, LG4, LG6, LG7, LG(8) or LG(9)). Some correspondences with the few published reports on quality traits in fodder grasses (Turner *et al.*, 2005; Cogan *et al.*, 2005) could be established: LG2, LG3, and LG4 for CP; LG2, LG3, LG6 and LG7 for WSC; and LG3, LG4 and LG7 for DMD.

Results of the MAS compositions at the two locations are presented in Table 1 (Belgium) and 2 (France). For most traits, several of the eight MAS compositions scored better than the reference varieties (REF) and than compositions of the same origin as the F1-populations generated by the classical breeding program (TB). However, the composition(s) did not necessarily rank as expected for the different traits. No significant differences were found between the phenotypic scores of the positive and negative selections for most traits and in many cases the negative selections revealed even better phenotypic scores than the positive selections, which was completely unexpected. Furthermore, the intercrossings of the F1-parents selected in this study for self-pollination (P-$\bar{X}$) were as good if not better than the MAS. Significant differences were observed between the results of the two experiment locations. This was probably a reflection of the differences in field management and in environmental conditions, leading to a different number of cuts performed. The differences reflected the normal conditions of both locations. Analysis of variance revealed acceptable coefficients of variation for the experiments and no further undesirable interaction between factors. Total or means of all cuts for the different traits were in the known range of values for both locations.

DISCUSSION

The present study is the first in which several mapping populations of different origins have been combined for QTL identification in fodder grasses. Significant differences between populations were apparent but QTLs located on the same LG were identified in the different populations, as well as similarities with published studies. These similarities indicated that there might be major conserved genome regions responsible for the expression of yield and quality quantitative traits in ryegrass. However, a majority of QTLs were located in chromosome regions specific to each population. Additionally, even though common regions were identified, the associated markers were in most cases different. Thus, the efficacy of the markers for MAS in another genetic background is not certain.

Different explanations are possible for the ambiguous results obtained by the MAS. In the first place, the QTL markers used and their relatively high number could be questioned. However, the same markers used for virtual MAS at the F1-level allowed the identification of genotypes leading to a mean for the trait higher (or lower) than the population means (particularly for DMY and WSC) and gave quite good correspondence with the phenotypic

selection. After self-pollination and recombination, the favourable linkages and positive allelic interactions at QTL positions for the different traits seem to be 'broken'. Some epistatic effects not present at the F1-level might have skewed the results of the MAS compositions. The recombination of S1s of different origins might have generated some heterosis effects which overruled the expected QTL-piling in the offspring populations. However, the compositions including plants out of the different self-pollinated populations not retained by MAS (SB in Table 1) achieved poor results, which indicated that the MAS allowed overcoming the negative effect of selfing. Another point is that, in some cases, the MAS highlighted the same S1 for more than one trait and an order of priority had to be given to the traits (from high to low priority: DMY, WSC, DMD, CP). Thus, the optimal MAS could only be performed for one of the traits (DMY). The results of the CP selections were in fact most inconsistent. However, the DMY selections did not produce the best response to MAS. Finally, the multiplication of the compositions to seed of second generation (in order to allow the testing at two locations) may have had an impact on the results but it was variable from one trait to another; this seemed a drawback for DMY while for the other traits the impact was reversed (tested in Belgium only, see results of G1 versus G2 in Table 1).

The selection of only elite F1s as parents of the S1-populations probably narrowed the possibility of generating a heterosis effect in the MAS. This could only be tested if we had also carried out a 'negative' selection in parallel. The selection for specific marker alleles was probably not completely effective due to the dominant nature of most associated markers (AFLPs) or to the presence of allelic combinations in the S1-populations absent at the F1-level (the weight assigned to each allelic combination might have skewed the results obtained). The opportunity of using self-pollination in the experiment may be questioned as no heterosis effect was stated. Selecting for several cuts at a time did perhaps hamper obtaining significant results. However, identification of material which would be valuable in only one cut was not the scope of the present study.

The compositions obtained by MAS represent valuable material for breeding purposes, generally better than the references and will certainly be used for new variety creation. However, the gain obtained by MAS was not as expected and no significant heterosis effect was generated. Overall, WSC and DMD had the best response to MAS. The opportunity to apply MAS for such complex traits from a whole season of observations in a species like ryegrass could be questioned, but many factors and many parameters linked to the scheme applied and choices made would have to be checked before making such a conclusion. The application of MAS at the level of F1s would have allowed answering some of these questions. However, this was not performed due to practical constrains. The choices made were greatly influenced by a strict time schedule and by the non persistent nature of the Italian ryegrass.

Table 1. *Total DMY (ton/ha), mean CP (g kg⁻¹DM), mean WSC (g kg⁻¹DM) and mean DMD (%) from the yield and quality trial at the Belgian location (five cuts, three replicates) of the standard varieties (REF), the traditional breeding composition (TB), the multiplications of the F1-seeds (F1G2), the eight groups of the MAS (DMY+, DMY-, DMD+, DMD-, CP+, CP-, WSC+ and WSC-), the positive and negative selection of a trait together (DMY, DMD, CP, WSC), all MAS selection groups together, for multiplication groups of the self-pollinated plants without MAS (SB), Italian ryegrass varieties (VAR) and of the multiplication groups of the F1-genotypes which generated the S1-populations (P-DMY, P-CP, P-WSC and P-DMD). G1 = seeds of first generation, G2 = seeds of second generation.*

		Total DMY		Mean CP		Mean WSC		Mean DMD	
REF		19.8	e	149.4	g	112.4	bc	59.9	bcd
TB		21.5	c	153.6	cde	115.5	h	60.6	fg
F1G2		19.5	f	156.3	ab	112.0	gh	59.4	efg
G2	DMY+	**20.1**	d	147.6	fg	116.5	efg	60.5	bcde
	DMY-	**22.0**	b	151.1	cdef	120.5	cdef	61.0	bc
	CP+	21.0	d	**149.6**	def	112.2	gh	58.7	g
	CP-	21.3	cd	**150.6**	cdef	111.9	gh	59.9	cdefg
	WSC+	21.7	bc	153.3	bcd	**116.0**	fg	60.0	cdef
	WSC-	22.5	a	147.2	fg	**125.0**	cd	59.6	defg
	DMD+	21.1	d	148.4	efg	122.5	cde	**60.7**	bcd
	DMD-	20.9	d	154.1	bc	131.1	b	**62.3**	a
	DMY	**21.5**		149.4		118.5		60.8	
	CP	21.2		**150.1**		112.0		59.3	
	WSC	22.1		150.3		**120.5**		59.8	
	DMD	21.0		151.2		126.8		**61.5**	
	All MAS	**21.4**		**150.2**		**119.5**		**60.4**	
G1	DMY+	**22.4**		150.7		123.2		61.1	
	DMY-	**22.7**		147.3		114.4		59.3	
	CP+	21.7		**145.7**		115.5		59.2	
	CP-	22.1		**146.1**		120.0		59.6	
	WSC+	21.6		151.0		**120.8**		60.5	
	WSC-	22.0		147.4		**115.6**		59.0	
	DMD+	21.9		151.6		116.1		**60.4**	
	DMD-	22.1		146.7		125.2		**60.3**	
	DMY	**22.5**		149.0		118.8		60.2	
	CP	21.9		**145.9**		117.7		59.4	
	WSC	21.8		149.2		**118.2**		59.8	
	DMD	22.0		149.1		120.6		**60.3**	
	All MAS	**22.1**		**148.3**		**118.8**		**59.9**	
SB		20.1	d	151.5	cdef	109.1	h	58.9	fg
VAR		19.7		153.1		106.1		58.8	
P-DMY		**21.1**	d	148.0	fg	120.9	cdef	59.8	cdefg
P-CP		20.4	e	**158.0**	a	119.1	def	61.6	ab
P-WSC		22.0	b	145.6	g	**121.3**	cdef	59.2	fg
P-DMD		21.0	d	147.2	fg	137.5	a	**61.7**	ab
All P-x		**21.1**		**149.7**		**124.7**		**60.6**	

Note: *Means with same letters within a column are not significantly different (P>0.05)*

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Table 2. Total DMY (ton/ha), mean CP (g kg^{-1}DM), mean WSC (g kg^{-1}DM) and mean DMD (%) from the yield and quality trial in France (four cuts, three replicates) of the standard varieties (REF), the traditional breeding composition (TB), the multiplications of the F1-seeds (F1G2), the eight groups of the MAS (DMY+, DMY-, DMD+, DMD-, CP+, CP-, WSC+ and WSC-), the positive and negative selection of a trait together (DMY, DMD, CP, WSC), all MAS selection groups together. MAS groups: seeds of second generation (G2).

	Total DMY		*Mean CP*		Mean WSC		Mean DMD	
REF	21.9	ab	124.3	e	142.2	ab	56.3	bc
TB	21.3	bc	134.3	b	143.7	ab	58.2	a
F1G2	20.4	d	130.7	c	132.9	c	55.5	c
DMY+	**21.3**	bc	125.2	e	131.5	c	55.8	c
DMY-	**22.4**	a	136.6	a	129.2	c	55.5	c
CP+	21.7	abc	**128.5**	d	142.4	ab	57.1	b
CP-	22.0	ab	**132.8**	b	134.8	bc	57.1	b
WSC+	21.7	abc	130.7	c	**131.8**	c	53.5	d
WSC-	21.5	abc	120.0	f	**151.8**	a	57.3	ab
DMD+	21.0	cd	124.7	e	129.5	c	**54.2**	d
DMD-	20.3	d	124.3	e	142.7	ab	**56.3**	bc
DMY	**21.9**		130.9		130.4		55.6	
CP	21.9		**130.7**		138.6		57.1	
WSC	21.6		125.3		**141.8**		55.4	
DMD	20.7		124.5		136.1		**55.3**	
All MAS	**21.5**		**127.9**		**136.7**		**55.8**	

Note: Means with same letters within a column are not significantly different (P>0.05)

Our priority for future work will be given tentatively to highlight the ambiguous results of the MAS gave ambiguous results. Therefore, genotypes from the MAS compositions will be fingerprinted with the QTL markers associated to the traits. There should be an enrichment of QTL-alleles in each group and the difference between positive and negative selection should clearly appear. If this is not the case we should conclude that MAS for specific marker alleles was not successful (due to the weights attributed to each allele combination or due to the nature of associated markers). The different steps performed prior to the identification of the associated markers without the timing constrain imposed by the MAS will be improved. Saturation of the maps with additional co-dominant markers and functionally associated markers will be a priority, in at least one of the populations and preferably two of them.

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NIR-Spectroscopy of non-dried forages as a tool in breeding for higher quality – laboratory tests and online investigations on plot harvesters

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ABSTRACT

Near infrared spectroscopy (NIRS) based on diffuse reflectance measurements on dried and ground samples has been established as a reliable and cost effective analytical tool for testing forage quality. An important new approach for the rationalisation of forage field trials consists in the compositional analysis of forage samples already during harvesting. This becomes feasible by integration of robust near infrared diode array spectrometers directly on plot harvesters. Based on a considerable body of experience this new methodology has been set up during the last years for forage breeding. Today NIRS on line measurements on forage plot harvesters allow significant cost reductions in forage testing for dry matter (DM) %. They permit higher precision (heritability) of DM assessment than conventional measurements by oven drying. Even higher repeatability and lesser susceptibility to malfunction is anticipated when new spectrometers with internal / dark white referencing become available. However, inherent limitations of NIRS online measurements tend to lower precision for compositional analysis of freshly harvested forages as compared to NIRS analysis on dried and ground forages in the lab. Therefore online measurements on plot harvesters suffice for approximate quality testing in the early phases of breeding but need to be followed up by NIRS on dried and ground forages in the final phase of breeding. A user group called NOFUG (NIRS online forage user group) has been formed to exchange information on this new methodology and to promote its use.

Key words: cost reduction, dry matter, heritability, Lolium perenne, NOFUG, forage quality, sample preparation, spectral range

INTRODUCTION

Near infrared spectroscopy (NIRS) using diffuse reflectance measurements was introduced by Norris *et al.* in 1976 as a new method of forage analysis. Since then, its cost effectiveness has made this analytical approach indispensable in plant breeding and variety evaluation schemes for forage quality (Paul, 1999). However, due to previous limitations in NIRS instrumentation this analytical technology has essentially been limited to applications under stationary laboratory conditions. High speed diode array sensors for the near infrared with high temperature stability and mechanical robustness developed in the late 90ies now permit fresh forage analysis even on harvesters under field conditions (Dardenne and Femenias, 1999; Rode and Paul, 1999).

This new development was implemented in a government-aided collaborative project (GFP) between breeding companies (NPZ and DSV), constructors (Haldrup and Zeiss) and scientists (FAL) in 1999. As an outcome of this project NIRS-online estimation of dry-matter by a conveyor belt module in DSV and NPZ started in 2002.In 2005 a newly designed piston module was developed and successfully introduced at the breeding stations.Today calibration maintenance and management is performed commercially by VDLUFA (Association of German Agricultural Testing and Research Institutes).

NIRS online use in practice
The number of plots harvested with NIRS on line could be increased steadily over years (Figure 1). In 2007 over 20,000 plot cuts were harvested at the EURO GRASS Breeding station Hof Steimke with only one harvester. Parallel to the increase in throughput the time needed to harvest a single plot was decreased from over 100 seconds per plot in 2002 to less than 50 seconds in 2007.
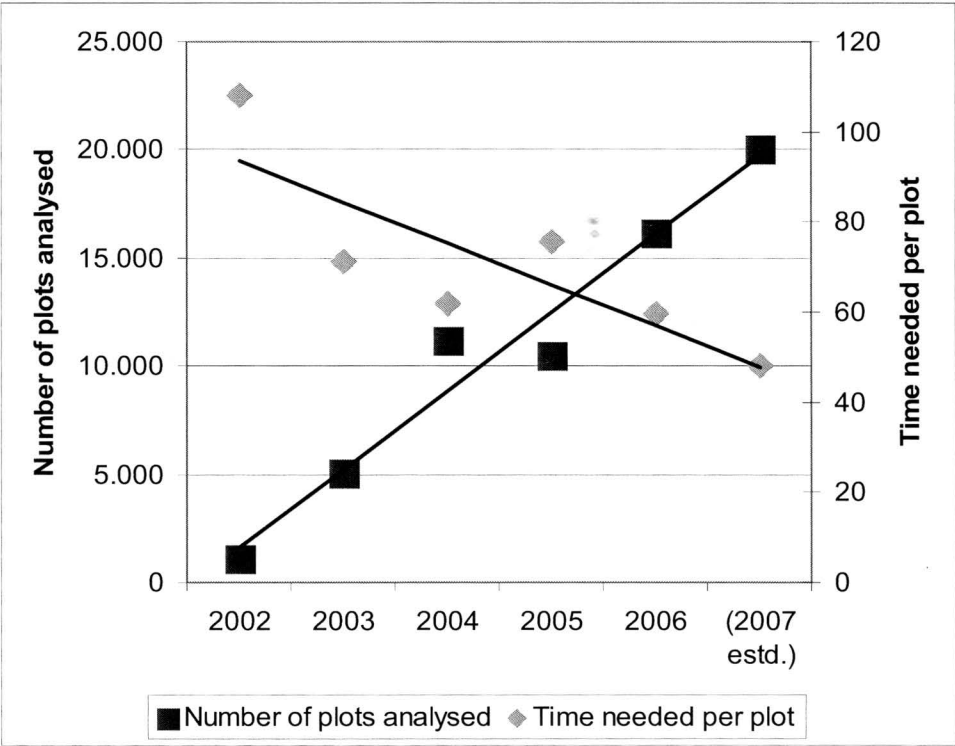
Fig. 1. NIRS Online Usage and Harvesting Time per Plot.

After proof of principle of the online method in practice, supervision and maintenance of the calibration was handed over to VDLUFA in 2007. At the same time an international working group was founded (NOFUG – NIRS online forage user group) with the goal of advancing the NIRS online technology in forage breeding and testing.

Significant cost reductions for work and energy are now possible because it is no longer necessary to take samples manually and to estimate dry matter content by conventional oven drying. In Figure 2 the results of DM-estimation by conventional oven drying are compared with the results of NIRS online estimation.

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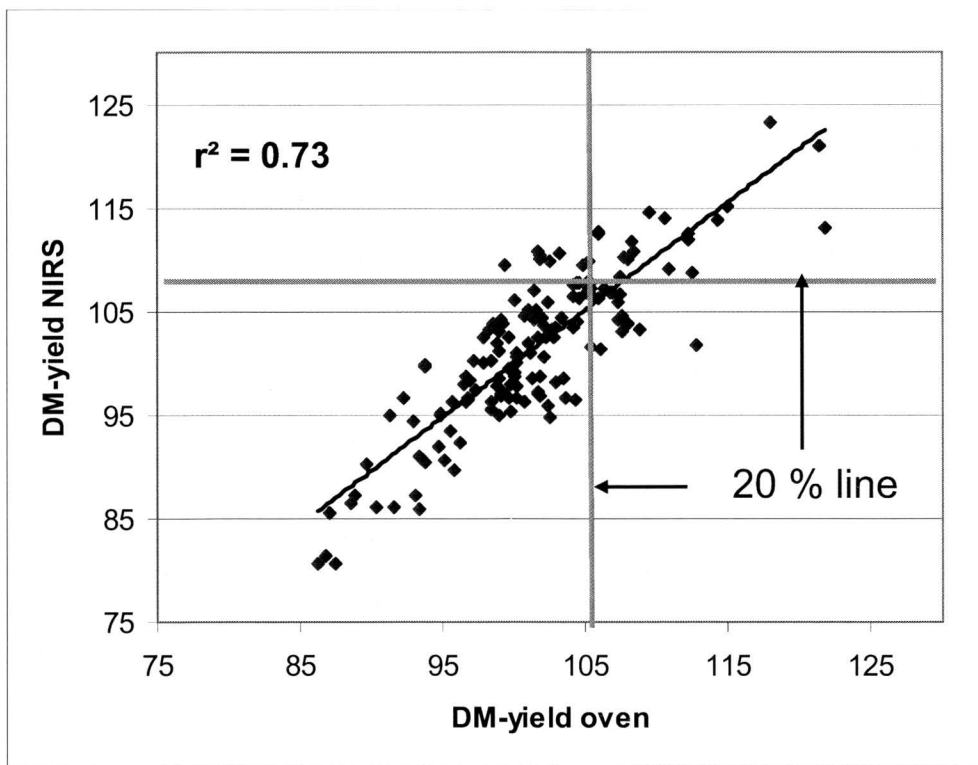
Fig. 2. Selection Based on Oven DM Yield versus NIRS DM Yield.

The experiment included samples from 150 entries with three replications analysed both for DM with NIRS online in the field as well as in the station by oven method. The results were highly correlated; however, selection decisions (candidates above the 20% line in Figure 2.) would have been different between the two methods for about half the cases. From a breeding point of view it is important that the heritability of the NIRS online method was significantly higher than the one achieved by oven drying (h^2 of 0.60 rather than 0.50).

NIRS- bench top analyses and NIRS online measurements of forage quality
NIRS bench top instruments are generally equipped with different detectors and span a different spectral range compared to online-instruments for field use. Bench top instruments are equipped with lead sulfide detectors for the range from 1050 nm to 2500 nm while online field instruments are equipped with different types of Indium – Gallium – Arsenide detectors, which cover a more or less reduced wavelength range (Figure 3).

The reduction in wavelength range has a direct influence on the precision of the measurements (Table 1). In this comparison 219 samples were measured in the undried as well as in the dried state on a dual-detector bench top instrument for the range between 400nm to 2500nm. This allowed to assess the effect of variable wavelength range on derived quality characteristics such as content of protein and water soluble carbohydrates (WSC).

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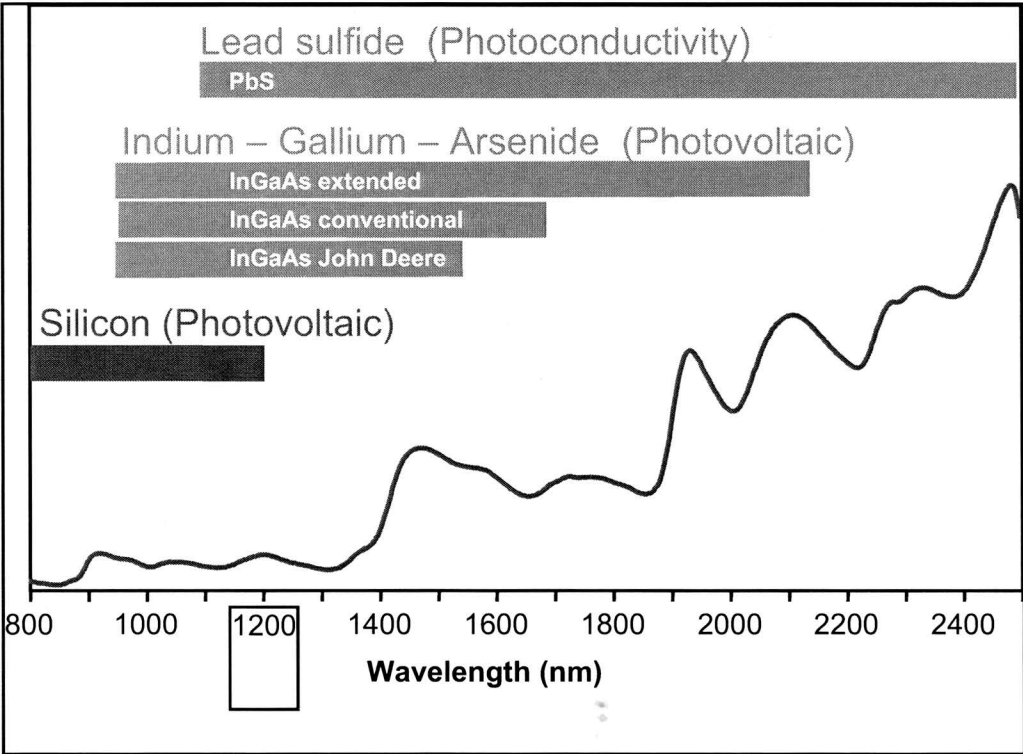
Fig. 3. Different Near Infrared Detectors.

Table 1. NIR Predictability of Crude Protein (% of DM) in Forage Grasses (n = 219).

Wavelength Range	Standard error of crossvalidation (SECV)		r^2 of crossvalidation	
	Dried & ground	Undried	Dried & ground	Undried
PbS	0.38	0.94	0.99	0.94
InGaAs extended	0.40	0.99	0.99	0.93
InGaAs conventional	0.54	1.16	0.98	0.90
InGaAs John Deere	0.71	1.22	0.96	0.89

With the bench top instrument (PbS) an SECV value of 0.38% protein was obtained with dried and ground samples. This error indicator increased with every reduction of the wavelength range for the various types of InGaAs detectors common for online devices. This comparison indicates that the extended InGaAs range is preferable compared to the short range InGaAs for diffuse reflection measurements on dried and ground samples when assessing protein content. A second dimension in Table 1 relates to dried and ground samples vs undried samples. Measurements on undried sample generally resulted in much higher SECV values at a given wavelength range compared to dried and ground samples. This result indicates that water in high concentrations conceals the absorption bands which are attributed

to protein or other constituents (results not shown). Figure 4 demonstrates the threefold higher prediction error ($S_{y.x}$) for water soluble carbohydrates when assessed via conventional InGaAs range in undried samples as compared to PbS-range in dried and ground samples.
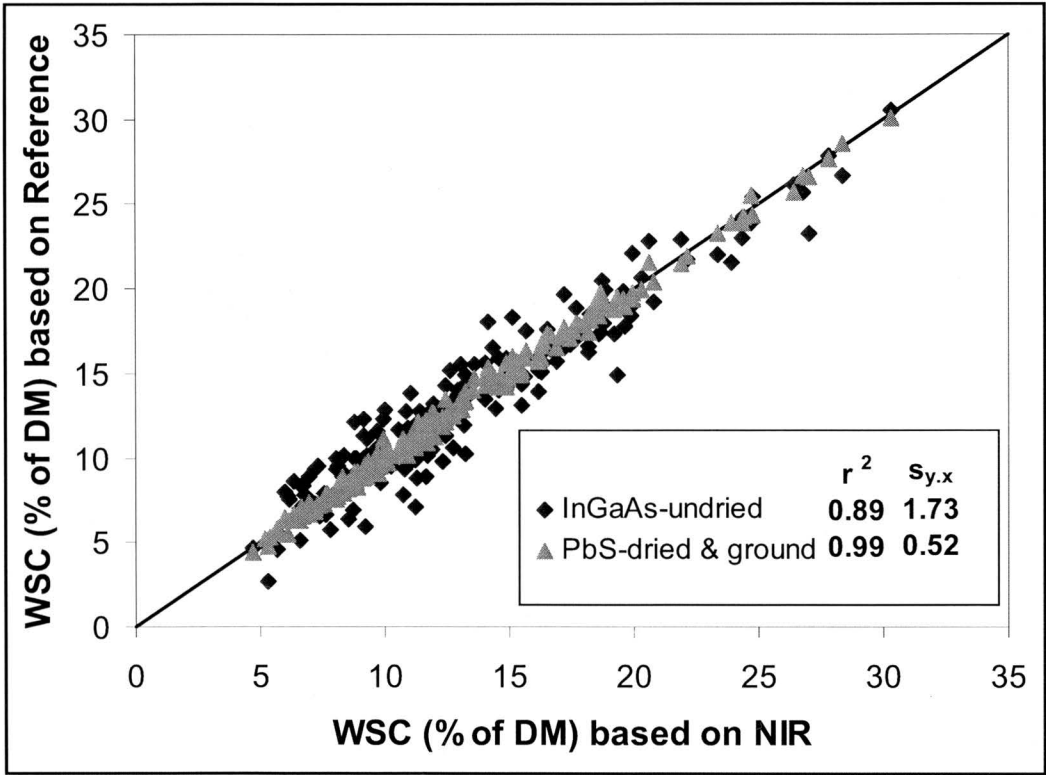
Fig. 4. NIR-Prediction Error for WSC in Forage Grass: Influence of Sample Preparation and Spectral Range.

From the results in Table 1 and Figure 4 the limitations faced by NIRS online users on the harvester in the field become obvious. When using the presently available NIRS online instruments quality assessments exhibit a level of precision that may be just sufficient for the first cycles of forage breeding. In case of higher precision requirements drying and grinding of samples prior to NIR measurements in the lab using bench top instrumentation are still needed.

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NIRS and chemometrics – exploration of grass forage quality

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ABSTRACT

Near infrared reflectance spectroscopy (NIRS) has been successfully implemented as a method to quantify forage quality parameters e.g. crude protein in grasses. Calibration models have also been developed on lignin and ergovaline. The objectives of the present study were to describe the successful NIRS calibration model we have developed on total nitrogen (N) and to evaluate the development of NIRS calibration models on ergovaline and lignin in Danish grass samples. Reflectance spectra of dried and ground grass samples were collected in the NIR region from 1100 to 2500 nm with data collection at every 2 nm. Chemometric methods were used to investigate the correlation between spectra and warranted concentrations of total N, ergovaline and lignin. For the total N concentration the error in the calibration model is acceptable as described by Gislum *et al.* (2005). In the case of ergovaline the principle component analysis scatter plot clearly shows that it is not possible to distinguish between samples with or without ergovaline. This is likely to be due to the fact that ergovaline is only present in small concentrations (ppm). The error on the lignin partial least squares regression model was 0.4 mg lignin g^{-1} dry weight in a range from 3.6 to 5.8 mg lignin g^{-1} dry weight which is not acceptable. In this case NIRS is not accurate enough to predict the lignin concentration. We concluded that NIRS can be used for measuring clear N peaks as seen in the spectra and high correlation coefficients as we obtained in the calibration model. Others have been able to correlate NIRS with ergovaline and lignin, however these correlations could not be establish by the authors in the present work. It should be further investigated if the PLSR model on lignin can be improved and if it is possible to develop a PLSR model for ergovaline by including samples with a larger variation in lignin and ergovaline concentration or by making local calibration models.

Key words: ergovaline, lignin, nitrogen, PCA, PLSR

INTRODUCTION

Near infrared reflectance spectroscopy (NIRS) is a fast and low cost spectroscopic method, which is based on measuring absorbance or reflectance of NIR light (wavelength range 700 to 2500 nm). NIRS is a secondary method that requires calibration to some primary reference method by using a calibration set of typical specimen representative of all future unknown samples (Andrés *et al.*, 2005b). If the calibration set does not include all future variability the prediction will not be accurate.

Chemometrics is the application of mathematical or statistical methods to chemical data. Chemometric methods can be used to develop calibrations between spectra (X-variables) and warranted (Y-variables). Besides being able to predict Y from X of future samples the Chemometric methods facilitates interpretation of X in relation to Y. In most cases the

purpose is to replace the more expensive chemical measurement of Y with a cheaper and faster spectroscopic measurement.

The use of NIRS to predict grass forage quality is well-documented, e.g. by Nousiainen *et al.* (2004), Andrés *et al.* (2005a) and Andrés *et al.* (2005b). The total number of projects with the aim to develop NIRS calibration models to predict different grass forage quality parameters is expected to be large due to the importance of knowing the quality of forage grasses and the advantage of optimising feeding of cows and cattle. Gislum *et al.* (2005) showed correlation coefficients of 0.97 to 0.98 when predicting total nitrogen in dried and grounded grass samples. Roberts *et al.* (1997) were able to predict ergovaline in tall fescue while Andrés *et al.* (2005a) used NIRS to predict the lignin concentration in permanent meadows. Encouraged by the positive results found in the latter papers, lignin and ergovaline calibrations have been developed on Danish grass samples. This work is described in the present paper.

An important issue in NIRS is to take the NIRS instrument to the sample and not take the sample to the NIRS instrument, which can be described as the 'proximity' principle opposite to the distance principle. This principle is strongly dependent on advanced chemometrics, cost-effective standardization and robust NIRS instruments. When predicting grass forage quality it makes sense to measure the quality during the growth while it is possible to alter the quality by e.g. additional application of nutrients or irrigation, than to measure the quality when it is impossible or very expensive to alter the quality. In accordance with the proximity principle NIRS is currently used to predict dry matter and water on-line in harvesting of grasses.

The objectives of the present study were to describe the successful NIRS calibration model we have developed on total nitrogen and to evaluate the development of new NIRS calibration models on lignin and ergovaline.

MATERIALS AND METODS

NIRS measurements

Reflectance spectra of dried and ground grass samples were obtained using a QFA-Flex 400 (Q-interline, Roskilde, Denmark) for the samples used to predict ergovaline and lignin and a NIRSystems 6500 (Foss NIRSystems, Silver spring, Maryland, USA) for the samples used to predict total N concentration. In both cases the spectra were collected in the NIR region from 1100 to 2500 nm with data collection at every 2 nm. The spectra are reported as log(1/Reflectance).

Measurement of total N, ergovaline and lignin

Plant samples were cut at soil surface in the period from the initiation of spring growth until approximately one month before seed harvest. Plant N concentration were analysed on aliquots of the samples using Dumas (Hansen, 1989). The amount of total N is expressed as % dry weight^{-1}.

Ergovaline was extracted, analyzed and quantified by high performance liquid chromatography (HPLC) with fluorescence detection. 50 mg samples were extracted with isopropanol/water/lactic acid (50:49:1) and subjected to a gradient separation on HPLC (at 40°C on a C18 column 5 μm 4.6 x 150 mm, with acetonitrile/aqueous 0.1 *M* ammonium acetate and acetonitrile/aqueous 0.1 *M* ammonium acetate and fluorescence detection). Ergotamine was used as internal standard. Ergovaline was quantified using peak area comparison to that of the ergotamine internal standard. The amount of ergovaline is expressed as μg g^{-1} dry weight.

Leaf samples from bulks of five clones from different perennial ryegrass genotypes were harvested, frozen in liquid nitrogen and freeze-dried. Following homogenization, the

lignothioglycolic acid method was used for determination of the lignin content as described by Müsel *et al.* (1997). The amount of lignin is expressed as mg g^{-1} dry weight.

Chemometric analysis of data

A partial least squares regression (PLSR) (Martens and Næs, 1993) was performed on the lignin data using The Unscrambler version 9.6 (CAMO A/S, Norway). Multiplicative Signal Corrected (MSC) NIR spectra (Geladi *et al.*, 1985) were used. The calibration model was validated using a segmented cross validation with four segments.

To test the possibility of making a calibration for ergovalin a principle component analysis (PCA) analysis (Wold *et al.*, 1987) was made on the NIR spectra. Sample scores were numbered according to ergovalin concentration; 0 = no ergovalin and 1 = ergovalin concentration above 0, thus facilitating visual evaluation of clustering according to concentration.

RESULTS

The MSC pre-processed NIR spectra used to develop the PLSR calibration model to predict total N concentration in grass samples are shown in Figure 1. Peaks are seen at 1510 nm, 1980 nm, 2050 nm and 2180 nm, which correspond to N absorption bands.
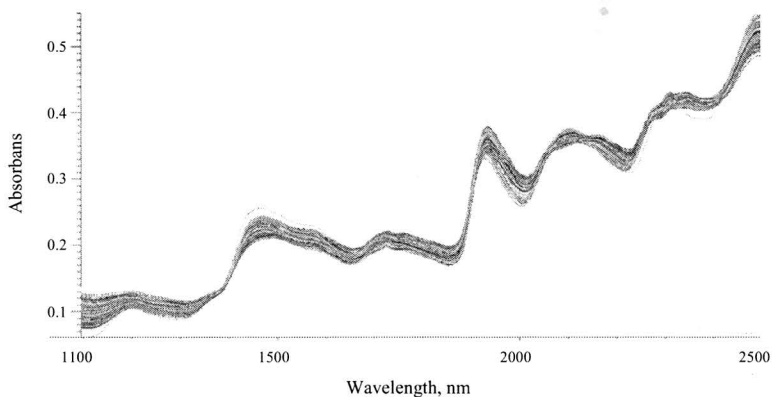
Fig. 1. NIR spectra (1100-2500 nm) of 849 samples used in the total nitrogen calibration model.

The correlation coefficient between measured and predicted lignin concentration was 0.64 with an error at 0.4 mg lignin g^{-1} dry weights. In the range from 3.6 to 5.8 mg lignin g^{-1} dry weights this equal 18 % (Figure 2).
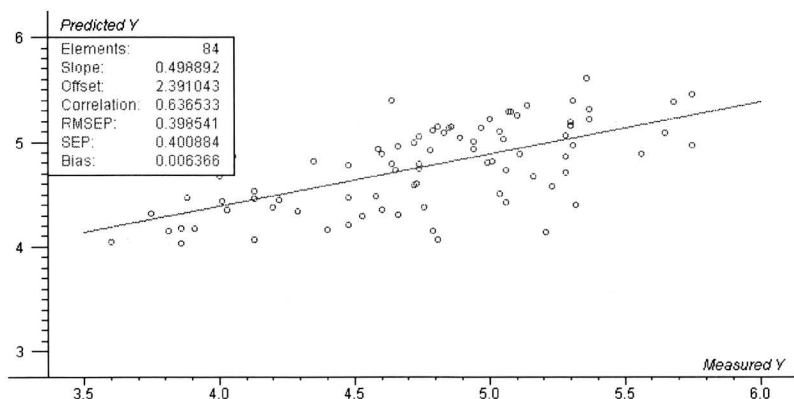
Fig. 2. Measured lignin concentration and predicted lignin concentration for the 84 samples. Lignin concentration is predicted from the MSC pre-processed NIR spectra (1100-2500 nm) using a PLSR model based on 9 PLSR components.

Most of the samples with an ergovaline concentration above 0 are clustered together, but samples without ergovaline are also in this cluster (Figure 3). The score-plot of the ergovaline data clearly shows that the information from the MSC pre-processed NIR spectra were not able to distinguish between the two groups (Figure 3).
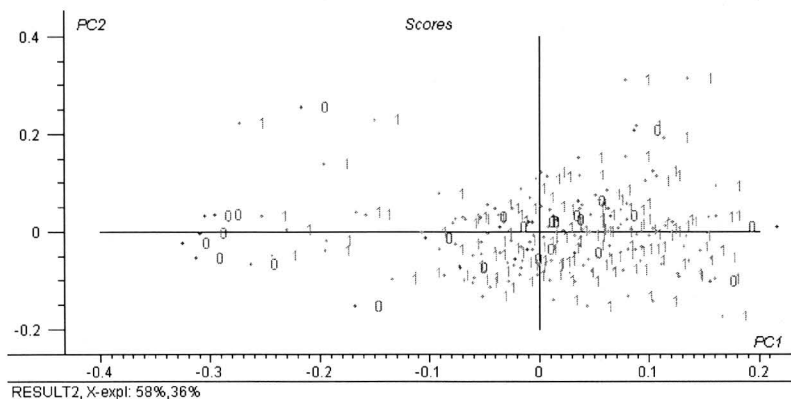
Fig. 3. Score plot of the first two principal components of MSC pre-processed NIR spectra of 167 plant samples.

DISCUSSION

A very important issue in the evaluation of a calibration model is to define if the current error on the NIRS calibration is acceptable. For the total N concentration the error is acceptable as described by Gislum *et al.* (2005). In the case of ergovaline the PCA scatter plot clearly shows that it is not possible to distinguish between samples with or without ergovaline. This is likely to be due to the fact that ergovaline is only present in small concentrations (ppm). This may be lower than the sensitivity of NIRS, which generally works best on constituents present in percentages, which is the case for total N. However, Roberts *et al.* (1997) showed correlations coefficients of 0.93 for the calibration model with a range in ergovaline from 0.05

to 0.50 ppm of ergovaline. We will continue our work on ergovalin and test the possibility of making local calibration models.

The error on the lignin PLSR model was 0.4 mg lignin g^{-1} dry weight in a range from 3.6 to 5.8 mg lignin g^{-1} dry weight which is not acceptable. In this case NIRS is not accurate enough to predict the lignin concentration. The range in lignin is low in the present experiment compared to e.g. Hodge *et al.* (2004) who showed a range from 22.2 to 32.3 mg lignin with standard error of prediction of 0.43 to 0.54 mg lignin. This may be the explanation for the two latter papers to make acceptable conclusions whereas we can not accept the error in the present experiment. It should be further investigated if the PLSR model can be improved by including samples with a larger variation in lignin concentration or by making local calibration models.

The nutritive value of forage depends not only on its chemical composition, but also on its digestive utilisation (Blaxter, 1956). To obtain the real biological value of forage and feed it is therefore also necessary to take the losses during the digestion, absorption and metabolism processes into account as described by Andrés *et al.* (2005b). A possible fast and low cost method to obtain the real biological value of the forage would be repeated spectroscopic measurements during these processes. In this case the proximity principle would have to be implemented. Even though the principle is quite problematic and several obstacles must be overcome before a method is developed, the importance of assessing the nutrient supply to the animal justifies further investigations.

A possible strategy for future NIRS work would be to use a combination of proximity and distance principles. The proximity principle can be applied for very robust calibration models. New calibration models require an error, which is as low as possible and development of new calibrations can be acquired using NIRS instrument nearby.

In conclusion, it is well documented that NIRS can be used for measuring clear N peaks as seen in the spectra and high correlation coefficients as we obtained in the calibration model. Others have been able to correlate NIRS with ergovaline and lignin, however these correlations could not be establish by the authors in the present work. It should be further investigated if the PLSR model on lignin can be improved and if it is possible to develop a PLSR model for ergovaline by including samples with a larger variation in lignin and ergovaline concentration or by making local calibration models.

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Forage quality of irrigated pasture species as affected by irrigation rate

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ABSTRACT

The USDA-ARS Forage and Range Research Lab has initiated a breeding program to improve tall fescue (*Festuca arundinaceae* Screb.), orchardgrass (*Dactylis glomerata* L.) and meadow bromegrass (*Bromus riparius* Rehm.) in semiarid pasturelands, which are characterized by extreme temperature fluctuations and limited water resources. An understanding of how irrigation level affects concentrations of crude protein (CP), neutral detergent fiber (NDF), digestible NDF (dNDF), and in vitro true digestibility (IVTD) is critical in pasture forage management. Cultivars of tall fescue, orchardgrass, and perennial ryegrass (*Lolium perenne* L.) were established under a line-source irrigation system to evaluate the effect of five water levels (WLs) and multiple harvest dates on CP, NDF, dNDF, and IVTD. Within all species, the most notable trend across WLs was a near linear increase in CP going from the wettest to the driest WL. Orchardgrass maturity (early vs. late) had little effect on forage quality across WLs. Tetraploid perennial ryegrass cultivars averaged higher concentrations of CP, IVTD, and dNDF and lower NDF values compared with diploid cultivars. Endophyte-free cultivars of tall fescue had lower NDF and higher IVTD concentrations than their endophyte-infected counterparts at the higher WLs.

Key words: crude protein, digestible neutral detergent fiber, in vitro true digestibility, neutral detergent fiber, orchardgrass, tall fescue, water levels

INTRODUCTION

Water use by forage crops is an important component of water management in the western United States. Forage crops account for 57% of the total irrigated area in the eight western states of Colorado, Idaho, Montana, Nevada, New Mexico, Oregon, Utah, and Wyoming (National Agricultural Statistics Service, 1998). Plant growth in most of these areas is frequently limited by the lack of soil water during later portions of the growing season. Forage plants that have adaptations that allow them to maintain plant growth with adequate nutritive quality under water-limiting conditions are critical to maintaining a constant source of high-quality forage throughout the growing season. This presentation reports on a series of line-source irrigation studies describing trends in nutritional quality with fluctuating irrigation levels across the growing season.

Forage quality has been defined as the relative performance of animals when forage is fed ad libitum (Buxton *et al.*, 1996). It is influenced largely by nutrient concentration, intake potential, and digestibility of the forage. In the absence of feeding trials, forage nutritive value is evaluated by measuring such characteristics as crude protein (CP), in vitro true digestibility

(IVTD), neutral detergent fiber (NDF), and digestible NDF (dNDF) (Asay *et al.*, 2002; Jensen *et al.*, 2003). Cell wall constituents are the major contributors to NDF (Fisher *et al.*, 1995). Laboratory measures of NDF are correlated with voluntary intake (Casler and Vogel, 1999). Fisher *et al.* (1995) reported that energy is closely correlated to dNDF in forage grasses.

The main objectives of the studies being presented were to determine trends in CP, NDF, dNDF, and IVTD in tall fescue, orchardgrass, and perennial ryegrass at five levels of irrigation and three harvest dates. Secondary objectives were to evaluate the effect of the endophyte fungus [*Neotyphodium coenophialum*] in tall fescue, diploid versus tetraploid perennial ryegrass, and early versus late maturing orchardgrass on these trends. These proceedings combine data from Asay *et al.* (2002) on tall fescue (CP, IVTD, and NDF) and Jensen *et al.* (2003) on orchardgrass and perennial ryegrass (CP, IVTD, dNDF, and NDF).

MATERIALS AND METHODS

The experiment was conducted under a line-source sprinkler design at the Utah State University Evans Research Farm located approximately 2 km south of Logan, UT (41°45' N, 111°8' W, 1350 m above sea level). Plant materials included cultivars of tall fescue, orchardgrass, perennial ryegrass, and meadow bromegrass, previously described by Asay *et al.* (2002) and Jensen *et al.* (2003). The line-source irrigation system is described by Hanks *et al.* (1980) and provides an environment to evaluate forage yield and nutritional characteristics across an irrigation gradient. The line-source sprinkler plot irrigation system produces a nearly linear water application pattern with irrigation declining with distance from the sprinkler line. Individual plots, consisting of six rows 15 cm apart and 15 m long, were drilled perpendicular to and on both sides of a line-source irrigation pipe. The grass entries were established in the plots as a randomized complete block with four replications, two on each side of the irrigation pipe. Plots were divided into five 2-m long subplots or WLs that were separated by 1-m alleys. Plots nearest to the sprinkler line, receiving the most water, were designated as WL-1, and those further from the sprinkler line were designated as WL-5. Combined over the study, WLs 1 to 5 received an average of 4.2, 3.7, 3.2, 2.8, 2.3 mm d^{-1}, respectively.

During the establishment year (1995), plots were irrigated as needed, and 112 kg N ha^{-1} were applied as a split application during midsummer and fall. After establishment year, fertilizer applications (56 kg N ha$^{-1)}$) were made prior to the first harvest and after Harvests 2, 4, and 6 in 1996 and 1997; and prior to the first harvest and after Harvests 2, 4, and 5 in 1998. Plots were harvested at the boot stage of plant development at the first harvest and thereafter when the regrowth height was 25 to 30 cm. Plots were harvested six times in 1997, and samples for determinations of forage quality were obtained at Harvest 2 (4 June), Harvest 4 (22 July), and Harvest 6 (23 September). Of the five harvests made in 1998, forage quality was determined at Harvest 2 (18 June), Harvest 4 (17 August), and Harvest 5 (1 October). These sampling dates were designated as early-, mid- and late-season for each year, respectively.

Samples for determination of forage quality were dried at 60°C, double-ground with a Wiley and Cyclone mills to pass through a 1-mm screen, and scanned with a Model 6500 near- infrared spectroscopy (NIRS) instrument (Pacific Scientific Instruments, Silver Spring, MD). The r^2 values for validation, computed from the six combinations of year and harvest, ranged from 0.96 to 0.99 for CP, from 0.82 to 0.93 for NDF, and from 0.72 to 0.91 for IVTD. Data were analyzed within and across years and WL using the GLM procedure (SAS Institute Inc., 1999) with grass entry, WLs, and year as fixed effects and replications as random. Mean separations were made on the basis of the Fisher's protected least significant difference (LSD) at the 0.05 level of probability. Linear, quadratic, and cubic trends of CP, NDF, and IVTD

across WL were determined for each cultivar using orthogonal polynomials with unequal intervals (Gomez and Gomez, 1984).

RESULTS

Under a line-source sprinkler design, perennial ryegrass consistently had higher CP levels than orchardgrass and tall fescue regardless of the irrigation level (Jensen *et al.*, 2003). A strong linear trend suggested increased CP as irrigation declined in perennial ryegrass, orchardgrass, and tall fescue (Asay *et al.*, 2002; Jensen *et al.*, 2003). That trend increased during the late season harvest (Figure 1 and 2) (Asay *et al.*, 2002; Jensen *et al.*, 2003). A possible cause of the increase in CP may be greater N content in drier soil, as with less intensive irrigation. Buxton *et al.* (1996) reported that CP concentration in forage is strongly influenced by available soil-N. Increased CP in tall fescue and perennial ryegrass was reported by Collins (1991) with increasing soil-N contents. Crude protein increased in maize (Crasta and Cox, 1996) and alfalfa (Halim *et al.*, 1990; Deetz *et al.*, 1996) when exposed to water stress. At each irrigation amount, tetraploid perennial ryegrass had higher CP than the diploids (Jensen *et al.*, 2003). Asay *et al.* (2002) reported no association between the presence or absence of the endophyte and CP.
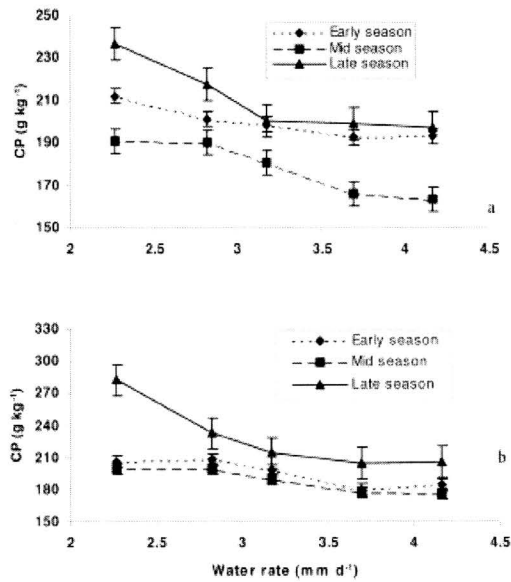
Fig. 1. Trends in crude protein concentrations across five irrigation amounts at three harvest dates (mid-June, July/August, Late-September): (a) orchardgrass and (b) perennial ryegrass. (Adapted from: Jensen et al., 2003).

Within each irrigation level, perennial ryegrass had higher dNDF concentrations than orchardgrass (Figure 2). Across irrigation amounts, the late-maturing orchardgrass cultivar Latar averaged 1% higher dNDF than the mean of the early-maturing orchardgrass cultivars (Jensen *et al.*, 2003). There was a general trend towards increased digestible fiber with decreased irrigation in perennial ryegrass and orchardgrass. At irrigation rates of 3.2, 3.7, and 4.2 mm d^{-1}, CP and dNDF were highly correlated (Jensen *et al.*, 2003) in perennial ryegrass

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and orchardgrass. At irrigation amounts of 2.8, 3.7, and 4.2 mm d⁻¹, tetraploid perennial ryegrasses had higher dNDF concentrations than the diploids (Jensen *et al.*, 2003).

At each irrigation rate, perennial ryegrass had higher IVTD than orchardgrass and tall fescue. As irrigation increased, trends towards lower IVTD values were observed, most notable at the mid-season harvest in orchardgrass; however, no consistent trends in IVTD were detected in perennial ryegrass and tall fescue with increased irrigation (Asay *et al.*, 2002; Jensen *et al.*, 2003). Endophyte-free Ky31 tended to have higher IVTD than its endophyte-infected counterpart with increased irrigation (Asay *et al.*, 2002). Bush and Burrus (1988), Collins (1991), and Emile *et al.* (2000) concluded that the presence of the endophyte does not consistently affect CP, NDF, or IVTD in tall fescue.

There were no apparent associations between irrigation amount and NDF concentration in perennial ryegrass, orchardgrass, and tall fescue (Asay *et al.*, 2002; Jensen *et al.*, 2003). In general, correlations between NDF and CP and between NDF and IVTD were negative at lower WLs. Tetraploid perennial ryegrass had lower NDF concentrations at irrigation rates of 2.3, 2.8, 3.2, and 3.7 mm d⁻¹ than diploids (Jensen *et al.*, 2003).
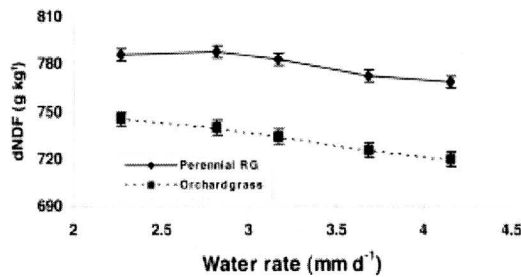
Fig. 2. Trends in digestible neutral detergent fiber (dNDF) of orchardgrass and perennial ryegrass across five water levels. (Adapted from: Jensen et al.*, 2003).*

DISCUSSION

The most notable trend in nutritional quality of forage grasses was a near linear increase in CP as irrigation rates declined. However, the increased nutritional value associated with water stress is in most cases negated by the associated reduction in dry matter yield with increased water stress. Due to positive correlations between CP, dNDF, and IVTD, similar trends were observed for dNDF and IVTD across different irrigation rates. Based on the literature, similar trends in nutritional quality were not as apparent in alfalfa and other legumes. As the amount of available irrigation water declines, it becomes critical that forage grasses and legumes be managed to achieve their optimal production and quality potential while minimizing the amount of irrigation water needed. If one is to be successful in utilizing forages, it is critical to match the appropriate forage species with the available water, soil texture, and management objectives.

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Association of candidate genes with flowering time and forage quality traits in *Lolium perenne*

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ABSTRACT

Association mapping is a complementary approach to mapping based on F_2 or backcross populations. It uses populations of unknown pedigree to exploit the recombination events that have occurred over many generations, in theory enabling a more refined mapping than is usually possible with conventional mapping families. Many of the challenges with this approach include correction for multiple testing, distinguishing real associations from false ones, and genetic structure in populations. We have used a candidate gene approach to association mapping in perennial ryegrass (*Lolium perenne*), since this minimises some of these issues. We have used 864 genotypes originating from nine populations to find associations between single nucleotide polymorphisms (SNPs) and flowering time, water soluble carbohydrates (WSC) and other forage quality traits. Some of the alleleic variants in the neutral/alkaline invertase gene *LpcAI* associated with WSC, nitrogen content (N) and dry matter digestibility (DMD), but none were found in phenotypic data from both years. In contrast, a SNP located 265 bp upstream of the homologue in *L. perenne*, *LpHD1*, of the photoperiod control gene *CONSTANS* in *Arabidopsis thaliana* and *HD1* in rice, was consistently associated with flowering time. We also found a significant association between allelic variants at one locus at the homologue of the *HD3a* gene in rice (*FT* or flowering time locus in *A. thaliana*). We have attempted to verify these associations by crossing selected genotypes from the association mapping population with genotypes of late flowering turf grass varieties. The "late allele" in the *LpHD1* locus appeared to delay flowering by 2-3 days. The effect of alleles at the *HD3a* locus was more difficult to interpret due to the presence of multiple alleles, but "late alleles" did appear to delay flowering by 2-3 days as well. Furthermore, genotyping of populations off or on paths appear to support the effect of the late alleles at these two loci. These results are encouraging for the prospects of association mapping at least for some traits.

Key words: association mapping, dry matter digestibility, heading date, linkage disequilibrium, nitrogen, perennial ryegrass, water soluble carbohydrates

INTRODUCTION

Association mapping based on linkage disequilibrium (LD) is an approach to mapping, in which populations in principle with unknown or complicated pedigree, are used. The advantage is that the many meioses that these populations have undergone, allows a more refined mapping resolution than F_2 or backcross populations. The extent of this advantage depends upon the breeding system. While there is likely to be intra-genomic variation in

extent of LD, effective recombination is generally lower in inbreeding species leading to more extensive LD than in self-incompatible species such as perennial ryegrass (*Lolium perenne*). Whole genome association studies are most likely to be successful in species with extensive LD, so that haplotype tagging can be used to minimise the number of markers needed for adequate coverage of the genome. In plants, it has been used in the inbreeding model species *Arabidopsis thaliana* (Aranzana *et al.*, 2005), but there are challenges with this approach, particularly correction for multiple testing. By focussing on specific candidate genes, the size of this problem is reduced, but the approach is dependent upon having suitable candidates to test.

In perennial ryegrass flowering time and water soluble carbohydrate content are important heritable traits, due to their fundamental influence on plant growth and development, as well as forage quality. They are thus targets for genetic improvement. The self-incompatible breeding system of perennial ryegrass would suggest that LD decays rapidly. This, and the size of its genome, makes it impracticable to obtain adequate coverage with a whole genome association analysis. A number of candidate genes are available for both traits. The perennial ryegrass orthologues of the photoperiod control gene *CONSTANS* and the *FT* (flowering time locus) in *A. thaliana* (the *Hd1* and *Hd3a* genes in rice) are closely linked and located within a QTL on linkage group (LG) 7 with a major effect on flowering time (Armstead *et al.*, 2004, 2005). They are therefore obvious candidate genes for association analysis of this trait. A number of genes encoding enzymes with likely involvement in metabolism of the major soluble carbohydrates have been identified in *L. perenne*, including fructosyl-transferases, fructan exohydrolases and invertases. In this work we focus on a soluble neutral/alkaline invertase, which is located on LG 6 within a QTL for glucose and fructose content (Gallagher and Pollock, 1998). Here we describe an association analysis of flowering time and WSC with these candidate genes, and subsequent attempts to validate significant associations of flowering time and allelic variants in the candidates.

MATERIALS AND METHODS

Plants were grown in a completely randomised design in 6 inch diameter pots in a polytunnel for the first year, and tillers were planted in the field as spaced plants for the second year's phenotypic analyses. A total of 864 genotypes were used, encompassing 96 from each of nine populations, which represented a wide spectrum in flowering time. WSC, N and DMD analyses was performed by near infrared reflectance spectroscopy. Extraction of DNA and analysis of amplified fragment length polymorphism (AFLP) was carried out as described (Skøt *et al.*, 2005). An ABI 3100 Genetic Analyzer was used for DNA sequencing. The TaqMan assay or KBioscience (http://www.kbioscience.co.uk) was used for SNP genotyping.

The STRUCTURE program (Pritchard *et al.*, 2000) was used for analysis of population structure, and PHASE (Stephens *et al.*, 2001) for inferring haplotypes. Treescanning (Templeton *et al.*, 2005) was used to perform the haplotype based association analysis.

RESULTS AND DISCUSSION

A two-way analysis of variance (ANOVA) showed that there were significant differences between the nine populations, between years and population x year interaction for flowering time, WSC, N and DMD. The association analyses were therefore carried out for each year separately. The population structure analysis separated the 864 genotypes into nine clusters with little evidence of ancestry from more than one cluster. The nine inferred clusters coincided with the nine given populations. Further analysis indicated that there was little structure within populations. Three strategies were used to find associations: within

population ANOVA, a linear mixed model and a haplotype tree analysis. Several of the 15 SNPs that were genotyped within the neutral/alkaline invertase locus were associated with WSC, N or DMD in some of the populations in one or other of the two years according to the ANOVA analysis, but none of them were consistent. Similarly, one SNP was significantly associated with WSC in one year in the linear mixed model analysis, and no significant associations were found in the haplotype tree analysis.

In contrast, all three analysis methods highlighted a significant association between flowering time and a C/A polymorphism located 265 bp upstream of the translation start site of the *LpHD1* gene. The C allele associated with earliness, and the A allele with lateness. For the haplotype tree analysis, the PHASE program was used to infer 17 haplotypes from the eight SNPs analysed in and around the *LpHD1* gene, and the most likely diplotype for each genotype was used as input in the Treescanning program. The Treescanning analysis extended this to include another SNP a further 2326 bp upstream. These two SNPs were also in significant pairwise LD. The analysis of the *Hd3a* gene included only one multi-allelic polymorphism, which obviously precluded a haplotype tree analysis, but the ANOVA and the linear mixed model both indicated a highly significant association with flowering time. Five alleles were detected, with alleles a and b associated with lateness, c with earliness, while d and e did not have a significant effect on flowering time.

Table 1. *Flowering times of progeny derived from two crosses between genotypes from the association mapping population and late flowering genotypes. Cross 1 was Ba10732 (CC/ce) x CA/aa) (turf genotype 'Bargold'). Cross 2 was Ba10732 (CC/ae) x (CA/bb) (turf genotype 'Hugo'). The* HD1 *alleles are capitalised. Flowering times are mean values in days after April 1st. Allelic effects are significant (P < 0.05).*

	Cross 1: (CC/ce) x (CA/aa)				Cross 2: (CC/ae) x (CA/bb)			
Genotype	CC/ac	CC/ae	CA/ac	CA/ae	CC/be	CC/ab	CA/be	CA/ab
Flowering time	36.6	38.1	39.0	40.4	45.5	47.5	46.4	49.3
Genotype	CC	CA	ac	ae	CC	CA	ab	be
Flowering time	37.3	39.6	37.6	39.2	46.3	48.1	45.9	48.3

Two crosses were carried out in an attempt to validate the associations with flowering time. Both crosses involved a genotype from the association mapping population Ba10732, and late flowering turf grass genotypes. The crosses are listed in Table 1 together with the flowering time phenotypes of the progeny. There are modest, but significant effects of early and late alleles at both the *HD1* and *Hd3a* loci. The allelic effects at each locus were approximately two days. The effects are much smaller than what we observed for the total population, where for example, the effect of an C/A allelic change is at least 10 times larger. This illustrates the importance of population structure.

More support for the effect of the alleles at the *HD1* and *Hd3a* loci was obtained by genotyping two pairs of populations from on or off paths in Poland and the UK. The on path population from Poland was 15 days later in flowering time, while the difference in the UK pair was 6-8 days. In the Polish pair the off path population was homozygous for the CC genotype at the *HD1* locus, while the genotypic frequencies were 0.5, 0.4 and 0.1 for the CC, CA and AA genotypes, respectively in the on path population. Similarly, there was also a

significant shift towards the two late alleles a and b in the on path population at the *Hd3a* locus. The same trend was apparent in the UK pair, but less dramatic.

The results reported here are encouraging and suggest that even in the presence of significant population structure, association analysis in self-incompatible plant species can be successful, at least for certain traits. Its high heritability makes the flowering time trait a useful one in this respect.

ACKNOWLEDGEMENTS

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Genetic and seasonal variations of fibre content in lucerne

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ABSTRACT

The digestability (quality) of lucerne depends on its fibre content. Its level is dependant on the stage of lucerne development and environmental conditions and on genetic factors (genotype) as well. The objective of this paper was to determine the levels of crude fibre (CF), neutral detergent fibre (NDF), and acid detergent fibre (ADF) in five lucerne varieties developed at the Novi Sad institute for different purposes and using different breeding methods. The CF content varied significantly according to cut and was lowest in the cooler part of the year (331.3 g kg^{-1}, 1st cut) and highest in the warmer part (413.6 g kg^{-1}, 3rd cut). Low crude fibre contents were found in the varieties Danka and NS Alfa. The NDF content was also lowest in the 1st and highest in the 3rd cut (411.9 g kg^{-1} and 508.3 g kg^{-1}, respectively). The lowest NDF content and highest animal intake were recorded in the variety Danka (454.3 g kg^{-1}), followed by NS Mediana ZMS V (472.0 g kg^{-1}), and then NS Alfa (477.3 g kg^{-1}). The highest NDF content was found in Niagara (486.7 g kg^{-1}) and Banat VS (481.7 g kg^{-1}).

The lowest (320.9 g kg^{-1}) and highest (409.3 g kg^{-1}) ADF contents were also recorded in the cooler and warmer parts of the year, respectively. The lowest ADF content was measured in Danka (374.4 g kg^{-1}) and the highest in NS Alfa. The variety Niagara had the highest hemicellulose content (NDF-ADF), while Danka and NS Alfa had the lowest (79.9 and 82.1 g kg^{-1}, respectively).

Key words: ADF, CF, genotype, lucerne, NDF

INTRODUCTION

In addition to increasing yield, increased forage quality is another top goal of lucerne breeding. Structural carbohydrates (fibre) are less digestible because of their complex chemical bonds, so decreasing their levels will lead to improved forage quality. Riday *et al.* (2002) argue that the genetic variation of fibre content is one of the main reasons for variation in forage quality. Variation in lucerne forage digestibility and forage intake is correlated with the variation in the cell wall content. The cell wall, i.e. fibres not soluble in neutral detergent (NDF), contains cellulose, lignin, hemicellulose, protein, wax, cutin, minerals, and pectin, all of which are 40-70% digestible by fibre digesting bacteria. Fibre is food for the rumen microbes, and help the animal maintain rumen health (cud chewing; saliva, higher rumen pH) (www.cfap.org).

The acid detergent fibre fraction (ADF) consists of cellulose and lignin. The secondary cell wall comprises a web of cellulose fibres embedded in an amorphous matrix comprised of hemicellulose, lignin and pectin.

Cellulose is the main plant structural polysaccharide and quantitatively the most important biological substance, as it accounts for more than 50% of all organic compounds found in the biosphere. Cellulose is a glucose polymer with elongated molecules, and hydrogen bonds that form between the adjacent molecules create a microfibril structure, which contributes to resistance to extension and gives plants their elasticity but is also resistant to digestion. Incorporation of lignin into the cell wall produces resistance to extension (cellulose) and resistance to pressure (lignin), often drawing comparisons to reinforced concrete. Lignification takes place in cells performing a specific function, such as transport of water or mechanical support. However, lignin is the main factor limiting cell wall digestibility, as it inhibits the digestibility of polysaccharides.

Ideally, lucerne forage should have digestibility of about 80% and a neutral detergent fibre (NDF) content of 300-360 g kg $^{-1}$, but this is seldom found in practice (Riday *et al.*, 2002). Lennsen *et al.* (1991) reported significant differences in NDF content among nine lucerne germplasms from North America. Breeding lucerne for higher digestibility and a lower lignin concentration produces higher leaf/stem ratios and lower NDF and ADF contents.

Sheaffer *et al.* (1998) found significant differences in NDF and ADF contents among lucerne cultivars of low, medium and high quality. Hall *et al.* (2000) reported that selection in lucerne for improved quality is successful without the unwanted correlated selection responses of slower development and maturity. Rotili *et al.* (2001) suggested a change of plant morphological structure in order to obtain reduced internode length and an increased leaf/stem ratio. Lamb *et al.* (2006) found no link between NDF content and the time a particular cultivar was developed during the 1940-1995 period in the U.S.

The objective of this paper was to study the structural carbohydrate content of five lucerne cultivars in cuts 1 through 4 of the second year of cultivation.

MATERIALS AND METHODS

Forage quality was studied using five lucerne varieties developed at the Institute of Field and Vegetable Crops in Novi Sad, using different breeding methods and directions.

Field trials with five replicates were conducted in 2005/2006 at the Experiment Field of the Institute of Field and Vegetable Crops in Novi Sad (45° N, 19° E, 80 m altitude). The soil was a chernozem with good physical properties and neutral soil reaction (pH 7.25 in KCL) containing 2.36% CaCO, 0.154% N, 21.81 mg P_2O_5/100g soil, and 31.2 mg K_2O/100 g soil in the 0-30 cm layer.

The average annual temperature in Novi Sad is 11.0°C, while the annual rainfall sum is 597 mm. For chemical analyses the samples were taken in the second year of cultivation (2006) from the second and fourth replicates of the four cuts (40 samples). The samples were cut at 5 cm height and the sampled area was around 0.02 m^2, amounting to 500 g of green forage. Herbage materials were dried at 60°C for about 48 hours. Particular attention was paid to making sure that the samples were homogenized and ground to a particle size of $\varnothing$ = 0.8 mm. Chemical analyses were carried out using the standard methods, namely the AOCS-approved Ba 6a-05 procedure for crude fibre (CF) and the Filter Bag Technique by Ankom Technology Method for neutral detergent fibre (NDF) and acid detergent fibre (ADF). All of the analyses were performed on a Fibre Analyzer Ankom 2001 (Ankom, USA).

Table 1. Date of cutting and seasonal variation of meteorological parameters during 2006.

Cut	Date of cutting	Number of days between cuts	Sum of rainfall (mm)	Mean daily temp.°C	Mean max. temp.°C	Mean min. temp.°C
I	5/5	45	107.0	13.5	18.2	3.1
II	17/6	42	166.2	16.2	25.4	10.8
III	13/7	27	23.4	24.1	28.0	19.1
IV	18/8	36	87.1	22.1	27.0	11.4

The two-factorial analysis of variance was used with cultivar as factor A and cut as factor B. The LSD test was used for testing the significance of differences.

RESULTS

The results of the present study indicate the presence of significant genetic variation in the fibre (structural carbohydrate) content of lucerne (Table 2). Lower CF contents and hence better forage quality were found in the cultivars Danka and NS Alfa. Today, it is ADF that is more often used as the indicator of fibre content because of its greater accuracy. In our study, the cultivars Danka, Niagara and Banat VS had the lowest ADF contents. The lowest NDF content was observed in the cultivar Danka, meaning that the forage of this cultivar had the best quality and would be preferred by livestock.

When it came to the hemicellulose (NDF-ADF) content of forage, the cultivars Niagara and Banat VS stood out (Table 2). Hemicellulose is more easily digestible, so its higher content may be indicative of better quality.

Table 2. Variation of lucerne genotypes in fibre content (g kg^{-1}) during 2006 (4 cuts).

Cultivar	CF	ADF	NDF	Hemicellulose (NDF-ADF)
Niagara	393.1	375.4	486.7	111.3
Banat VS	395.9	375.9	481.7	105.8
Mediana	394.7	389.7	472.0	82.3
NS Alfa	367.7	395.2	477.3	82.1
Danka	362.6	374.4	454.3	79.9
CV%	5.88	7.95	5.56	22.9
0.05	11.0	13.0	14.0	7.0
LSD 0.01	15.0	17.0	18.0	9.0

Our results also showed that there was significant variation in fibre content among the different cuts during the season (Table 3). The CF, NDF/ADF, and hemicellulose contents were highest in the second and third cuts, i.e. in the summer during the driest and warmest part of the season (Table 1). Significantly lower CF and NDF/ADF levels were recorded in the first cut in the spring, when temperatures were lowest. The hemicellulose content was lowest in the fourth cut coinciding with lower temperatures. This content may be affected by day length and illumination.

Table 3. Seasonal variation of fibre content in lucerne (g kg $^{-1}$) during 2006.

Cut	CF	ADF	NDF	Hemicellulose (NDF-ADF)
1	331.3	320.9	411.9	72.7
2	396.3	401.6	499.3	77.1
3	413.6	409.3	508.3	78.0
4	390.1	396.7	478.2	64.3
CV%	7.9	7.9	5.6	92.2
0.05	10.0	12.0	12.0	6.0
LSD 0.01	14.0	16.0	16.0	8.0

The correlation coefficients showed interdependence among CF, NDF and ADF contents and no significant correlation between hemicellulose content and the other three traits (Table 4). The fibre (structural carbohydrate) content of lucerne forage can be estimated by determining either one of three of the above parameters, namely CF, ADF, or NDF. Hemicellulose content cannot be used for such purpose.

Table 4. Correlation coefficients among CF, NDF, ADF and hemicellulose (20 means).

	CF	ADF	NDF	Hemicellulose
CF	-	0.78	0.83	0,13
ADF		-	0.87	-0.22
NDF			-	0.28
Hemicellulose				-

DISCUSSION

Knowing the genetical and seasonal variation of the fibre content of lucerne forage is an important part of the strategy for developing new, high quality cultivars of this crop.

In Sheaffer *et al.* (1998), lower-quality cultivars differed significantly in ADF and NDF contents from medium- and higher-quality ones. Odoardi *et al.* (2001) suggests that lucerne cultivars can have higher yields and forage quality than the traditional varieties of this crop.

Breeding lucerne for a lower lignin concentration and higher digestibility, respectively, Kephart *et al.* (1990) and Shenk and Elliott (1971) obtained higher leaf/stem ratios and lower NDF and ADF contents.

According to Sheaffer *et al.* (1998), lucerne cultivars have been developed more recently that are resistant to diseases attacking this crop. Increased disease resistance increases forage yields and quality under environmental conditions promoting the development of diseases. However, when environmental conditions are not favourable for disease development, no

differences in yield and quality were observed among the cultivars. It is possible that the cultivars we studied have different levels of disease resistance.

Lucerne forage from the third cut in our study had higher CF, NDF and ADF contents than that from the first, second and fourth cuts. According to Hall *et al.* (2000), faster lucerne growth over the summer compared to spring or autumn may contribute to a lower quality of the forage obtained in the summer. Changes occurring in the chemistry of the cell wall as a result of higher temperatures contribute to the decreased quality of forage during the summer (Griffin *et al.*, 1994). The seasonal variation of fibre, NDF and ADF levels was under the influence of higher air temperatures and lower soil moisture. However, part of variation may also have been a result of the faster growth and maturity of lucerne plants in the summer. Hemicellulose content is at its lowest in the autumn, possibly due to a drop in temperature and a shortening of day length and illumination.

Significant positive correlations among CF, NDF and ADF levels were obtained. According to Sheaffer *et al.* (1998), significant correlations exist between NDF and ADF contents independent of cultivar, location or year. Fibre content could be determined using either one CF, NDF or ADF independant of cultivar, location or cut.

The determination of cellulose, lignin and hemicellulose contents in lucerne is important to predict forage quality and digestibility. However, these substances take part in the formation of the cell wall and vascular and mechanical tissues and give lucerne plants their elasticity and stature. Because of this, breeding for lower levels of these substances may have some undesirable effects, such as more lodging, reduced plant height and lower yield.

Selection for lower levels of fibre should be optimized so as to achieve maximum digestibility (quality) whilst retaining satisfactory levels of plant elasticity and lodging resistance.

Breeding for improved lucerne quality is a worthy challenge, because the development of higher-quality lucerne cultivars reduces the need to buy concentrates and thus contributes to increased economy of livestock production.

CONCLUSIONS

The genetic variation of CF, ADF, NDF and hemicellulose levels among lucerne cultivars was significant. The forage cultivar Danka was found to have the best quality, as it had the lowest levels of the above fibre components.

CF, ADF and NDF levels were higher in the summer because of the higher temperatures and more rapid plant development.

Correlations among CF, ADF, and NDF contents were significant, so either one of these three indicators could be used for fibre content determination in lucerne.

Fibres are less digestible for animals but they are important components of the cell wall and vascular and mechanical tissues.

Breeding for optimum fibre content should strike a balance between digestibility and plant elasticity and resistance to lodging.

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Biomass QTL analysis in a perennial ryegrass inbred line derived population

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ABSTRACT

A genetic map of perennial ryegrass (*Lolium perenne*) was constructed from SSR and AFLP markers based on a large inbred line derived F2 population. The F1 plants showed strong heterosis for biomass. We aimed to map quantitative trait loci (QTL) for biomass heterosis on the F2 population. An alpha lattice design was used for both greenhouse and field experiments. In the greenhouse three independent replicates were planted as single plants and three harvests were done from the experiment. In the field, the genotypes were planted in mini swards in two replicates and two harvests were carried out in 2006 and two in 2007. In preliminary analysis, three QTL areas were identified on linkage group 2, 3 and 7 with LOD scores between 5 and 10. The percentage of explained variance ranged between 5% and 20%. QTL for biomass is an important agronomic trait and increased biomass is still one of the most important traits in perennial ryegrass breeding programmes. Furthermore, with the interest in biofuel crops, understanding the genetic basis of biomass production is important not only for animal feed production but for potential fuel use. This study gives an indication of the position of biomass QTL and will benefit future molecular breeding programmes.

Key words: *Lolium perenne*, Quantitative trait loci, yield, heterosis, mapping

INTRODUCTION

Biomass yield is one of the most important agronomic traits in forage grasses and is controlled by quantitative trait loci (QTL). QTL analysis requires the availability of a genetic map and phenotypic data for the trait of interest. Because of the outbreeding nature and high level of heterozygosity in perennial ryegrass the action of recessive genes remains often undetected. Using a segregating population based on two highly inbred parents would enable the detection of recessive loci contributing to the expression of certain traits. In a study of Yamada *et al.* (2004) in a heterozygous perennial ryegrass mapping population QTL for biomass yield were found on linkage groups four and five. However only a proportion of the phenotypic variation was explained, thus other genomic regions contributing to biomass yield are still to be discovered. The aims of this study were to (1) identify QTL locations for biomass heterosis, (2) compare data from different environments, harvests and replications and, (3) provide breeders with knowledge of genomic areas controlling biomass yield in ryegrass genome for future molecular breeding programmes.

MATERIALS AND METHODS

SSR and AFLP markers were mapped on a linkage map based on 360 genotypes of a F2 inbred line derived population. A single F1 plant was raised and self-pollinated in pollination bags to generate the segregating F2 population.

For the biomass harvests, experiments were set up with three independent replicates of single plants in the greenhouse. Three harvests were done for the greenhouse experiment. Two replicates were planted as mini swards in the field consisting of six clonal replicates each per plot. Four harvests were carried out in the field over a period of two years. An alpha lattice design was used for the greenhouse and field experiments. Dry weight, dry matter content, fresh weight and leaf width were measured and heading date was recorded. Estimates were calculated for all traits using PROC MIXED in SAS® 9.1 software (SAS 2004). The linkage map was calculated with JoinMap® 3.0 (Van Ooijen and Voorrips, 2001) and QTL locations were identified with MapQTL® 4.0 (Van Ooijen et al., 2002) using Interval Mapping and Multiple QTL Models (MQM) mapping. We focused on dry weight biomass as a trait since this trait can be measured most accurately excluding influences of dampness in the morning or different weather conditions during the harvests.

RESULTS

SSR and AFLP markers were used to construct a perennial ryegrass genetic map. A genetic map with a total length of 600 cM and an average marker density of 8 cM was developed.

Three QTL locations for biomass yield were identified on linkage group 2, 3 and 7 with LOD scores between 5 and 10. In preliminary analyses, the percentage of explained variance ranged between 5% and 20%. Overall 30% of the variance for biomass were explained as suggested by the QTL analysis, and the QTL positions were consistent over, multiple replications, locations and harvests.
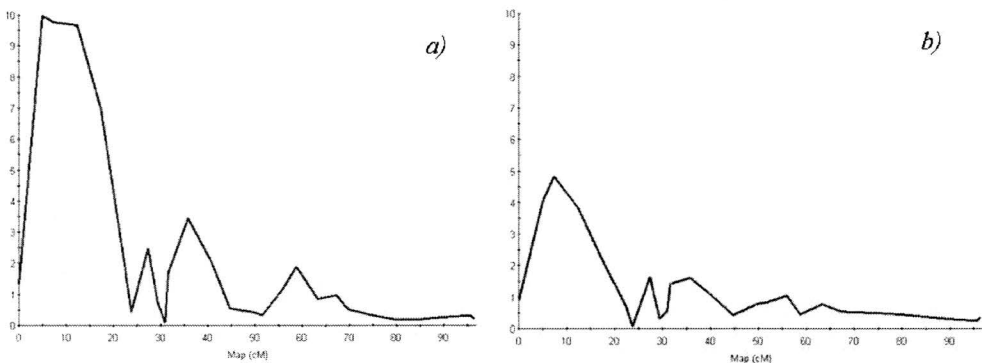

Fig. 2. Position of dry weight biomass QTL (LOD scores) on LG 3 generated in MapQTL® 4.0 using MQM mapping function for two harvests in the greenhouse and two in the field in 2006. a) Dry weight biomass QTL for greenhouse experiment and b) dry weight biomass QTL for field experiment.

DISCUSSION

This study provides insights in genomic regions contributing to biomass yield in perennial ryegrass. Yamada *et al.* (2004) had previously identified biomass QTL for fresh weight which explained 13% (LOD 3) of the variance for the QTL on LG 4 and 23% (LOD 6) for the QTL on LG 5 in the ILGI p150/112 population derived from a cross between a di-haploid plant and a hybrid F1 plant. Our study complements the study of Yamada *et al.* (2004) indicating additional QTL for biomass yield. In our study QTL were stable across environments and replications. As demonstrated by comparable heritability values, assays based on single plants were as valuable as assays derived from genotypes grown in mini swards. This demonstrates the robustness of the presented data. The future prospect of this study will be the fine mapping of the QTL in the F2 generation and the identification of single markers linked to the QTL for biomass yield, re-mapping of biomass QTL in recombinant inbred lines, and comparative genomics approaches. Furthermore, with the interest in biofuel crops, understanding the genetic basis of biomass production is important not only for animal feed production but for potential fuel use. Future molecular breeding programmes will benefit from the knowledge of this study to breed for enhanced biomass yield.

ACKNOWLEDGEMENTS

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Quality traits for bioenergy in grasses

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ABSTRACT

The potential of grasses for energy is limited because plant varieties have not been selected for this purpose. There are distinct challenges to determine and improve quality traits to increase ultimate energy yields. Perennial grasses offer the potential to be utilised through either thermal or biological conversion methods to generate heat, electricity or transport fuels; the route chosen being largely determined by the calorific value, moisture content and the availability of carbohydrates. Chemical composition underlies these characteristics and can be measured to associate phenotype to genotype. For such studies it is necessary to develop both molecular markers in candidate genes and high throughput methods for phenotyping of composition. In the EU project GRASP, SNP based markers have been developed in carbohydrate associated genes which map to water soluble carbohydrate (WSC) quantitative trait loci (QTL) and these have been used in association studies in a synthetic population of perennial ryegrass to measure allele shifts when under selection pressure for high and low WSC content. Furthermore infrared spectroscopy methods, calibrated with wet chemistry, have been developed in a number of energy grasses to predict lignin, cellulose and hemicellulose contents. These calibrations can enable forecasts of quality and subsequent conversion efficiency in energy grasses.

Key words: cell walls, cellulose, water soluble carbohydrate, hemicellulose, lignin, infrared spectroscopy, QTL, SNP

INTRODUCTION

The international concern over the impact of global warming has lead to the demand that traditional fossil fuels should be replaced, where possible, with renewable forms of energy, thereby reducing global carbon emissions. Furthermore, many countries are dependent on energy supplies that are largely beyond their control, and so have identified the adoption of renewable sources as a strategy for increasing their fuel security. Several grass species have been identified as appropriate for the production of bioenergy, and scientific resources are now being focused on improving the agronomic performance and chemical constituents of these crops to make them realistic alternatives to gas, oil and coal. For example, the amount of energy released from a given quantity of biomass can be increased by tailoring the chemical composition of the crop. Thus, there are obvious parallels between the improvement of biomass composition quality, in energy crops and forages. Biomass can be thermally converted using combustion, gasification or pyrolysis into heat, electricity or liquid fuels, or

alternatively be fermented to alcohols, methane or hydrogen. Plant biomass with a high calorific value is more suited to thermal conversion, whilst biomass with more available sugars derives a greater range of fermentation products. Such differences in calorific value and available sugar content is directly related to plant chemical composition, and varies both between and within crops depending on the age of the plant, the time of year and the variety grown. Breeding programs rely on allelic variation between genotypes. In order to exploit this natural variation, DNA based genetic markers can be identified to enable accelerated marker-assisted selection of suitable individuals for breeding. Many of the important traits which are targets for grass plant breeding are complex with a number of quantitative trait loci (QTL) controlling phenotype.

A number of perennial grasses are suitable as energy crops including traditional temperate forage grasses such as *Lolium perenne* and also tropical giant grasses such as *Miscanthus*. In the Engineering and Physical Sciences Research Council (EPSRC) funded Sustainable Power Generation (SUPERGEN) project (http://www.supergen-bioenergy.net), the effect of crop biomass quality on downstream thermal conversion processes are being assessed (Fahmi *et al.*, 2007a, b). In this project, high throughput methods to measure plant cell wall composition in grasses, including *Lolium* and *Miscanthus* species have been developed. For components such as lignin, which are present at relatively high concentrations in biomass, analytical methods based on infrared spectroscopy with chemometric data analysis enable the rapid assessment of many samples by reducing the requirement for time consuming wet chemistry.

MATERIALS AND METHODS

SNPs for Marker Assisted Selection

A linkage map of *L. perenne* based on an F2 mapping population was generated for the genetic analysis of water soluble carbohydrate (WSC) accumulation, and has been used to identify quantitative trait loci (QTL) associated with WSC in the leaves and leaf sheaths of *L. perenne* (Turner *et al.*, 2006). Interestingly, some QTL are already known to overlie or fall close to the location of genes with known function identified in earlier studies (eg. Gallagher *et al.*, 2004). The most reliable and straightforward approach to clone genes underlying such QTL is by map-based cloning, which is dependent on the availability of suitable large insert DNA libraries. In the EU project GRASP (http://www.grasp-euv.dk), a bacterial artificial chromosome (BAC) library of *L. perenne* (Farrar *et al.*, 2007), was constructed and has allowed the isolation of upstream and other non-coding regions in candidate genes for the identification of SNPs. Gene specific primer pairs were designed using information in DNA databases and their utility tested by PCR using a template of *L. perenne* genomic DNA followed by DNA sequencing of candidate amplimers. Validated primers were used to identify BAC clones containing genes of interest in a PCR based screen. Full length gene sequences including promoter and intron regions were obtained and new primers pairs designed to allow amplification of internal sequences for the identification of SNPs. Each of these primer pairs was used to amplify the respective alleles from 20 diverse *L. perenne* genotypes and alignment of the sequences revealed molecular markers including SNPs. These markers are being used to trace shifts in allele frequency in *L. perenne* populations undergoing selection pressures for high and low WSC.

Analytical Chemistry and Infrared Spectroscopy

A total of 366 individual samples taken from *Miscanthus* plants originating from the European *Miscanthus* Improvement (EMI) project (Clifton-Brown *et al.*, 2001) were oven dried and ground to pass a 1 mm screen. The EMI project comprised 15 different replicated *Miscanthus* genotypes grown in five countries across Europe, sampled on two harvest dates. These 15 genotypes included the most commonly planted genotypes, *M.* x *giganteus,* together with representatives of the two parental species of this hybrid, *M. sinensis* and *M. sacchariflorus*. All 366 samples were scanned over the NIR spectral range (1100 to 2500 nm at 2 nm intervals) using a NIRSystems 6500 spectrophotometer, operating with WINISI^{TM} software (FOSS UK Limited, Warrington, UK). On the basis of spectral data a representative subset of 76 samples was selected and analysed to determine lignin, acid detergent fibre (ADF) and neutral detergent fibre (NDF) concentrations in the dry matter (DM). Lignin content was determined using the modified Van Soest (1963) method. Ash free, ADF and NDF contents were determined by the methods described by Van Soest *et al.* (1991) using the Fibrecap system (Kitcherside *et al.*, 2000). In the case of NDF, both amylase and sodium sulphite were omitted.

To predict the concentrations of biomass components by infrared spectroscopy methods, it is necessary to first calibrate spectral data from a range of samples of dried and ground plant material (Figure 1) against chemical assessments of cell wall component contents made in the laboratory using wet-chemical techniques.
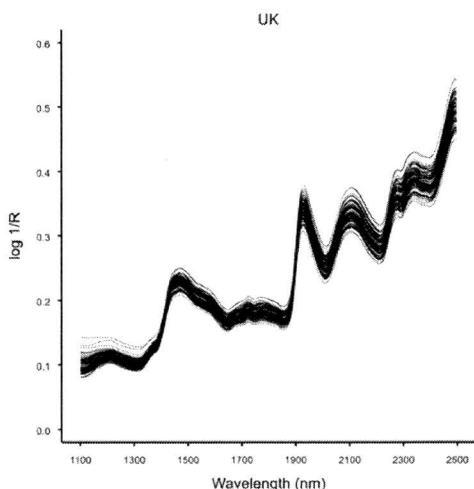

Fig. 1. Examples of NIRS spectra from Miscanthus.

RESULTS

Molecular Markers

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                 400        *        420         *        440         *        460
Lolium1a  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium1b  : GCTCGGTGATGTACGTATATGGGTAAAAACACCGCTGGCTTGTGCATATATAT--ATATATAGATAATGACGCAAGTT : 464
Lolium2   : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium3   : GCTCGGTGATGTACGTATATGGGTAAAAACACCGCTGGCTTGTGCATATATATATATATATAGATAATGACGCAAGTT : 466
Lolium4   : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium5   : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium6   : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium7a  : GCTCGGTGATGTACGTATATGGGTAAAAACACCGCTGGCTTGTGCATATATAT--ATATATAGATAATGACGCAAGTT : 464
Lolium7b  : GCTCGGTGATGTACGTATATGGGTAAAAACACCGCTGGCTTGTGCATATATAT--ATATATAGATAATGACGCAAGTT : 460
Lolium8   : GCTCGGTGATGTACGTATATGGGTAAAAACACCGCTGGCTTGTGCATATATAT--ATATATAGATAATGACGCAAGTT : 463
Lolium9   : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium10  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium11  : GCTCGGTGATGTACGTATATGGGTAAAAACACCGCTGGCTTGTGCATATATATATATATATAGATAATGACGCAAGTt : 466
Lolium12  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium13  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium14  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium15  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium16  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium17  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 418
Lolium18  : GCTCGGTGATGTACGTATATGGGTAAAAACACCGCTGGCTTGTGCATATATATATATATATAGATAATGACGCAAGTT : 465
Lolium19  : GCTCGGTGACCTACGTATATGGGTAACAACAGCGCTGGGCTGTGCATATATA----------GATAATAACGCAAGTT : 415
Lolium20  : GCTCGGTGATGTACGTATATGGGTAAAAACACCGCTGGCTTGTGCATATATATATATATATAGATAATGACGCAAGTT : 466
            GCTC GTGA  TACGTATATGGGTAA AACA CGCTGGG TGTGCATATATA            GATAAT ACGCAAGTT

            *  **         *   *    *        ††††††    *
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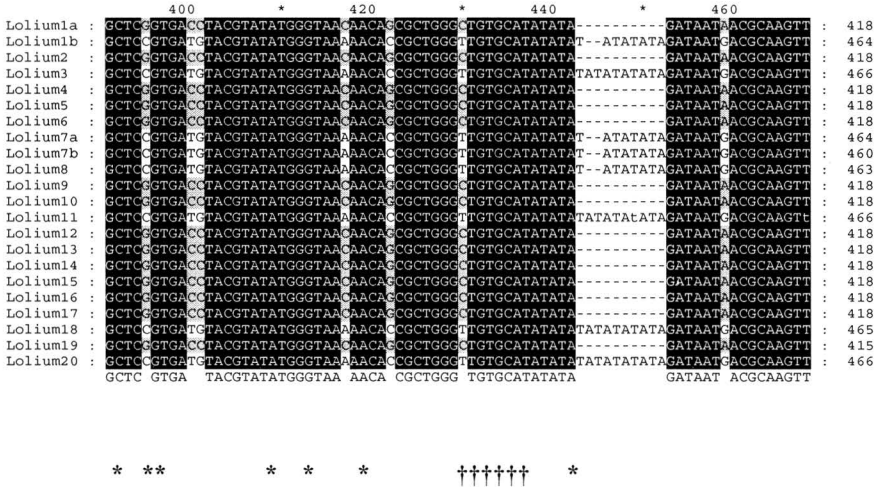
Fig. 2. Example of molecular markers (SNPs and InDels) as revealed by alignment of DNA sequences from 20 L. perenne *genotypes. Asterisks indicate position of SNPs and crosses indicate position of InDel. In this region, three haplotypes were identified: haplotype 1 (*Lolium *1a, 2, 4, 5, 6, 9, 10, 12, 13, 14, 15, 16, 17, 19), haplotype 2 (*Lolium *1b, 7a, 7b, 8), haplotype 3 (*Lolium *3, 11, 18, 20).*

Genes associated with carbohydrate metabolism and regulation were identified and full length sequences isolated from the *L. perenne* BAC library. New primers were designed and a region of the gene amplified and sequenced in 20 genotypes (Figure 2). Comparison of allele frequencies between populations of *L. perenne* plants which were unselected, or selected for high or low WSC content, over several generations, indicates some candidate gene alleles may contribute to high or low WSC phenotypes. Such alleles may in the future be selectable by PCR using the molecular markers unique to the allele of interest without the need for laborious phenotypic selection.

Chemical Phenotyping

Calibrations were developed for lignin (Figure 3), ADF and NDF in *Miscanthus* using modified partial least squares techniques and concentrations (g/kg DM) of these fractions were predicted in all 366 samples from the EMI project.
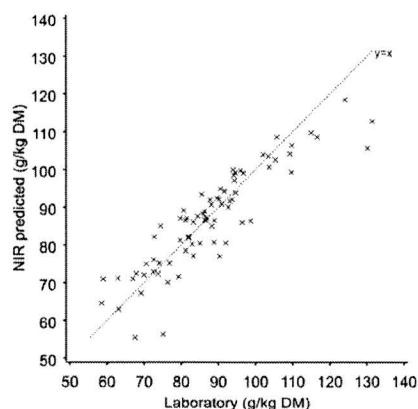
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Fig. 3. Comparison of NIR predicted and laboratory lignin contents.

Hemicellulose and cellulose contents were derived as NDF – ADF and ADF – Lignin respectively. The biggest differences detected in chemical composition were between *M. sinensis* and *M. sacchariflorus*, whilst *M.* x *giganteus* was more similar in chemical composition to *M. sacchariflorus* (Table 1).

Table 1. Average lignin, cellulose & hemicellulose contents (g kg-1 DM) in M. x giganteus, M. sacchariflorus *and* M. sinensis.

Genotype	Lignin	Cellulose	Hemi-cellulose
Gig	106	484	249
Sac	105	491	265
Sin	91	460	309

DISCUSSION

The identification of grasses with different chemical compositions enables the calibration of fuel specification and matching for different conversion technologies and end product quality. Many of the genes involved in the biosynthesis of plant polymers are known, it is therefore possible to determine the location of these genes or associated genomic DNA within the genomes of energy grasses, and associate specific genetic differences with variation in chemical composition. Such molecular marker information can then be used to predict the chemistry of the mature crop at the seedling stage. For thermal conversion of biomass, such as would occur when biomass is combusted to generate electricity, crops with greater lignin content would be selected for their higher heating or calorific value, whereas for biological conversion of biomass, crops with more available sugars would be chosen due to their greater potential to produce fermentation products such as ethanol or biogas.

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Linkage disequilibrium and associations with forage quality at loci involved in monolignol biosynthesis in breeding lines of European silage maize (*Zea mays* L.)

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ABSTRACT

During recent decades, breeding efforts have lead to a substantial increase in whole plant yield of silage maize. However, during the same period of time there has been a steady decrease in cell wall digestibility, and, consequently, in feeding value of elite hybrids. Cell wall digestibility is influenced by both lignin content and lignin structure. Thus, genes involved in the lignin biosynthetic pathway are considered promising candidate genes for improving digestibility of silage maize. Partial genomic sequences of 10 genes involved in biosynthesis of monolignols have been obtained in a number of inbred lines currently employed in European silage maize breeding. Different levels of nucleotide diversity and linkage disequilibrium (LD) were found, indicating different levels of selection pressure on individual genes of the monolignol pathway. Individual polymorphisms were tested for association with four quality-related traits to identify candidate functional markers for forage quality. Significant associations were identified, both when including and excluding population structure in the analysis. However, discrimination of effects of individual polymorphism was in some cases not possible due to extended LD. Studies in larger and/or broader sets of maize germplasm could decrease LD and validate candidate functional markers for forage quality identified in the present study.

Key words: diversity, feed, LD-mapping, lignin, phenylpropanoid

INTRODUCTION

The biosynthesis of monolignols (p-coumaryl-, coniferyl-, and sinapyl alcohols) are controlled by the phenylpropanoid pathway and oxidative polymerization of monolignols subsequently leads to the formation of lignin. The *brown-midrib* (*bm*) mutants of maize are characterized by decreased lignin content, altered cell wall composition, and a reddish brown pigmentation of the leaf midrib. In addition, improved forage quality is observed for the *bm* mutants. Of the four known *bm* mutants, *bm3* (altered in the second exon of the *COMT* gene) exhibits the strongest effect on plant phenotype, including a reduction in total lignin and an altered lignin composition (Barrière *et al.*, 2004). While a positive effect of the *bm3* mutant has been observed on intake and digestibility of forage maize, inferior agronomic performance such as lodging and lower biomass yield result from this mutation as well, restricting the use of *bm3* mutants in maize breeding programs. Characterization of genetic

diversity associated with forage quality traits in genes of the phenylpropanoid pathway might facilitate identification of alleles more applicable for development of functional markers (Andersen & Lübberstedt, 2003) for forage quality. We have studied sequence diversity, linkage disequilibrium (LD), and associations between individual polymorphisms in 10 phenylpropaniod pathway genes and phenotypes related to forage quality in 40 maize breeding lines.

MATERIALS AND METHODS

A collection of 40 maize inbred lines consisting of 22 Flint and 18 Dent lines were included in this analysis. These lines were selected based on digestibility of neutral detergent fiber (DNDF) values to represent a broad range of variability for this trait in central European germplasm employed in forage maize breeding. The inbred lines were evaluated in Grucking (sandy loam) in 2002, 2003, and 2004, and in Bernburg (sandy loam) in 2003 and 2004. The experiments included 49 entries in a 7×7 lattice design with two replications. Plots consisted of single rows, 0.75 m apart and 3 m long with a total of 20 plants. About 50 days after flowering the ears were manually removed and the stover was chopped. Quality analyses were performed with near infrared reflectance spectroscopy (NIRS) based on previous calibrations on the data of 300 inbred lines (unpublished results). Plants were grown for DNA isolation in the greenhouse and leaves were harvested at three weeks after germination. Genomic DNA was extracted and polymerase chain reaction (PCR) primers were developed for the candidate genes based on maize mRNA sequences identified in GenBank. Amplicons were purified and sequenced directly. Alignments were built up using the Clustal program version 1.8 (Thompson *et al.*, 1994). Nucleotide diversity (π) was estimated by using DNASP Version 4.10 (Rozas *et al.*, 2003). Linkage disequilibrium (LD) was estimated by the TASSEL software, version 1.9.0 (http://www.maizegenetics.net/bioinformatics/tasselindex.htm). Population structure was inferred from 101 simple sequence repeat markers (SSRs), providing an even coverage of the maize genome, by the Structure 2.0 software (Pritchard *et al.*, 2000; Falush *et al.*, 2003). Association analysis was carried out as a general linear model (GLM) analysis in TASSEL to test for associations between individual polymorphisms and mean phenotypic values across five environments. The Q matrix produced by Structure was included as covariate in the analysis to control for populations structure. Associations were further tested by the unified mixed model method for association mapping (MLM) in TASSEL (Yu *et al.*, 2006).

RESULTS AND DISCUSSION

Genomic DNA sequences of ten phenylpropanoid pathway genes (*PAL*, *C4H*, *C3H*, *4CL1*, *4CL2*, *CCoAOMT1*, *CCoAOMT2*, *F5H*, *COMT*, and *CAD*) were obtained in a set of 40 maize inbred lines of Flint and Dent pedigree (Andersen *et al.*, 2007a; Andersen *et al.*, 2007b; Zein *et al.*, 2007; Lübberstedt *et al.*, 2005). The length of the resulting alignments ranged from 460 base pairs (bp) for *C4H* to 3150 bp for *PAL*. Total nucleotide diversity (π) at these loci ranged from 0.00049 at the *CAD* locus to 0.01025 at the *4CL2* locus, which is within the π values previously reported for maize. For most loci included in this study, a rapid breakdown of LD (within few hundred bp) was observed. However, extended LD was identified at the *4CL1* locus at which all polymorphisms, with the exception of two 1-bp deletions, were in complete LD across the entire amplified sequence (1.3 kb). Similarly, at the *PAL* locus, an extended LD block was identified spanning the 3' half of the intron and the second exon (Figure 1).
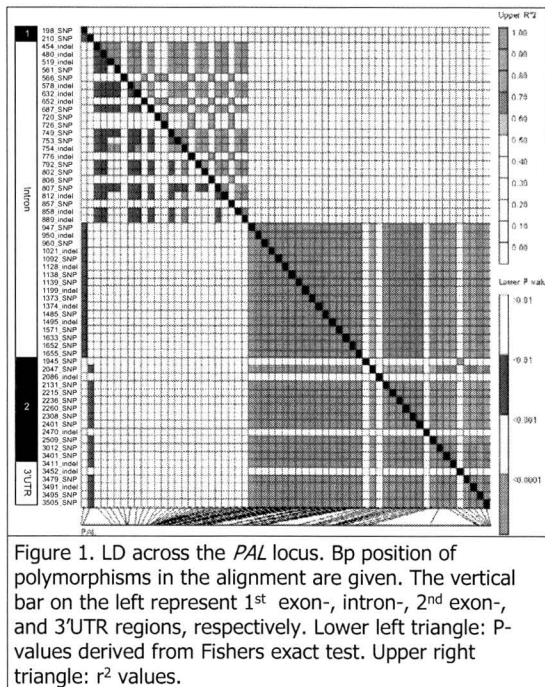
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Figure 1. LD across the *PAL* locus. Bp position of polymorphisms in the alignment are given. The vertical bar on the left represent 1st exon-, intron-, 2nd exon-, and 3'UTR regions, respectively. Lower left triangle: P-values derived from Fishers exact test. Upper right triangle: r^2 values.

Including population structure estimates in the association analysis, one to several polymorphisms at five genes were significantly associated with one or more traits (Table 1). In relation to forage quality, digestibility of neutral detergent fiber (DNDF) is the major factor, and polymorphisms at the *C3H*, *F5H*, and *COMT* genes showed associations with DNDF. Several genes were associated with more than one trait. At the *4CL1* locus, an indel was associated with water soluble carbohydrates, neutral detergent fiber (NDF), and *in vitro* digestibility of organic matter (IVDOM). Associations to multiple traits could be explained by a high correlation between traits (data not shown). It might also be speculated that allelic variation in genes upstream in the phenylpropanoid pathway affects several metabolic pathways as shown in *Arabidopsis* (Rhode *et al.*, 2004). When population structure was excluded from the analysis, a higher number of associations were identified. At the *PAL* locus, all polymorphisms in the 3' LD block (Figure 1), excluding singletons, and all singletons in the 5 part of the intron were associated with NDF when excluding population structure from the analysis. When including population structure, only one association was identified: a 1 bp indel (introducing a premature stop codon) was associated with IVDOM. For other genes we included both the overall population structure and finer scale relative kinship in the analysis (Yu *et al.*, 2006). This further reduced the number of identified associations as compared to accounting only for overall population structure (data not shown). It is expected that accounting for more levels of relatedness reduces the number of (false) associations. However, these results underline the importance of validating associations in multiple samples and/or by multiple methods. In summary, a number of candidate causative polymorphisms, with respect to forage quality traits, has been identified in several genes in the phenylpropanoid pathway of breeding lines of maize. However, the power of association analysis is limited by the number of individuals included. Thus, the associations should be validated in larger and/or broader sets of maize germplasm. Alternatively, series of point mutations could be produced by tilling, allowing for comparison of individual polymorphisms in isogenic backgrounds.

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Table 1. Associations between polymorphisms in genes of the phenylpropanoid pathway and four quality related traits in maize. WSC: water soluble carbohydrates, NDF: neutral detergent fiber, IVDOM: *in vitro* digestibility of organic matter, DNDF: digestibility of neutral detergent fiber.	
Trait	**Associated Gene(s)**
WSC	*4CL1, F5H*
NDF	*4CL1, F5H*
IVDOM	*PAL, 4CL1, C3H*
DNDF	*C3H, F5H, COMT*

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Vernalization response in ryegrass involves orthologues of wheat *VRN1* and rice *Hd1*

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ABSTRACT

Flowering time is important when adapting crop plants to different environments. In the breeding of perennial ryegrass (*Lolium perenne* L.) a dual requirement for flowering time exists. Flowering should be repressed to produce high-quality grass for feed while flowering should also be inducible to facilitate grass seed production. Consequently, the identification and characterization of the genes controlling flowering time in perennial ryegrass is of great interest. Three candidate genes for vernalization response genes in perennial ryegrass were identified based on DNA sequence homology to *TmVRN1* and *TmVRN2* of diploid wheat (*Triticum monococcum*), and *Hd1* of rice (*Oryza sativa*). High sequence similarity between *LpVRN1* and *TmVRN1*, co-localization of *LpVRN1* with a major quantitative trait loci (QTL) for vernalization response in perennial ryegrass, synteny between map-positions of *LpVRN1* and *TmVRN1*, mRNA expression analysis of *LpVRN1* alleles during vernalization, and the correspondence between *LpVRN1* mRNA expression levels and flowering time leads us to conclude that *LpVRN1* is orthologous to *TmVRN1* and that its function is conserved between diploid wheat and perennial ryegrass. Of the remaining two candidate genes, a putative *Hd1* orthologue, *LpCO*, co-localized with a second QTL for vernlization response. *LpCO* has recently been shown to be involved in the photoperiodic regulation of flowering time. While epistasis, at the level of *LpVRN1* transcription, was observed between the *LpVRN1* and *LpCO* genomic regions, no differential expression of *LpCO* transcripts was observed during vernalization. While orthologous genes controlling flowering time can thus be identified, future allele sequencing efforts will reveal if causative polymorphisms are conserved across the grasses.

Key words: flowering time, vernalization, ryegrass, *VRN1, VRN2, CO*

INTRODUCTION

Genetic variation for vernalization and photoperiod response is necessary when adapting crop plants to different latitudes and cropping seasons. Most temperate grasses, including perennial ryegrass (*Lolium perenne* L., *2n = 2x = 14*) require a prolonged period of low temperatures, i.e. vernalization, followed by an increase in photoperiod, i.e. longer days, to induce flowering. The objectives of the present study were to (I) identify and develop allele-specific markers for *LpVRN1* and *LpVRN2* candidates in perennial ryegrass, (II) map these candidates on the genetic map of perennial ryegrass, and (III) study the mRNA expression pattern of *LpVRN1* and *LpVRN2* candidates in selected genotypes.

MATERIALS AND METHODS

A mapping population (184 F_2 genotypes) was developed from an initial cross between the Italian variety Veyo and the Danish ecotype Falster. Plants were vernalized in climate chamber for six weeks (6°C, 8h daylength) and moved to the greenhouse (April to August) for subsequent phenotyping of vernalization response measured as days to heading. Map construction was carried out with 117 SSR and AFLP markers using the Haldane mapping function of JoinMap. QTL analysis was performed using multi QTL model analysis of MapQTL. Transcript levels of *LpVRN1* and *LpVRN2* candidate genes were studied by real time PCR in four pools of genotypes from the mapping population. These pools were selected based on their allelic composition at the *LpVRN1* and *LpVRN2* locus. Transcript levels of *LpVRN1* and the two *LpVRN2* candidates were studied before and during vernalization and compared to non-vernalized plants.

RESULTS AND DISCUSSION

Candidate genes *LpVRN1*, *LpVRN2_2* and *LpVRN2_3,* identical to *LpCO* (Martin *et al.*, 2004), were identified in perennial ryegrass based on deduced amino acid sequence homology to *TmVRN1* and *TmVRN2* of diploid wheat, and *HD1* of rice, respectively. CAPS markers vrn-1, vrn-2_2 and vrn-2_3 were developed for the three candidate genes based on SNP polymorphisms detected between perennial ryegrass genotypes Veyo and Falster.
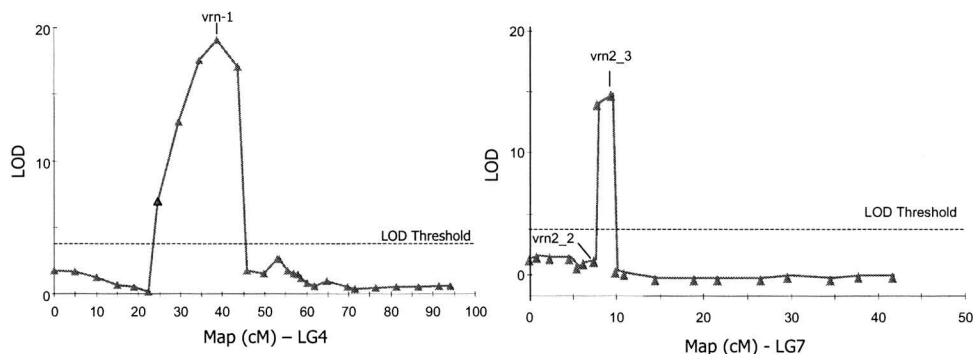

Fig. 1. LOD profiles of the vernaliziation responses on LG4 and LG7. CAPS markers vrn-1 and vrn2_3 co-localizes with maximum LODs at LG4 and LG7, respectively.

In total, five QTL for vernalization response measured as days to heading were identified and mapped to linkage groups (LG) 2, 4, 6 and 7 (Jensen *et al.*, 2005). The CAPS marker vrn-1 was found to co-segregate with a major QTL on LG4 for vernalization response explaining 28% of the total phenotypic variation. On LG7, a QTL explaining 14% of the total phenotypic variation was identified, with CAPS markers vrn-2_2 and vrn-2_3 mapping close to and at the peak of this QTL, respectively (Figure 1).

Transcription of *LpVRN1* is induced in all four genotype pools during vernalization, but more so in plants carrying the Veyo *LpVRN1* allele (Andersen *et al.*, 2006; Figure 2). In addition, an allelic shift at the *LpVRN2* locus also influences *LpVRN1* transcription rates

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during vernalization. In non-vernalized control plants, *LpVRN1* transcription is completely repressed in plants carrying the Falster allele at both loci (genotype pool 1).

Notably, in the non-vernalized control plants, an allelic shift at the *LpVRN2* locus seems to have a greater effect on *LpVRN1* transcription levels when compared to an allelic shift at the *LpVRN1* locus itself. Thus, the allelic composition at both loci influences the transcription level of *LpVRN1*.
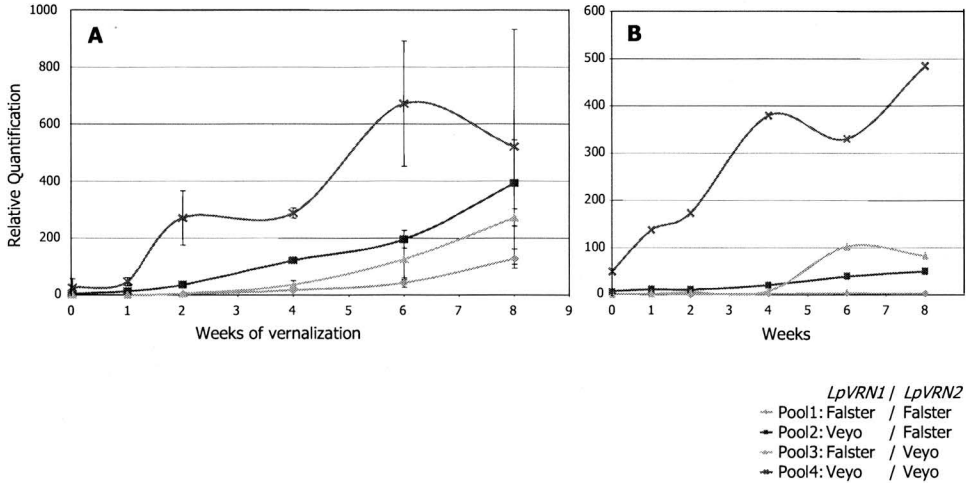
Fig. 2. Expression levels of LpVRN1 *in vernalized (A) and non-vernalized (B) plants of four different allelic pools.*

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Comparison between two breeding methods in tetraploid *Lolium perenne*: polycross versus F2

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ABSTRACT

Most of the tetraploid perennial ryegrass cultivars are synthetics, composed of at least 3 (up to 20 or more) clones. More components mean less inbreeding but also reduced selection intensity and a higher risk of heterogeneity. Is it possible to create high yielding varieties based on only 2 top clones? To be able to compare synthetics with F2 populations based on the same starting material we created two synthetics, one based on 3 and the other on 4 selected clones. We carried out pair-crosses (F1) with the components within each synthetic (respectively 3 and 6 combinations). We also produced inbred lines (I1) of the components. The syn1, F1 and I1 were multiplied to the next generation (syn2, F2 and I2) in 2003. The progenies have been sown in a herbage yield trial in 2004. In 2005 and 2006 five and four cuts resp. were harvested. There was no significant difference in herbage yield between the F2 and syn2 populations. Even the herbage yield of most of the multiplied inbred lines was not significantly different from the multiplied pair-crosses. The seed yield of all the F2 and I2 populations was lower than the syn2. These results confirm roughly the previously reported results of identical trials carried out with diploid perennial ryegrass. However the inbreeding effect in the F2 and I2 populations on the herbage yield was less clear at the tetraploid level.

Key words: pair-cross, perennial ryegrass, polycross, yield

INTRODUCTION

Most of the perennial ryegrass cultivars are synthetics composed of at least 3 (up to 20 or more) clones. The optimal number of clones depends on their general combining ability and their inbreeding depression. More components means less inbreeding but also reduced selection intensity and a higher risk of heterogeneity. Is it possible to create high yielding varieties based on only 2 similar top clones? Ghesquiere *et al.* (2007) already presented an answer for diploid perennial ryegrass. The present study deals with tetraploid perennial ryegrass.

MATERIALS AND METHODS

In 2002 we created two synthetics, one based on 3 (synthetic C) and the other on 4 (synthetic D) selected tetraploid clones of perennial ryegrass. In the same year we carried out pair-crosses (F1) with the components within each synthetic (respectively 3 and 6 combinations) and we produced inbred lines (I1) of the components. One clone of synthetic C didn't produce seeds after selfing. The syn1, F1 and I1 were multiplied in isolaton to the next generation (syn2, F2 and I2) in 2003. These progenies were sown in a herbage yield trial in 2004. We

harvested five cuts in 2005 and four in 2006 and determined the fresh and dry matter yield for each cut.

RESULTS

Dry matter yield didn't differ significantly between syn2 and F2 (Figure 1). The yield of most multiplied inbred lines was comparable to the multiplied pair-crosses. The inbreeding depression was rather low. The seed yield of all the F2 populations was lower than the syn2 (Figure 2). The F2 populations with the highest herbage yield had the lowest seed yield. Seed yield of I2 was on average lower than F2 for origin C but higher for origin D.
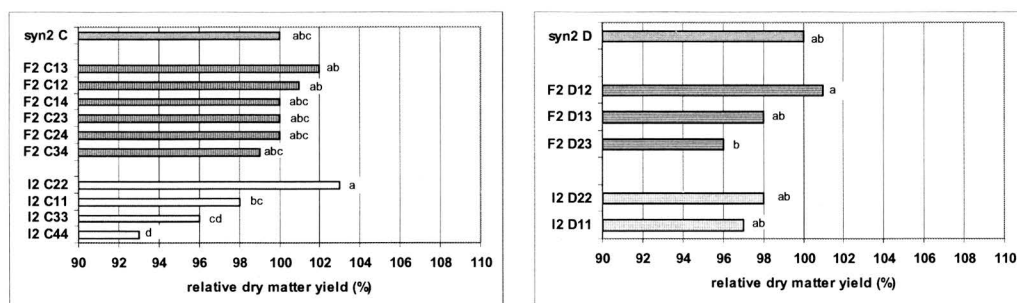

Fig. 1. Relative dry matter yield of syn2 C (left) and D (right) and of the multiplied pair-crosses (F2) and inbred lines (I2), based on the same clones.
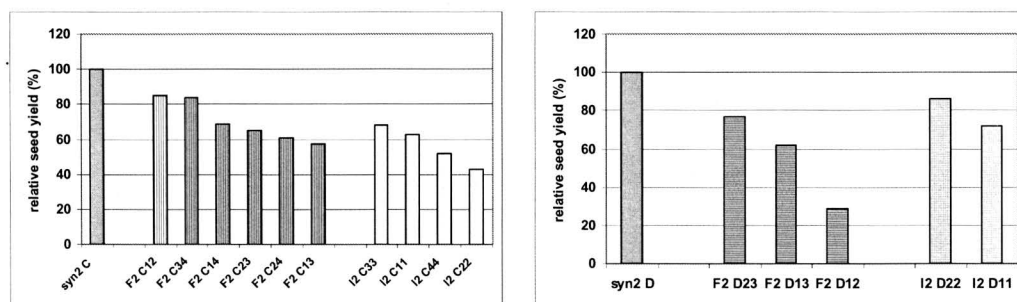
Fig. 2. Relative number of viable seeds of the multiplied pair-crosses (F2) and inbred lines (I2) compared to the syn2 of(synthetics C and D, based on 4 and 3 clones).

DISCUSSION

According to Posselt (2000) the optimum number of parents for a synthetic variety lies in the range of 5-15. With increasing inbreeding depression the optimum number increases. Above results show that inbreeding depression related to herbage yield in ryegrass is not so high. On the other hand all F2 populations had a lower seed yield than the syn2.

These results confirm the results obtained with diploid perennial ryegrass (Ghesquiere *et al.*, 2007). The inbreeding depression seemed to be slightly higher at the diploid level.

In conclusion: Tetraploid varieties based on only 2 parents may produce as much herbage as varieties based on more components. However their seed yield may be much lower.

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Selection for high molecular marker diversity to improve agronomic performance of *Lolium perenne* synthetics

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ABSTRACT

Agronomic performance of *Lolium perenne* synthetics was studied at three sites over two seeding years, each followed by two main harvest years (H1 and H2). Genetically narrow and wide polycrosses with 6 clones each were constructed based on Euclidean distances (E2), using 184 polymorphic AFLP markers. In a first approach, comparing polycrosses with the narrowest and widest possible combinations of 6 clones for each of three maturity groups consisting of 30 to 34 clones, a significantly higher forage dry matter yield (+ 1.7% in H1 and +2.1% in H2) of the wide synthetics was observed. In a second approach, where 18 elite clones were arranged either in three wide or in three narrow polycrosses, no effect of greater genetic diversity on yield was observed. The results support the hypothesis that selection for high genetic diversity can improve agronomic performance of grass synthetics, but the difference in diversity must be large to obtain statistically significant results.

Key words: AFLP, genetic diversity, heterosis, perennial ryegrass, polycross, synthetics

INTRODUCTION

Selection of polycross parents for high molecular marker diversity is a possibility to exploit heterosis in the construction of forage grass synthetics (Kölliker *et al.*, 2005). We used two approaches to test this concept in our breeding material of diploid perennial ryegrass (*Lolium perenne* L.).

MATERIALS AND METODS

Genetic diversity of 98 diploid *Lolium perenne* genotypes was analysed using 184 polymorphic AFLP markers (details see Kölliker *et al.*, 2005). Based on cluster analysis, we selected groups of 6 genotypes each with either low or high genetic diversity. In Approach 1, 30 to 34 clones were assigned to three maturity groups, and polycrosses with the narrowest and widest possible combinations of 6 clones for each group were formed. In Approach 2, 18 best performing clones were selected from the intermediate group and then arranged in three narrow and three wide 6 clone combinations. Combinations of both approaches were polycrossed and seed was increased to syn-2 lots which were used for evaluation in standard plot trials at three locations in Switzerland.

RESULTS

For Approach 1 which maximized the difference in genetic diversity between wide and

narrow polycrosses within maturity groups, average Euclidean distance (E^2) between parental clones was 55% higher in wide than in narrow polycrosses. There was a consistent and overall significant tendency for higher DM yields (Table 1) of wide synthetics, as well as for better scores for vigour, (Table 1), persistence and diseases (data not shown).

Conversely, average E^2 for the wide polycrosses of Approach 2 was only 26% higher than that of the narrow polycrosses. Individual synthetics differed significantly in DM yield and visual scores but there was no tendency for a higher DM yield of the wide synthetics (data not shown). Only vigour scores of H1 were significantly better for wide synthetics.

Table 1. Euclidean distance (E^2) between parental clones and annual dry matter yield (dt/ha) in first (H1) and second (H2) full harvest year of syn-2 generation of narrowest and widest 6-clone synthetics selected from 30 to 34 clones per maturity group (Approach 1). Means of 6 environments (3 locations, 2 seeding years 2003, 2004).

| Maturity group | diversity | average E^2 | DM yield (dt/ha, sum of 5cuts) | | | | Visual scores (1=best) | | | |
			H1		H2		vigour H1		vigour H2	
early	narrow	34.1	107.9	c	80.4	c	3.01	bc	2.91	bc
	wide	60.4	119.0	a	89.3	a	2.67	ab	2.46	ab
intermediate	narrow	42.2	115.1	ab	84.7	b	2.83	bc	2.93	c
	wide	59.3	117.0	ab	86.4	ab	2.28	ab	2.23	ab
late	narrow	42.4	111.3	bc	86.6	ab	3.07	c	3.03	c
	wide	64.5	114.2	ab	86.6	ab	3.14	c	3.21	c
all groups	**narrow**	**39.6**	**112.6**	**B**	**83.2**	**B**	**2.97**	**B**	**2.96**	**B**
	wide	**61.4**	**114.5**	**A**	**85.0**	**A**	**2.70**	**A**	**2.63**	**A**

DISCUSSION

These comprehensive results based on multiple cuts in first and second harvest years after two consecutive years of sowing at three locations each confirm previous, less widely based results (Kölliker *et al.*, 2005) that selection for high molecular diversity can improve agronomic performance of grass synthetics. The positive effect of increased genetic diversity among parent clones of the synthetics can be attributed to increased heterosis or to avoidance of inbreeding depression. However, the difference in diversity must be large to obtain statistically significant results.

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SNP genotyping for axillary tiller formation in perennial ryegrass

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ABSTRACT

In order to study genetic control of tiller formation a synthetic ryegrass population was created by intercrossing five genotypes within EU FP5 project 'GRASP'. Two rounds of selection (both positive and negative) for axillary tiller formation were performed. The mean number of axillary tillers per plant was 3.90 for plants from positive selection, 0.22 for plants from negative selection and 2.18 for population with no selection. Five ryegrass genes with putative functions in plant architecture and hormone response were chosen as candidate genes for genotyping. SNP genotyping in five ryegrass genes was done in all three populations. Three genes (*IAA1*, *RUB1 conjugating enzyme* and *BRI1*) out of five revealed significant deviations in allelic distributions across three populations under study. These results indicate possible role of the studied genes in ryegrass axillary tiller formation.

Key words: *Lolium perenne* L., plant architecture, hormone signaling

INTRODUCTION

Tiller formation in perennial ryegrass (*Lolium perenne* L.) is of primary importance both for forage and turf grasses. Ryegrass with increased tillering has higher density and persistency. SNP genotyping in five genes with putative function in plant architecture was employed to study formation of axillary tillers in perennial ryegrass.

MATERIALS AND METHODS

In order to study genetic control of tiller formation a synthetic ryegrass population was created by intercrossing five genotypes (LTS) within EU FP5 project 'GRASP'. LTS3 and LTS4 were parent genotypes of VrnA mapping population (Jensen *et al.*, 2005), LTS15 and LTS16 were ecotypes and LTS11 was ryegrass mutant with enhanced tillering (Figure 1 and Figure 2). Two rounds of selection (both positive and negative) for axillary tiller formation were performed. Five ryegrass genes with putative functions in plant architecture and hormone response were chosen as candidate genes for genotyping (Table 1). SNP genotyping in five ryegrass genes was done in all three populations. Two genes (G02 and G06) were genotyped by TaqMan (ArosAB) and the remaining three (G01, G05 and G07) by MassArray (Cigene) approach. Haplotypes were inferred from SNP data with PHASE v.2.1.

RESULTS

Axillary tiller formation was evaluated in three populations: one founder population (100 plants) and two selected populations (34 plants each). The mean number of axillary tillers per primary tiller was 3.90 (3.00 – 6.20) for plants from positive selection (POS), 0.22 (0 - 0.45) for plants from negative selection (NEG) and 2.18 (0.10 – 4.35) for population with no selection (C0). SNP genotyping with 25 SNPs in five genes was performed in all three populations and parental genotypes (LTS). Three to six haplotypes were identified for each gene (Table 1). Three genes (G01, G02 and G05) out of five revealed deviations in allelic distributions across the populations under study.

Table 1. Summary of genotyping.

Gene code	Annotation	Putative function	LG	No. of SNPs	No of haplotypes
G01	*IAA1*	Auxin signalling	4	5	3
G02	*RUB1 conj. enzyme*	Auxin signalling	5	5	6
G05	*BRI1*	Brassinosteroid signalling	3	6	6
G06	*SHOOT1*	Outgrowth of axillary buds	4	7	3
G07	*TB1*	Outgrowth of axillary buds	2	2	3

DISCUSSION

Modern breeding in perennial ryegrass for high yield has led to a tendency towards swards having a lower density of larger tillers. However, persistency of perennial ryegrass depends on the equilibrium between the relative rate of tiller initiation and tiller death (Bahmani *et al.*, 2000). Three genes were identified in this study as possible candidates for plant architecture control in perennial ryegrass. SNPs developed for these genes show promising results for the MAS application in ryegrass breeding. Further studies are needed to validate these relations in broader populations.

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Investigating drought tolerance in a set of *Lolium perenne* inbred lines

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ABSTRACT

A set of *Lolium perenne* inbred lines have been characterized for their response to PEG induced drought in a hydroponics system. A Suppression Subtractive Hybridisation (SSH) approach has been employed to elucidate the genetic basis of the contrasting drought response. Leaf and root tissues were taken from two inbred lines with a contrasting response to the PEG induced drought stress. Subtracted libraries were then generated to identify genes under differential expression between the two lines in each tissue. Subsequently, a macroarray approach was used to monitor the expression profiles of selected candidate genes at various points in the drought regime. This allowed the identification of a number of genes whose expression profile is indicative of them having a role to play in responding to a PEG induced water stress.

Key words: suppression subtractive hybridisation, PEG, perennial ryegrass

INTRODUCTION

With climate change predicted to lead to warm and dryer summers in the British Isles a goal of our group is to develop tools to assist the breeding programme develop superior varieties with respect to drought tolerance. A number of genomic studies have been carried out in the model plants to elucidate genetic factors regulated under drought stress but a limited number of studies have been carried out in the economically important forage grasses. The objective of this research was to generate subtracted libraries between two partially inbred lines with a contrasting response to PEG induced drought stress.

MATERIALS AND METODS

Two inbred lines of perennial ryegrass, *Pma* and *Pfa*, were grown in an aerated hydroponics system using MS medium for nutrient supply. Plants from each line were subjected to a PEG induced drought stress by placing in a 15% PEG 6000 solution. Samples were taken for RNA isolation, relative water content (RWC) and a Trypan blue assay after one week of stress. Suppression Subtractive Hybridization (SSH) was performed according to the protocol of Desai *et al.* (2000). Subtracted libraries were generated between the lines for both root and leaf tissues. SSH libraries were cloned and a proportion was selected for differential screening. Amplified inserts were blotted onto nylon membrane and hybridized with labeled

probes representing the original mRNA complement from both plant lines under drought stress.

RESULTS

After eight days of PEG induced drought stress, the *Pma* line had a significantly lower RWC than the *Pfa* line ($P<0.05$). Additionally, eleven days in to the drought stress, the *Pma* line was dead while the *Pfa* remained alive. Trypan blue staining of root material from both lines under drought stress and controls showed an increase in cell death in the roots of stressed plants. No obvious differences in cell death levels could be identified between the inbred lines under stress.

Both forward and reverse subtracted libraries were generated to identify genes differentially expressed between *Pfa* and *Pma* after one week of PEG induced drought stress in both leaf and root tissues. 384 colonies from each of the four libraries were screened on nylon membranes to confirm differential expression (Figure 1). 192 out of 1536 clones showing differential expression between the two lines under drought stress were sequenced.
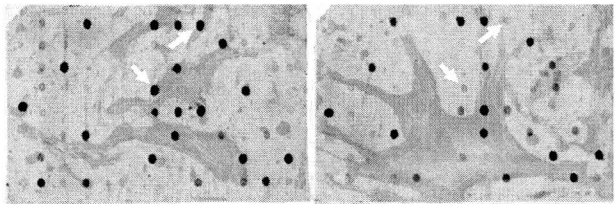

Fig. 1. Differential screening of SSH libraries on nylon membranes. White arrows indicate examples of differentially expressed transcripts.

Table 1. Most significant matches in rice for a selection of transcripts showing differential expression between the Pfa and Pma lines under PEG induced drought stress.

Library	Most significant match in Rice	E Value
Pfa Leaf	glutamine synthetase, chloroplast precursor	1.40E-40
Pfa Leaf	chlorophyll a-b binding protein, chloroplast precursor,	1.90E-78
Pfa Root	calmodulin, putative, expressed	5.80E-05
Pfa Root	3-isopropylmalate dehydratase large subunit 2	8.40E-81

DISCUSSION

The *Pfa* line is more tolerant to PEG induced drought stress than the *Pma* line. This was indicated by the greater ability of the *Pfa* line to retain water along with greater survival ability. The SSH technique identified a number of transcripts under differential expression between both lines following one week of stress (examples in Table 1). A number of these transcripts have been putatively associated with a role in drought tolerance. Ongoing work is looking at characterising the expression profiles of a number of candidate genes in various plant lines with contrasting responses to drought stress.

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Study of correlations among seed production characters in birdsfoot trefoil (*Lotus corniculatus* L.)

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ABSTRACT

Seed production is affected by various characteristics with positive or negative influence on seed yield. Thus, the direct correlations between seed yield and the following characters were investigated: number of inflorescences, number of pods, number of seeds/pod and rate of seed setting. Dehiscence of the pods acts indirectly on seed yield. Though seed yield of birdsfoot trefoil is very much negatively influenced by the high degree of dehiscence in the pods, a indirect significant correlation between the dehiscence and the erect position of the shoots was found. As in the majority of studied cases the sense and intensity of the correlations were similar for two seeding years it could be concluded that the relationships between the characters studied are genetically conditioned.

Key words: birdsfoot trefoil (*Lotus corniculatus* L.), seed production, correlations, seed production characters

INTRODUCTION

The study upon correlations among seed yield characters represents an indispensable procedure for amelioration works in plants. Theoretically, it enables the combination of plant biological and dimension aspects by indirect selection within a mathematical methodology, passing from a subjective appreciation to a quantitative expression (Dragomir, 1982).

In birdsfoot trefoil, the correlations among various production characteristics are largely unknown. Moreover, the variability of these characteristics determined by hereditary factors or by the environment has not been studied to date.

The research performed in this field revealed the presence of some correlations between plant vigor and some seed characteristics, leading to the elaboration of a methodology for the study upon the causality of many pairs of characteristics in birdsfoot trefoil (Bologna *et al.*, 1996; Grant, 1996; McGraw and Beuselinck, 1983; Seaney and Henson, 1970; Winch *et al.*, 1985).

This paper work intends to study the main characteristics determining seed production in birdsfoot trefoil by calculating correlation coefficients.

MATERIALS AND METHODS

Correlations among characters determining birdsfoot trefoil seed production has been assessed during 2002 to 2004 at the Banat's University of Agricultural Sciences and Veterinary Medicine Timisoara using three Romanian varieties of birdsfoot trefoil (Livada,

Nico, Oltim) as biological material. From each variety, 50 plants were studied in the first and second year of vegetation. So the determination of correlation coefficients in both years allowed a verification of their accuracy and enabled identifying the optimal moment for selection of elite material for the respective characteristics. Due to the variability of the vegetation conditions from one year to another, we have supervised the relationships between the correlation coefficients per cycles of seeding. Moreover, this fact should make evident the availability and the accuracy of the results achieved.

RESULTS AND DISCUSSION

The amount of seeds per plant, a characteristic of special importance for the extension of birdsfoot trefoil crops, depends upon a large range of other production elements. So significant positive correlations were found to almost every character under investigation with the exception of husk length and dehiscence average degree, for which the correlation coefficients were not significant or revealed negative values (Table 1). The closest positive correlations were observed between seed amount and the following characteristics: number of inflorescences per sprouts and per plant, average number of seeds in husk and average degree of fructification.

Table 1. Calculation of correlation coefficients among seed production characteristics.

Characteristics	Veget. years	Aver. of infl. per shoot	Aver. nr. of infl. per plant	Aver. nr. of pods per infl.	Aver. nr. of pods per plant	Pods lenght	Aver. nr. of seeds per pod	Aver. degree of fructific.	Aver. degree of dehis.	Thousand grain weight
Seed quantity per plant	I	0.402	0.824 **	0.582 **	0.284	-0.116	0.673 **	0.811 **	-0.723 **	0.486 *
	II	0.841 **	0.898 **	0.731 **	0.594 **	0.236	0.732 **	0.874 **	-0.649 **	0.413
Average nr. of infl. per shoot	I		0.473 *	0.417	0.632 **	0.351	0.075	0.524 *	-0.017	0.099
	II		0.784 **	0.448	0.374	0.299	0.436	0.420	0.110	0.142
Total nr. of infl. per plant	I			0.711 **	0.621 **	0.022	0.194	0.289	0.289	0.122
	II			0.724 **	0.783 **	0.218	0.102	0.217	0.330	-0.381
Average nr. of pods per infl.	I				0.822 **	-0.482 *	0.513 *	0.758 **	0.089	-0.512 **
	II				0.845 **	-0.610 **	0.464 *	0.743 **	0.054	-0.473 **
Average nr. of pods per plant	I					-0.322	0.236	-0.633 **	0.393	0.026
	II					-0.404	0.161	0.729 **	0.214	-0.153
Pods length	I						0.473 *	0.135	0.463 *	0.487 *
	II						0.498 *	0.478 *	0.472 *	0.566 **
Aver. nr. of seed per pod	I							0.816 **	-0.089	-0.489 *
	II							0.875 **	0.121	-0.511 *
Aver. fructification degree	I								0.218	0.143
	II								0.033	0.088
Aver. degree of dehiscence	I									-0.502 *
	II									-0.484 *

Significance: *0,05; **0,01

Seed production is largely conditioned by husk dehiscence degree. This characteristic was negatively correlated to total seed amount and revealed no close relationships to any other characteristics. However, there is a positive significant correlation between dehiscence degree and husk length. Seed weight correlates negatively and significantly with the average dehiscence degree.

The intensity of the correlation between husk length and the average dehiscence degree has a special importance in the achievement of some birdsfoot trefoil forms with high-degree husk indehiscence.

There is a positive significant correlation between plant weight and seed amount in the two years of vegetation. The selection of some elite plants with high green mass yields may guarantees the achievement of some higher seed amounts in the same time. Moreover, the importance of this finding is also supported by the fact that the selection of elite plants may begin in the first year of vegetation (Table 2).

Table 2. Calculation of correlation coefficients among the characteristics of green mass production and the characteristics of seed production.

Characteristics for green mass	Veget. years	Characteristics for seed production									
		Seed quantity per plant	Aver. of infl. per shoot	Total nr. of infl. per plant	Aver. nr. of pods per infl.	Aver. nr. of pods per plant	Pods length	Aver. nr. of seeds per pod	Aver. fructific. degree	Aver. degree of dehis.	Thousand grain weight
Wheight of plant	I	0.562 **	0.180	0.501 *	0.356	0.465 *	-0.244	0.532 *	0.519 *	0.146	0.473 *
	II	0.813 **	0.211	0.576 **	0.496	0.448 *	-0.325	0.479*	0.624 **	0.072	0.394
Average nr. of shoot	I	0.734 **	0.336	0.531 *	0.349	0.543 *	0.099	0.131	0.470 *	0.052	-0.144
	II	0.762 **	0.309	0.603 **	0.447 *	0.593 **	0.138	0.450 *	0.633 **	0.313	-0.304
Shoot position -Erect	I	0.589 **	0.322	0.244	0.309	0.492 *	0.009	0.499 *	0.472 *	-0.530 *	0.034
	II	0.715 **	0.479 *	0.583 **	0.239	0.637 **	0.147	0.534 *	0.591**	-0.589 **	0.283
Shoot position -Semierrect	I	0.372	0.375	0.322	0.187	0.393	0.299	0.381	0.417	-0.129	0.031
	II	0.480 *	0.392	0.456 *	0.277	0.451 *	0.293	0.467 *	0.501 *	-0.482 *	0.145
Shoots position -Traileres	I	0.513 *	0.073	0.157	0.185	0.244	0.221	0.413	0.198	0.417	0.003
	II	0.459 *	0.296	0.438	0.366	0.397	0.314	0.461 *	0.384	0.394	0.014
Average nr. of ramif. per shoots	I	0.789 **	0.723 **	0.463 *	0.437 *	0.372	0.155	0.146	0.386	-0.022	-0.235
	II	0.732 **	0.759 **	0.517 *	0.487 *	0.423	0.089	0.396	0.471 *	0.071	-0.443 *
Average nr. of ramif. per plant	I	0.716 **	0.489 *	0.762 **	0.366	0.417	0.268	0.299	0.513 *	0.138	-0.419 *
	II	0.773 **	0.465 *	0.667 **	0.472 *	0.430	0.089	0.228	0.589 **	0.103	-0.508 *
Average nr. of leaves per shoot	I	0.502 *	0.189	0.498 *	0.400	0.384	0.148	0.376	0.036	0.003	-0.218
	II	0.439	0.297	0.513 *	0.359	0.317	0.082	0.480 *	0.118	0.127	-0.451

Significance: *0,05; **0,01

Green plant weight was positively correlated with almost all production characteristics, excepting husk length and average dehiscence degree. There was a negative correlation between sprouts' erect position and the average dehiscence degree.

CONCLUSIONS

- Seed amount correlates positively and significantly with almost all characteristics determining this trait, excepting husk length and husk dehiscence degree.
- The analysis of the two main characters, plant weight and seed amount per plant, led to the conclusion that there is a positive and significant correlation in the two years of vegetation between them. This allows the selection of some elite plants in the beginning of the first year of vegetation, which should be characterized by high green mass production and also by the achievement of a higher seed amount.
- The presence of a negative, significant correlation between the husk dehiscence degree and the sprouts' erect position allows the improvement of seed production in birdsfoot trefoil by selecting some forms with a sprout position as erect as possible.

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Increasing biomass for bioenergy in perennial grasses

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ABSTRACT

Dedicated energy crops such as *Miscanthus x giganteus* are becoming extensively planted in Europe. *M. giganteus* is a sterile triploid arising through natural hybridisation of a tetraploid *M. sacchariflorus* and a diploid *M. sinensis*. *M. giganteus* displays a good balance of traits from each parent, combining rapid growth with a tolerance to low temperatures, making it an ideal biomass crop for Northern Europe.

Understanding of the genetic control of biomass performance traits is required to identify markers in order to accelerate breeding efforts and increase land use efficiency. *Miscanthus* takes three years to reach maturity in the field and so could benefit from marker assisted breeding. Target traits include the control of flowering time and plant architecture. The transition to flowering influences biomass yield as it determines the length of the growing season: delayed flowering generates increased yields. This additionally affects quality as flowering triggers senescence and nutrient reallocation from the aerial parts to the underground rhizome. Plant architecture consists of stem height, number and diameter, all of which are under genetic control and can be optimised for increased biomass yield. The ratio of stem to leaf may also affect biomass quality as the chemical components of these organs are different. The UK *Miscanthus* breeding programme is based at IGER, and research tools are being developed to enable the association of phenotype to genotype.

Key words: energy crop, genetic control, flowering time, marker assisted breeding, Miscanthus, plant architecture

INTRODUCTION

Despite a dearth of resources with which to study *Miscanthus* directly, much can be made of the information obtained in the model species *Arabidopsis, Brachypodium*, and rice, as well as other grasses such as perennial ryegrass, maize, millet, *Sorghum* and the closely related sugarcane. Genes of interest may be selected by homology and synteny, and their orthologues identified in *Miscanthus*. It is expected that morphological traits of interest may not be fully addressed by the *Arabidopsis* model as monocotyledonous and dicotyledonous plant architecture are fundamentally different, although there are numerous well characterised genes regulating plant development such as the MAX genes in *Arabidopsis*, two of which have been demonstrated to have orthologues in rice (Ishikawa *et al*., 2005; Zou *et al*., 2005). The genetic control of branching has been studied in millet and has revealed that tillering and axillary branching are partially controlled by different loci (Doust *et al*., 2004). Ric, *Sorghum* and other monocots to be sequenced shortly (e.g., maize, switchgrass and *Brachypodium*) will be a useful source of candidate gene information as synteny will enable alignment or comparison to full genome sequence. Sugarcane is very closely related to *Miscanthus* and has been under selection pressure for high yield for many years.

MATERIALS AND METHODS

In order to identify the genes controlling architecture and flowering traits, wide genetic crosses between *Miscanthus* genotypes of dissimilar morphologies are being undertaken to generate suitable mapping families to identify QTL. Data on the inheritance of morphological characteristics obtained by Atienza *et al.* (2003) was based on a *M. sinensis* x *M. sinensis* cross with a small number of individuals (89). Use of the IGER collection will allow access to wider genetic diversity and a higher resolution of mapping by increasing the number of individuals (>250 progeny). QTL maps will be complemented by a genetic map. Genetic markers will particularly be targeted at regions of the genome where candidate genes are predicted from closely related species such as the *MONOCULUM, OsTB1* and *Os PIN1* genes of rice (Li *et al.*, 2003; Yakeda *et al.*, 2003; Xu *et al.*, 2005). For major QTL regions where no candidate genes are identified, the region will be fine mapped, a BAC contig will be constructed, and the region sequenced to identify novel underlying genes.

Homologues of genes implicated in plant architecture and flowering time in other species will be genetically mapped to test for association with QTL (for yield; stem number, diameter and height; and flowering time) and the allelic variation present in the IGER collection studied and compared to morphological characteristics in association studies. One of the key traits to be studied in this project is basal branching (tillering) which is unique to perennial grasses and, like axillary branching, involves two developmental processes: initiation of meristems and subsequent growth. *MONOCULUM* is involved in bud formation and *OsTB1* is involved in the outgrowth step (Sakamoto *et al.*, 2004). Allelic variation for QTL associated candidate genes will be studied in a wider population of phenotyped *Miscanthus* to identify SNPs. Such SNPs will provide molecular markers to allow the use of marker assisted selection (MAS) at an early stage of plant development before the mature plant characteristics are visible.

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Phenotypic and molecular characterization of genetic resources of Nordic timothy (*Phleum pratense* L.)

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TIMOTHY AS THE NORDIC FORAGE SPECIES

Timothy is the most important forage grass species in the northern part of the Nordic countries due to it's adaptation to the cool and relatively humid northern climate. In Finland, 60-70% of pastures are sown with timothy. Correspondingly, in Sweden 50% and in Norway 52% of the seed production acreage of forage grass is covered with this species. This figure is even higher in Iceland where timothy is the dominating grassland species. Most of the forage grass production is utilised in milk and meat production, which counts 48, 30, 64 and 17% of the agricultural income in Finland, Sweden, Norway and Denmark, respectively[3]. Thus, the importance of grassland production including timothy is indisputable within the Nordic region.

However, serious winter damages, which significantly lower forage yields, occur regularly in the Nordic region. Winter-hardiness of timothy involves tolerance to many environmental stresses including ice-encasement, frost, and low-temperature pathogens. Areas with sparse snow cover may suffer from frost and ice-encasement. As an example, a typical loss of sward due to winter damage at the Lapland experimental station in Finland has been 24% for the first year sward. According to a report of the Nordic Council dating back to 1988, winter damages caused economical losses of 317 million NOK (466 million NOK when indexed to year 2005 level) each year in Finland, 71 million NOK in Sweden and 94 million NOK in Norway (Gudleifson, 1989). In addition, farmer's inputs in fertilisers may not be fully exploited by the forage crop due to winter damages, and nutrients not utilised by the forage are causing environmental problems.

In other important crop species we can rely on publicly available international research and development. This is not the case for timothy since this species is primarily utilised at higher latitudes and research is conducted mostly within the Nordic region. Therefore, the Nordic countries should have a special obligation to fund research in this economically

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[3] http://tike.mmm.fi/, http://www.sjv.se/, http://www.ssb.no/, http://www.statistikbanken.dk/.

important species. The Nordic plant breeders have so far been quite successful in developing new, high-yielding varieties. However, the challenges today are to further improve winter hardiness and quality, as well as to produce varieties that might be exported to new markets, e.g. Russia.

GENETIC RESOURCES AND PLANT BREEDING

Plant breeding relies on broad genetic variation, which can be utilised to combine positive traits such as resistance to pathogens, environmental stresses, quality and high yield in new varieties. Therefore, plant breeders have to make sure that they have sufficient genetic variation available for their breeding programmes. The breeding history of timothy is relatively short and the present varieties originate from old local timothy varieties and natural populations. Therefore, modern varieties have not diverged much from the natural (local) populations and consequently, natural populations can be used successfully in timothy breeding.

The Nordic Gene Bank (NGB), the centre for conservation and utilisation of genetic resources from the Nordic region, has today a large collection of timothy with approx. 560 accessions. A large part of these accessions is composed of collected samples of natural populations. This collection has been characterised to some extent for morphological and agronomic traits[4]. However, neither the genetic structure nor the value of the collection for plant breeding purposes has been studied based on modern molecular tools, e.g. DNA markers. For plant breeding such information would be of great value since it would make the collection more accessible for plant breeders who will be able to improve specific traits more targeted and develop new varieties more efficiently. Consequently, the international challenge to utilise gene bank collections (according to FAO's Global Plan of Action) for sustainable agricultural production would be realised.

The geographical coverage of NGB's timothy collection is not yet complete[5]. Uncollected areas comprise almost all of central Sweden, parts of the provinces Kainuu and North Ostrobothnia in Finland and the Danish islands Fyn and Sjælland, as well as many small islands. Especially, old meadows and pastures may contain timothy types that are valuable for developing varieties with specific traits and adaptations. This aspect should be carefully studied and material from these areas collected or better: organise their *in situ* conservation (conservation in the natural surroundings).

EXOTIC GERMPLASM

Besides the collection at the NGB, there are exotic timothy accessions present in gene banks from other geographical regions as well. In forage breeding the utilisation of heterosis (the production of an exceptionally vigorous and/or productive hybrid progeny from a directed cross) is an essential tool in breeding of new varieties. Heterosis is based on the selection on genetically distant clones that will form the basis of a new synthetic variety. This demands molecular and phenotypic characterisation of timothy populations and individual genotypes to be used for selection of parental clones. Thus, the exploitation of exotic germplasm from other gene bank collections may provide a source of further progress for timothy breeding in our region. The prerequisite for this is the ability to estimate genetic differences between populations and identify superior populations and genotypes to be used in new crosses.

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[4] http://www.ngb.se/Databases/Activities/pro_detail.php?57
[5] http://tor.ngb.se/sesto/index.php?scp=ngb&thm=loc&dtatyp=mat

Analysis of the water soluble carbohydrate content in an unselected breeding pool of perennial ryegrass

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ABSTRACT

A high water soluble carbohydrate (WSC) content in perennial ryegrass leads to a more efficient use of the protein in the grass by the ruminant and hence to reduced nitrogen losses.

During 2004, 2005 and 2006 we systematically determined by NIRS the WSC content of each forage cut in the year after sowing of the progeny test plots of our perennial ryegrass breeding program. These plots included diploid and tetraploid families, synthetics and varieties and were partly fertilized at two nitrogen application levels.

The variation of the WSC content among families is described. We calculated the correlations between the WSC content of the single cuts and the WSC content on an annual basis. Also correlations between the WSC content and dry matter yield, disease resistance, digestibility and nitrogen content and the effect of nitrogen fertilization are shown.

Key words: correlation, NIRS, nitrogen

INTRODUCTION

A high WSC content in perennial ryegrass leads to a more efficient use of the protein in the grass by the ruminant and hence to reduced nitrogen losses. To breed perennial ryegrass with an improved WSC content it is important to know what is the variation within a breeding pool, how to measure it in an efficient, fast and reliable way and to know the correlation with yield and other quality parameters.

MATERIALS AND METHODS

During 2004, 2005 and 2006 we studied 315 different diploid and tetraploid families, synthetics and varieties belonging to our perennial ryegrass breeding pool. These entries were tested in 10 different yield trials on 8.4 m² plots in 3 replicates. The trials were mown 4 to 5 times a year. In each trial all cuts were analysed in the year after sowing, except for the third cut in 2006 because of the very warm and dry month of July. The trials received between 340 and 430 units of nitrogen (N) fertilizer per ha per year. Part (79) of the entries were also tested at a nitrogen fertilizer level reduced by one third (210-290 kg N/ha/year).

We determined the dry matter yield (DMY). A sample of about 300 g fresh weight was taken per plot and dried at 75°C. The dried samples of the replicates were mixed and ground. We analysed by NIRS the WSC content, the dry matter digestibility (DMD) and the crude protein (CP) content. We also scored crown rust resistance by visual observation.

We calculated the arithmetic mean of the WSC content over the cuts and the weighted average of WSC, CP and DMD (dry matter yield per cut as weighing factor). We looked at the variation of the weighted mean of WSC over the entries in each trial. The correlation

coefficients between the arithmetic mean of WSC and the WSC of each single cut were calculated to find the best prediction of the WSC on an annual basis by one cut. We studied the correlation between the weighted means of WSC and DMY, CP, DMD and rust resistance to detect undesirable relationships. Finally we compared the WSC content at high and reduced levels of nitrogen fertilization for 79 entries in 4 trials.

RESULTS AND DISCUSSION

The weighted annual mean of WSC content of all trials was 16.9% with a minimum of 14.3% and a maximum of 19.3%. The average WSC variation coefficient among entries of all trials was 7.9%. This means that when selecting the 5% best entries the overall mean WSC content will increase from 16.9% to 19.1%. The average WSC content of the tetraploids was 1.1% higher than that of the diploids.

The WSC content on an annual basis was in most cases significantly correlated with the WSC content of all single cuts although the WSC content of the single cuts were among themselves not significantly correlated (Table 1). The forth cut was the best related to the annual mean and the third the least but differences were small. Taking the two best related cuts (1 and 4) together, the correlation coefficient increased from 0.69 to 0.87. The coefficient of variation of WSC among entries within a single cut was the highest for cut 4 and cut 1 (respectively 12.6% and 12.0%) and the lowest for cut 2 (9.8%). This makes cut 4 or cut 1 the recommended cuts if one can analyse only a single cut.

Table 1. Correlation between water soluble carbohydrate(WSC) content on an annual basis and WSC content of single cuts or combination of cuts and average correlation among WSC content of single cuts.

trial	correlation coefficient between average annual WSC and WSC content of										mean correlation among all cuts	
	cut1		cut2		cut3		cut4		cut1+4			
a0304	0.67	***	0.76	***	0.64	***	0.64	***	0.80	***	0.29	ns
a0303	0.71	***	0.71	***	0.46	*	0.79	***	0.94	***	0.27	ns
a0305	0.73	***	0.68	***	0.56	***	0.79	***	0.89	***	0.30	*
a0306	0.70	***	0.37	ns	0.70	***	0.57	**	0.83	***	0.21	ns
a0401	0.72	***	0.57	***	0.78	***	0.62	***	0.86	***	0.28	ns
a0403	0.62	**	0.68	***	0.78	***	0.79	***	0.87	***	0.33	ns
a0402	0.68	***	0.78	***			0.70	***	0.85	***	0.29	ns
a0501	0.68	***	0.79	***			0.61	***	0.87	***	0.23	ns
a0502	0.84	***	0.66	***			0.69	***	0.93	***	0.31	ns
a0503	0.50	ns	0.64	*			0.72	*	0.85	***	0.08	ns
mean	0.68		0.66				0.69		0.87		0.26	

Also Reheul and Ghesquiere (1994) advised to consider the last cut for the determination of the digestibility if only 1 cut could be analysed. This analogy is confirmed in Table 2. The WSC content is mostly very significantly positively correlated with the dry matter digestibility. In all cases but not always significantly the WSC content is negatively correlated with the crude protein content. There is mostly no relationship between WSC content and yield or rust resistance.

Table 2. Correlation coefficient between the water soluble carbohydrate(WSC) content and dry matter yield (DMY), dry matter digestibility (DMD), crude protein (CP) and rust resistance.

trial	DMY		DMD		CP		rust resistance	
a0304	0.52	**	0.87	***	-0.35	ns	0.37	ns
a0303	0.16	ns	0.52	*	-0.40	ns	0.07	ns
a0305	-017	ns	0.62	***	-0.12	ns	0.18	ns
a0306	0.36	ns	0.76	***	-0.22	ns	0.03	ns
a0401	0.09	ns	0.76	***	-0.30	*	-0.05	ns
a0403	0.32	ns	0.82	***	-0.25	ns	-0.04	ns
a0402	0.29	ns	0.65	***	-0.50	***		
a0501	0.01	ns	0.72	***	-0.26	ns	0.06	ns
a0502	0.13	ns	0.80	***	-0.32	ns	-0.21	ns
a0503	0.03	ns	0.25	***	-0.27	ns	0.08	ns
mean	0.17		0.68		-0.30		0.05	

The WSC content is on average 5% units higher at the reduced nitrogen fertilizer level. The variation among entries is not higher at the lower N level. The mean correlation coefficient between the WSC content of the entries under high and low nitrogen fertilization is 0.61.

CONCLUSION

When selecting the 5% best material of our breeding pool the WSC content increased by 13% relatively.

The WSC content of all single cuts is well correlated with the mean annual WSC content. If one can analyse only a single cut one should consider cut 4 or cut 1 since these have the highest correlation with the annual mean and show the highest variation among the entries.

The WSC content is very well positively correlated with the digestibility and to a less extent negatively correlated with the crude protein content. No correlations were observed with yield or rust resistance.

The WSC content is higher at a low nitrogen application level but shows no more variation among entries.

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Occurrence and characterization of *Neotyphodium* endophytes in Bulgarian populations of *Lolium perenne*

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ABSTRACT

During a collection trip to Bulgaria 62 accessions of *Lolium perenne* were collected from 55 sites representing different lowland and mountain habitat types. Endophytes were detected in 59 accessions by microscopic analysis of seed. Infection level varied from > 5 - 100% with 50 out of 59 accessions showing infection levels higher than 50%. The morphological diversity of the endophyte isolates appeared to be greater than described originally for *Neotyphodium lolii*. Radial growth of many isolates was very slow (< 0.2 mm/day) but faster growing isolates were also found. Isolates were assigned to 6 groups according to the morphological characteristics of the colonies grown on PDA. Detection of alkaloid levels revealed that isolates were highly variable according to their levels of lolitrem B, ergovaline, and peramine. One group of slow growing isolates did not produce ergovaline but high levels of lolitrem B. Clusters of isolates according to their origin reflect the adaptation of endophyte/grass associations to specific climatic and geographic conditions.

Key words: adaptation, diversity, growth rate, fungal endophytes, infection rate, incidence, isolate morphology, perennial ryegrass

INTRODUCTION

Most grassland in Bulgaria is permanent semi-natural pasture with only very limited areas of sown pasture. The larger portion of the permanent pastures is situated on infertile soils in regions of insufficient moisture. Therefore, adaptation to these conditions is a prerequisite for grass species to survive and proliferate. As endophyte infection may improve stress response of grass plants, the aim of our study was (1) to investigate the incidence of fungal endophytes in perennial ryegrass collected from such habitats, and (2) to characterize the corresponding fungal isolates.

MATERIALS AND METODS

During a collection trip to Bulgaria 62 accessions of *Lolium perenne* were collected from 55 locations representing different lowland and mountain habitat types. After a generative propagation in Germany, 50 seeds per accession were screened microscopically for the presence of endophytic fungi using rose Bengal staining (Saha *et al.*, 1988).

From seed of all endophyte-infected accessions plants were cultivated to obtain leaf sheath tissue for endophyte isolation as well as biomass for alkaloid analysis. Isolates were grown on PDA at 22°C in the dark. Radial growth rates were measured every second day for a period of 20 days on four replicates per isolate. For comparison, one Romanian isolate and two New Zealand isolates were included.

Lolitrem B was analyzed by a modified method of Gallagher *et al.* (1985). Ergovaline and peramine were detected by methods of Panaccione *et al.* (2003) and Spiering *et al.* (2002).

RESULTS AND DISCUSSION

Endophyte infection was detected in 59 out of 62 *Lolium perenne* accessions. Infection levels varied from > 5 - 100%. No accession was found to be low infected, but 50 out of 59 accessions showed infection levels higher than 50%.

Endophyte isolates showed a morphological diversity of their colonies that appeared to be greater than described originally for *Neotyphodium lolii* (Latch *et al.*, 1984). Considering isolate morphology and radial growth rate, isolates were assigned to 6 morphological groups with slow growing rates (<0.2 mm/day) for group A up to very fast growing rates (0.7mm/day) for group F.

Isolates were also highly variable regarding their levels of lolitrem B, ergovaline and peramine. Isolates of group B did not produce egovaline but high levels of lolitrem B. The Romanian isolate in group A produced neither ergovaline nor lolitrem B but low concentrations of peramine. High levels of all three analyzed alkaloids were found for isolates of group D. A relationship between morphology and ergovaline deficiency seems possible but needs further investigation.

Isolates belonging to the same group originate mostly from sites which are situated geographically close together and/or which are characterized by similar growth conditions (altitude, maritime climate, water availability). These clusters of isolates depending on their origin reflect the adaptation of endophyte/grass associations to specific climatic and geographic conditions.

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Selecting genes in *Lolium* x *Festuca* hybrids for root growth to improve soil hydrology

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ABSTRACT

Plant roots can affect soil hydraulic properties and consequently may influence drainage patterns from agricultural land. Effects of rooting traits are being investigated at different scales using *Lolium* (ryegrass), *Festuca* (fescue), *Festulolium* hybrids and *Lolium* genotypes with introgressed *Festuca* genes in a new project called SuperGrass. Primers designed from the rice genome will be used to map markers to important chromosome segments.

Key words: forage grasses, markers, run-off, soil hydraulic properties, soil macro-porosity

INTRODUCTION

A new project - SuperGrass (http://www.iger.bbsrc.ac.uk/SGF/) - aims to investigate the potential to manipulate water-soil-plant systems in farmed and semi-natural landscapes to control runoff to, and diffuse pollution of, surface water courses. Plant roots can change the macro-porosity and hydraulic properties of soil by affecting soil structure and/or water repellence (Figure 1) (Whalley *et al.*, 2004).
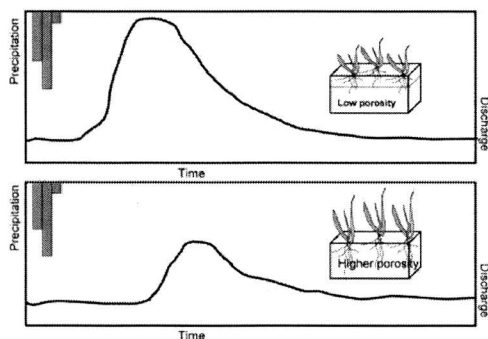

Fig. 1. Increased effective soil depth, f (rooting depth, porosity) increases time to peak discharge and decreases total discharge.

Little is known about how different grasses affect soil profile hydraulic responses during either water excess or shortage. *Festuca* species have deeper rooting and water extraction

characteristics than *Lolium* and this contributes to their greater drought resistance (Durand *et al.*, 2007). It is postulated that deeper, stronger roots from *Festuca* species will increase soil porosity and the hydraulic conductivity of soil at both plot and headwater catchment scales.

MATERIALS AND METHODS

Rooting traits are being transferred from *Festuca* to *Lolium* by inter-generic hybridization and by marker assisted selection using methods previously successful in transferring drought-resistance traits from *Festuca* species to *Lolium* (Humphreys J. *et al.*, 2005; Humphreys M *et al.*, 2006).

RESULTS

Genes for resistance against severe drought stress have previously been transferred from two different *Festuca* species (*F. arundinacea*, and *F. glaucescens*) onto chromosome 3 of drought sensitive *L. multiflorum* (Figure 2). Early results indicate that some of these introgressions also affect root growth and morphology.

Fig. 2. Genes from Festuca transferred onto L. multiflorum *chromosome 3. F. arundinacea total genomic DNA probes used to differentiate introgressed Festuca DNA (light region on chromosome) with counterstained L. multiflorum DNA (darker chromosome regions).*

DISCUSSION

Lolium chromosome 3 has conserved DNA sequences along its entire length in common with rice chromosome 1 (King *et al.*, 2002), which is implicated strongly in drought resistance and carries QTL for effective soil penetration traits. Primers designed from rice chromosome 1 QTL regions for rooting traits are being used to map functional single nucleotide polymorphism (SNP) markers to the introgressed *Festuca* chromosome segments. These will be screened on a *F. pratensis* BAC library, with selected BACs sequenced and compared to rice to identify putative genes for rooting traits for improved soil hydrology. These markers will also be of use for breeding programmes aimed at designing new *Lolium* cultivars for improved water-use-efficiency and soil hydrology.

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The evaluation of morphological variability in interspecific hybrids *Trifolium pratense* x *T. medium* and comparison with the parental species

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ABSTRACT

Trifolium pratense L. is a high-yield as well as a high-quality fodder crop. However, it shows a lower persistency, which could be overcome by hybridisation with species creating rhizomes. Hybrids between *T. pratense* and *T. medium* have been previously obtained by embryo rescue and the number of chromosomes was evaluated by flow cytometry. The aim of this study was to evaluate thirteen morphological traits (stem weight/length, internodes number, length/width of a central leaflet of the triple leaf under the top head, length/width of the central leaflet of the triple leaf on the 4th internodes, stem thickness on the 4th internodes, weight of plant, stem and head number per plant, intensity of white marks and average leaf area) in ten populations (550 plants) derived from F_1 hybrids and in the parental species. The significance of morphological differences was determined by ANOVA test. Nearly all examined traits were intermediate in hybrids; they reached higher values than in *T. medium* and lower values than in *T. pratense*. The stem number as well as head number per plants were significantly higher in ten hybrid populations compared with both parental species.

Key words: interspecific hybrids, morphological traits, *T. medium, T. pratense*

INTRODUCTION

Red clover (*Trifolium pratense* L.) is a widespread forage crop in the Czech Republic as well as in the rest of Europe. It is a high-yield and high-quality fodder crop from the point of view of both nutrition content and ensilage ability. It has high content of proteins, water soluble carbohydrates (WSC), tannins, polyphenol oxidase (PPO) and polyunsaturated fatty acids (PUFA). As a disadvantage we consider lower persistency which could be overcome by hybridisation with the more persistent *T. medium*. The hybrids between *T. pratense* (2n=4x=28) and *T. medium.* (2n=6x to 8x=48 to 80) were obtained by embryo rescue in The Research Institute for Fodder Crops, Ltd Troubsko by Brno, the Czech Republic in 1991 (Repkova *et al.*, 1991).

The objective of this research was to compare morphological traits of both parental species with plants derived from F_2 hybrids.

MATERIALS AND METODS

Hybrid plants of F_3 generation were planted in to nurseries and open pollinated within populations together with *T. pratense* tetraploid variety Amos. Subsequently, they were planted to nursery in 2005. In the second cut in 2006, the longest stem from each plant was collected (550 plants in total, divided into ten populations). The following morphological traits were evaluated for each plant:

Stem weight, stem length, internodes number, length/width of a central leaflet of the triple leaf under the top head, length/width of the central leaflet of the triple leaf on the 4th internodes, stem thickness on the 4th internodes, leaf area, intensity of white marks, plant weight, number of stems per plant, number of heads per plant.

The same measurements were performed for *T pratense,* variety Amos (60 plants) and *T. medium* (clone 10/8, 38 plants) which were used for the original interspecific crosses.

The measured data were statistically evaluated by one – way ANOVA, followed by a test of the least significant difference.

RESULTS

Differences between the progeny and the parental genotypes were found in the most evaluated morphological traits. Except the number of stems and heads per plant and the length/width of the central leaflet of the triple leaf under the top head, the progeny reached intermediate values in individual morphological traits as it is shown for the leaf area (Table 1).

Table 1. Leaf area of parental species and populations of hybrids in cm².

population	average											
Amos	15,21	x	2	*	*	*	*	*	*	*	*	*	*
H 5	13,41	2	x									2	*
H 2	13,03	*		x								5	*
H 1	13,02	*			x							5	*
H 4	12,76	*				x							*
H 7	12,39	*					x						*
H 9	12,31	*						x					*
H 6	12,23	*							x				*
H 8	12,11	*								x			*
H 3	12,01	*									x		*
H 10	11,56	*	2	5	5							x	*
T. medium	6,90	*	*	*	*	*	*	*	*	*	*	*	x

* LSD 0,1%

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In the number of stems (Figure 1) and the number of heads per plant, progeny in all ten populations reached higher values than both parental genotypes.
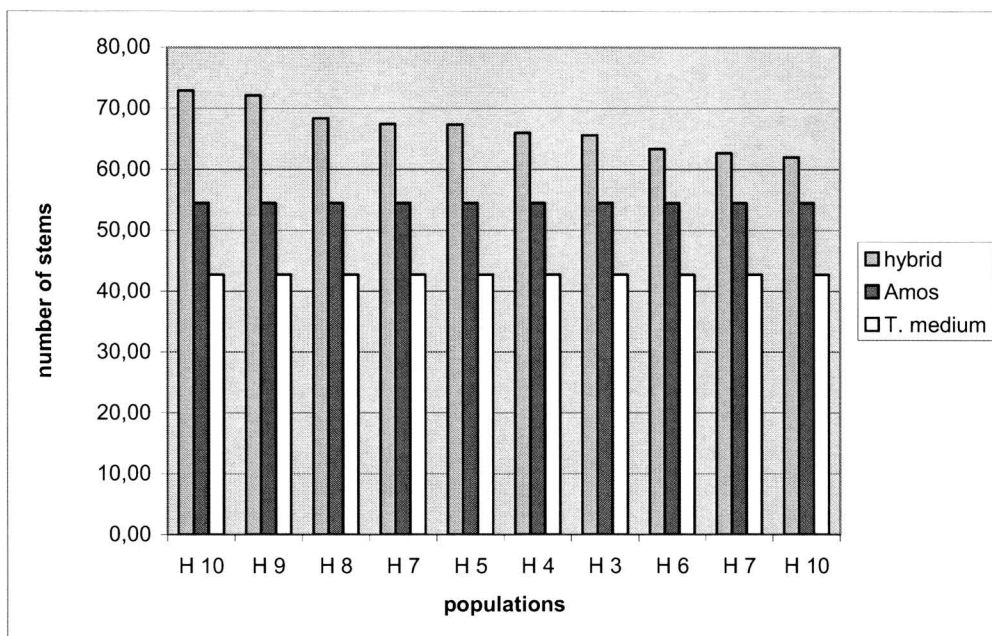

Fig. 1. Number of stems per plant of parental species and populations of hybrids.

DISCUSSION

The experiment confirmed the differences between the progeny of interspecific hybrids and its parental species in the morphological traits. Hybrids showed mostly intermediate level. It corresponds to the results of Isobe *at al.* (2001) who observed different leaf morphology in the same cross combination in comparison to the parental species.

The number of heads and stems per plant is a promising trait with a good agronomical value.

It is necessary to repeat this experiment in following years to verify above mentioned one year results.

ACKNOWLEDGEMENTS

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2D electrophoresis protein expression patterns of perennial ryegrass inbred lines and their F1 hybrid

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ABSTRACT

Proteomics has developed into an important experimental approach for the investigation of complex cellular processes and network functions. Heterosis is one of the most widely used phenomena in plant breeding, but among the least understood in its biological and genetic mechanism and function. Proteomics could be a useful tool to further our understanding of the basis of heterosis. In this study abundant soluble proteins of two perennial ryegrass inbred lines and their heterotic F1 hybrid were analysed by 2D protein electrophoresis in 5 different overlapping pH-ranges: 3-6, 4-7, 5.3-6.5, 6-11 and 3-10 at two harvest time points in 2006. The objective of this study was to create protein maps for each of the three plant lines and to identify similarly and differentially expressed proteins for further studies. Similar protein patterns were obtained for the two parental lines. This was in contrast to the protein pattern of the F1 hybrid line. Quantitative differences in the number of detected spots were observed among the two harvesting time points, mainly within the pH-range of 5.3-6.5. Mass spectrometric analysis of protein spots will further complement this study.

Key words: heterosis, proteomics

INTRODUCTION

Proteomics has developed into an important experimental approach for the investigation of complex cellular processes and network functions. Heterosis is one of the most widely used phenomena in plant breeding, but among the least understood in its biological and genetic mechanism and function.

Abundant soluble proteins of two perennial ryegrass inbred lines and their heterotic F1 hybrid were analysed by 2D protein electrophoresis in five different overlapping pH-ranges: 3-6, 4-7, 5.3-6.5, 6-11 and 3-10 at two harvest time points in 2006.

The objective of this study was to create protein maps for each of the three plant lines and to identify similar and differentially expressed proteins for further studies.

MATERIALS AND METHODS

Two inbred perennial ryegrass lines and their F1 hybrid were investigated. Plants were grown in the field and two harvests were done on 12.06.2006 and 18.08.2006. Proteins were isolated with the NucleoSpin Kit® RNA/Protein kit (Macherey-Nagel). Proteins were quantified using the BCA assay and protein quality was checked on 1D SDS electrophoresis. 2D electrophoresis was carried out on 10%SDS gels in 5 overlapping pH ranges and gels were

silver stained to visualize protein spots. Gels were scanned and images were overlayed using Proteomweaver software (Definiens). Spots were picked and a selection of spots was subjected to Maldi-TOF analysis. Identified peptides were matched against protein databases using the Mascot software (Matrix Science).

RESULTS

2D protein maps for the three plant lines were generated in five overlapping pH ranges from pH 3-11. Overlapping pH ranges led to a better resolution of regions containing the majority of the proteins in protein clusters, *e.g.* pH 4-6. Overlaying of gel images for plant lines at the same harvest time points allowed a comparative analysis of differentially expressed protein spots (Table 1).

Table 1. Number of detected spots for two pH ranges for parental and hybrid lines at one harvest time point.

Line	pH range	N gels	Average number of spots/gel	
			total number of detected spots	number of reliable spots
Maternal line	3-10	4	248	136
Paternal line	3-10	4	238	147
Hybrid line	3-10	3	260	191
Maternal line	4-7	2	148	83
Paternal line	4	7	337	238
Hybrid line	4	3	213	142

DISCUSSION

Technically the use of differential gel electrophoresis (DIGE) would lead to a better protein spot detection and would facilitate further studies.

A large number of highly abundant proteins across all three plant lines were found in this study. Protein patterns obtained for the two parental lines were frequently different from the pattern obtained from the F1 line, thus possibly involved in heterosis for biomass in perennial ryegrass. Only some protein sequences identified in this study by Maldi-TOF were found in the databases, e.g. a GDP-D-mannose-4,6-dehydratase.

ACKNOWLEDGEMENTS

Heritability of seed yielding capacity in tetraploid red clover (*Trifolium pratense L.*)

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ABSTRACT

Tetraploid red clover varieties are often characterised by an unsatisfactory level of seed yield leading to high production costs. Investigation of seed yield components showed a highly significant and positive correlation between the number of flower heads per plant and seed yield (Herrmann *et al.*, 2005). In the phenotypical recurrent family selection plants are harvested individually and only plants with sufficient seed yield per plant start the next cycle. To investigate the heritability of seed yielding capacity we applied a divergent selection. Five families of 5 high seed yielding plants were allowed to intercross in one field and five families of low seed yielding plants in another field. Seeds were harvested per offspring family during two years. Analysis of variance for the seed yield per offspring family of the high and low seed yielding plants showed a highly significant difference between the two polycrosses. The heritability was estimated as the report between the difference of the mean of the seed yields of the high and low seed yielding parents and the difference of the mean of the seed yields of their progenies. The heritability was high and amounted to 0.82 in the first year and to 0.88 in the second year. This indicates that eliminating plants with insufficient seed yield for the next cycle of recurrent family selection will improve the seed yield capacity of tetraploid red clover. The mean seed yield of the progenies of the high seed yielding plants showed a broad variation and inconsistency over the harvest years. Selection for seed yield among and within these families needs repeated observations.

Key words: heritability, seed yielding capacity, tetraploid red clover

INTRODUCTION

Tetraploid red clover varieties are often characterised by an unsatisfactory level of seed yield leading to high production costs. In the phenotypical recurrent family selection plants are selected for high number of flower heads per plant and are harvested individually. To improve seed yield, only plants with sufficient seed yield per plant start the next selection cycle. The objective of this research is to investigate the heritability of seed yielding capacity.

MATERIALS AND METHODS

In 2003 we planted 275 individual tetraploid red clover plants belonging to 11 families. In the nursery field all plants with insufficient growth and disease resistance were eliminated. In the summer of 2004, the seeds of 45 remaining tetraploid plants were individually harvested. To investigate the heritability of seed yielding capacity we applied a divergent selection. Five offspring families from 5 high seed yielding plants were allowed to intercross in one field and five offspring families from 5 low seed yielding plants in another field. The plants were

grouped in 6 blocks containing 5 plants of each of the 5 families. Seeds of each polycross were harvested per block in 2006 and 2007 and the mean seed weight per family was measured.

RESULTS AND DISCUSSION

Both in 2006 and 2007 the seed yield of the progenies from the low seed yielding parents was very significantly lower than the seed yield of the progenies from the high seed yielding parents.

Figure 1 shows the seed yield harvested in 2004 on the 10 selected parents and the mean seed yield of the 30 progenies per plant harvested in 2007 in the second year after sowing. The figures are standardized to eliminate year effects. The heritability was estimated as the report between the difference of the mean of the seed yields of the progenies from the high and low seed yielding plants and the difference between the mean of the seed yields of the high and low seed yielding plants. The heritability calculated in this way was very high and amounted to 0.88.

2004　　　　　　　**2007**
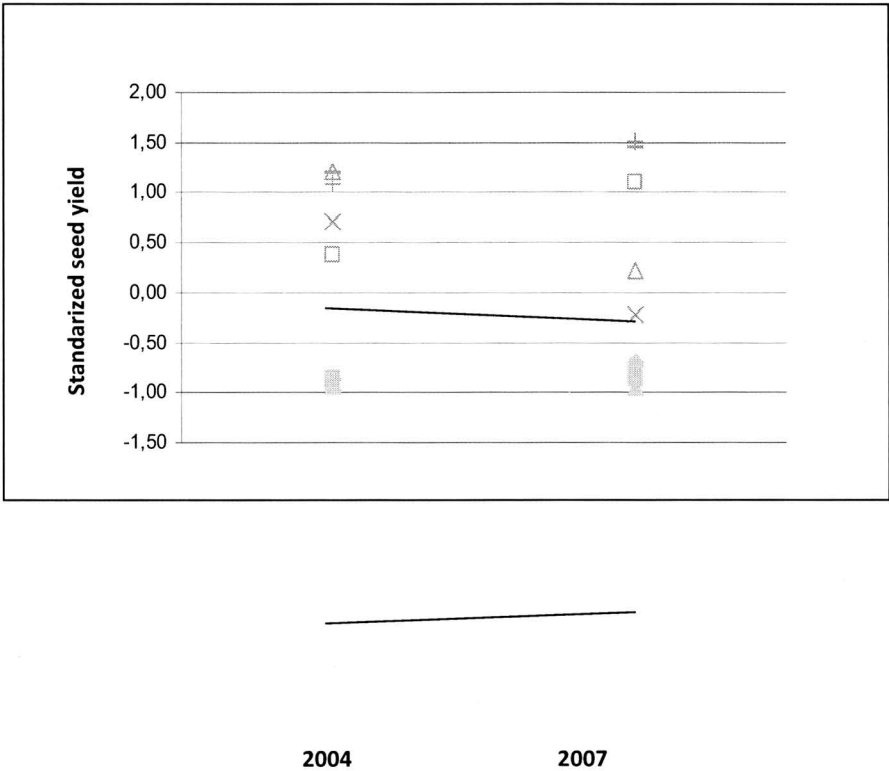
Fig. 1. Standarized seed yield of 5 high (□+△—x) and 5 low (▪▲♦●—) seed yielding plants in 2004 and the mean of their progenies in 2007. (The lines connect the means of high and low seed yielding plants in 2004 with the means of their progenies in 2007).

The correlation between the mean seed yield per plant of the 10 families in 2006 and 2007 was significant (r= 0.69) (Figure 2). Within the low seed yielding families and within the high seed yielding families, no correlation between seed yield in 2006 and 2007 was found.

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The 5 families of the polycross showing the lowest seed yielding capacity in 2006 also had the lowest seed yield in 2007. Eliminating these families in recurrent family selection will improve seed yielding capacity.

The mean seed yield of the progenies of the high seed yielding plants showed a broad variation and inconsistency over the harvest years. Selection for seed yield among and within these families needs repeated observations.
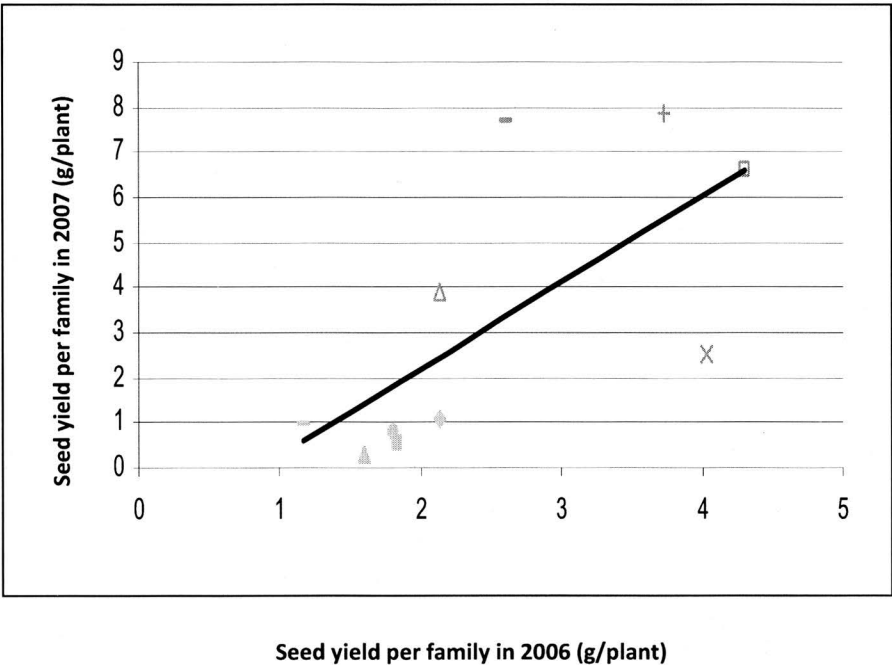

Seed yield per family in 2006 (g/plant)

Fig. 2. Relationship between 2006 and 2007 of the mean seed yield (g / plant) of the 30 progenies of 5 high (□+△− x) and 5 low (▪ ▲ ♦ ●—) seed yielding plants.

CONCLUSION

Eliminating all plants with insufficient seed yields for the next selection cycle of recurrent family selection will improve the seed yielding capacity of tetraploid red clover. Broad variation and year effect make selection among and within high seed yielding families a great challenge.

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Improving herbage nonstructural carbohydrates in alfalfa

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ABSTRACT

High herbage nonstructural carbohydrate (NSC) concentration improves ruminant digestion and ensiling. Concentration of NSC is genetically variable and increases during the day. We evaluated two populations from one cycle of divergent selection for NSC (soluble sugars and starch) in alfalfa (*Medicago sativa* L.) harvested in the morning (AM: 9h00) or the afternoon (PM: 15h00) under field conditions. Populations (NSC + and NSC -) were obtained by intercrossing 10 genotypes selected for high or low NSC concentrations from 500 genotypes of AC Caribou. Populations were evaluated for NSC, crude protein (CP), ADF, NDF, and dry matter yield (DMY). Concentrations of NSC were estimated by the sum of soluble sugars and starch. The NSC + population had higher NSC (109 vs. 94 g/kg DM), and lower NDF (321 vs. 337 g/kg DM) and ADF (248 vs. 262 g/kg DM) concentrations than the NSC - population. Herbage DMY and CP concentration were not affected by populations and time of harvest. The PM-harvested alfalfa had greater NSC (119 vs. 83 g/kg DM) and lower NDF (313 vs. 342 g/kg DM) and ADF (241 vs. 268 g/kg DM) concentrations than AM-harvested alfalfa. The increase in NSC concentration with NSC + population or PM harvest was mainly due to an increase in starch concentration. Alfalfa NSC concentration can be increased through breeding and harvest management.

Key words: soluble sugar, starch, crude protein, divergent selection, acid detergent fibre, neutral detergent fibre

INTRODUCTION

Concentration of nonstructural carbohydrates (NSC) in alfalfa is genetically variable and increases during the day (Burns *et al.*, 2005). High herbage NSC concentration improves ruminant digestion and ensiling. Our objective was to evaluate the response of one cycle of divergent selection for NSC in alfalfa (*Medicago sativa* L.) populations harvested in the morning or the afternoon under field conditions.

MATERIALS AND METHODS

Two populations (NSC + and NSC -) were obtained by intercrossing 10 genotypes selected for high or low NSC concentrations from 500 genotypes of the cultivar AC Caribou. A third population (NSC 0) was obtained from intercrossing 10 genotypes randomly selected. The three populations were compared in an experiment conducted near Quebec City; a split plot

design was used with time of harvest (AM: 9h00 and PM: 15h00) as main plots and populations as subplots. Populations were harvested at the early flowering stage of development. Forage samples were dried at 55°C immediately after each harvest and ground to pass through a 1-mm screen. Selected samples were analysed by colorimetry for soluble sugars and starch (Blakeney and Mutton, 1980). Concentrations of NSC were estimated by the sum of soluble sugars and starch. Concentrations of NDF and ADF were nonsequentially determined using the method of Van Soest *et al.* (1991).

RESULTS AND DISCUSSION

The NSC + population had higher NSC (109 vs. 94 g/kg DM), and lower NDF (321 vs. 337 g/kg DM) and ADF (248 vs. 262 g/kg DM) concentrations than the NSC - population. The NSC 0 population was intermediate. Alfalfa harvested in the afternoon had greater NSC (119 vs. 83 g/kg DM) and lower NDF (313 vs. 342 g/kg DM) and ADF (241 vs. 268 g/kg DM) concentrations than alfalfa harvested in the morning. The increase in NSC concentration with NSC + population or PM harvest was mainly due to significant changes in the accumulation of starch (Table 1). Populations or times of harvest did not differ in herbage DMY and CP concentration. The NSC + population harvested in the afternoon had higher NSC concentration (+51 g/kg DM), lower ADF (- 43 g/kg DM) and NDF (- 44 g/kg DM) than the NSC - population harvested in the morning. This increase in NSC associated with a decreased fibre concentration has the potential to improve daily dry matter intake of more digestible forage and the resulting animal performance. A management strategy of harvesting hay in the afternoon combined with genetically improved populations may provide an opportunity to increase total nonstructural carbohydrate concentrations in alfalfa herbage.

Table 1. Chemical composition and dry matter yield of divergently selected alfalfa populations harvested in the morning (AM) or in the afternoon (PM).

Alfalfa population	NSC -		NSC 0		NSC +	
Harvest	AM	PM	AM	PM	AM	PM
Chemical composition (g/kg DM)						
Soluble sugars	61.3*d*	74.9*b*	64.9*c*	78.6*a*	65.7*c*	78.9*a*
Starch	14.1*d*	38.5*b*	17.9*d*	38.2*b*	25.1*c*	47.4*a*
Nonstructural carbohydrates	75.4*e*	113.4*b*	82.8*d*	116.8*b*	90.9*c*	126.3*a*
Crude protein	354	354	358	352	354	349
Acid detergent fibre	277*a*	248*c*	264*b*	241*cd*	263*b*	234*d*
Neutral detergent fibre	351*a*	322*cd*	339*ab*	310*d*	335*bc*	307*d*
DM yield (g DM/m^2)	186	163	153	176	191	183

In the same line, means followed by different letters are different according to the Duncan test ($P < 0.05$).

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Forage yields in some Serbian urban populations of large-flowered vetch (*Vicia grandiflora* Scop.)

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ABSTRACT

A small-plot trial was carried out in 2005 and 2006 at Rimski Šančevi Experimental Field of the Institute of Field and Vegetable Crops with twelve urban populations of large-flowered vetch (*Vicia grandiflora* Scop.). Number of plants surviving until cutting ranged from 55 in NS-8 to 132 in NS-1 and NS-6. The highest green forage yield was in NS-3 (35.5 t ha⁻¹), while the highest forage dry matter yield was in NS-5 (11.6 t ha⁻¹). The lowest yields were in BG-4, with 21.4 t ha⁻¹ of green forage, and 4.7 t ha⁻¹ of forage dry matter.

Key words: forage yield, forage yield components, large-flowered vetch, potential, wild populations.

INTRODUCTION

The flora of Serbia and its province of Vojvodina is rather rich in *Vicia* species, with large-flowered vetch (*Vicia* grandiflora Scop.) as one of the most widely distributed (Krstić *et al.*, 2007). Apart from being a component of natural grasslands, this species is present in various urban environments, such as cities of Belgrade and Novi Sad. Large-flowered vetch often grows together with narrow-leafed (*Vicia sativa* L. ssp. *nigra* (L.) Ehrh.) and hairy vetches (*Vicia villosa* Roth), germinates in early autumn, flowers in April and produces seed in May.

The objective of our study was to evaluate the potential forage yields in urban populations of large-flowered vetch.

MATERIALS AND METHODS

A small-plot trial was carried out in 2005 and 2006 at the Experimental Field of the Institute of Field and Vegetable Crops at Rimski Šančevi. It included twelve populations of large-flowered vetch collected at various sites in the cities of Belgrade and Novi Sad. All populations were sown in early October, with a rate of 150 viable seeds/m² and were cut in the stages of full flowering and formation of the first pods (Mihailović *et al.*, 2005b).

Results were processed by analysis of variance (ANOVA) applying the Least Significant Difference (LSD) test using the computer software MSTAT-C.

RESULTS AND DISCUSSION

Number of plants that survived until cutting ranged from 55 in NS-8 to 132 in NS-1 and NS-6 (Table 1). The highest green forage yield was in NS-3 (35.5 t ha^{-1}), while the highest forage dry matter yield was in NS-5 (11.6 t ha^{-1}), confirming the preliminary results of the testing of this species (Ćupina *et al.*, 2007). The lowest yields were in BG-4, with 21.4 t ha^{-1} of green forage, and 4.7 t ha^{-1} of forage dry matter.

Table 1. Forage yield components and forage yields in twelve urban populations of large-flowered vetch at Rimski Šančevi in 2005 and 2006.

Population	Number of plants (m^{-2})	Plant height (cm)	Number of stems and lateral branches (plant^{-1})	Number of internodes (plant^{-1})	Green forage yield (t ha^{-1})	Forage dry matter yield (t ha^{-1})	Forage dry matter proportion
BG 1	115	80	7.0	103.0	22.6	8.5	0.37
BG 2	110	70	6.0	126.0	22.1	5.7	0.26
BG 3	88	92	9.0	196.0	35.1	11.1	0.32
BG 4	124	40	5.0	76.0	21.4	4.7	0.22
NS 1	132	45	6.0	121.0	25.5	5.6	0.22
NS 2	131	57	6.0	68.0	27.4	6.0	0.22
NS 3	82	75	9.0	173.0	35.5	9.9	0.28
NS 4	78	80	12.0	185.0	32.0	9.7	0.30
NS 5	128	62	6.0	72.0	30.5	11.6	0.38
NS 6	132	65	7.0	149.0	31.7	8.9	0.28
NS 7	118	62	13.0	255.0	26.3	7.6	0.29
NS 8	55	77	14.0	317.0	31.3	8.8	0.28
$LSD_{0.05}$	21	17	2.6	34.1	5.8	1.8	0.05
$LSD_{0.01}$	28	22	4.0	49.5	7.3	2.3	0.07

Wild populations of large-flowered vetch have shown a high level of variability of the characteristics of agronomic importance and may be considered as having a considerable potential for its utilisation in breeding for forage production and green manure.

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Development and mapping of Expressed Sequence Tags (ESTs) in *Lolium perenne*

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ABSTRACT

Several linkage maps, mainly based on anonymous markers are now available for *Lolium* perenne. The saturation of these maps with markers derived from expressed sequences would provide complementary information useful also for QTL-mapping. Therefore we initiated an study with the main aim to map candidate genes in *Lolium* spp. In a first step, EST markers were developed on sequences derived from a cDNA library, constructed from leaf tissue. 48 sequences with interesting homologies to genes with known gene function and involved in traits such as digestibility, nitrogen use efficiency, and disease resistance were selected. Seventeen markers were polymorphic in the CLO-DvP reference population. We also developed EST markers from candidate genes involved in self-incompatibility. Three sources of candidate genes were used (1) putative S and Z genes, (2) genes involved in the signalling cascade triggered by a SI response, both identified by the cDNA-AFLP technique, and (3) S thioredoxine and S receptor kinase homologues. These EST markers were mapped, in the ILGI population, where S and Z had previously been mapped and/or the CLO-DvP reference population. Success rates, levels of polymorphism and mapping results are discussed.

Key words: disease resistance, functional markers, linkage analysis, self-incompatibility

INTRODUCTION

Several linkage maps, mainly based on anonymous markers are now available for *Lolium perenne*. The saturation of these maps with markers derived from expressed sequences would provide complementary information useful also for QTL-mapping. Therefore we initiated a study with the main aim to map candidate genes in *Lolium* spp.

MATERIALS AND METODS

The following sequences were used for DNA-marker development and/or mapping in *L. perenne*: (i) 44 leaf-derived *L. perenne* cDNA clones (Muylle *et al.*, 2003), (ii) 475 sequences derived from cDNA-AFLP Transcript Derived Fragments (TDFs) related to SI (Van Daele *et al.*, submitted), (iii) a thioredoxin homologue, amplified in *L. perenne* using the primers SI3 (ATCAATCGAACCACTCGCTGCG) and SI4 (GGCGGGAAAGACACAGACACTGT) (Van Daele, unpublished), (iv) a SRK homologue amplified in *L. perenne* using the degenerated primers S-SRK2 (GGNTTYGGNATYGTNTAYGAR) and AS-SRK

(RATNCKNGCCATNCCRAARTC), derived from the conserved amino acid region of SRK-9 from *Brassica napus* (accession number: D88196) and a receptor kinase domain of *Arabidopsis thaliana* (acession number: M802387), (v) six primer pairs which amplify sequences flanking Z in rye (Hackauf and Wehling, 2005). All primer pairs were designed using the software Primer Express v. 2.0 (Applied Biosystems) using the default parameters, except for optimal primer length, which was set to 25 nucleotides. Two populations were screened for polymorphisms and used for genetic mapping: the *L. perenne* mapping family P150/112 (ILGI) (Jones *et al.*, 2002) and the CLO-DvP reference population TC1 x SB2 (Muylle *et al.*, 2005). Linkage analysis and mapping was done using Joinmap 3.0.

RESULTS AND DISCUSSION

The primer pairs were first used to screen one or both mapping populations for length polymorphisms. Of all the primer pairs tested 13% and 11%, were unable to amplify any DNA fragment in the ILGI and CLO-DvP populations respectively. Seventy-seven percent and 71% of the primer pairs amplified monomorphic bands in the ILGI and the CLO-DvP populations respectively. The rest of the primer pairs (10% for ILGI and 18% for CLO-DvP) detected clear length polymorphisms and were used for amplification in the complete ILGI and/or CLO-DvP population. Of the primer pairs which revealed polymorphism in the ILGI and CLO-DvP population, 41 and 83 produced single locus amplification products, respectively while 8 and 12 primer pairs amplified two or more loci, respectively. Fifty markers could be mapped in the CLO-DvP population and 33 markers were mapped in the ILGI population. Some of the genes mapped around the S (LG1) or the Z (LG2) locus, both involved in self-incompatibility. The thioredoxin marker maps close to S on LG1 in the ILGI population. In the CLO-DvP population a marker with an interesting expression profile and showing homology to an ATP binding protein of the ABC transport system of bacteria (Van Daele *et al.*, submitted) maps in the neighbourhood of the S locus. Ten EST derived markers map to LG2 but none of them co-segregates with Z. Some EST-derived markers with interesting homology to known resistance genes mapped around the QTLs involved in crown rust resistance in the CLO-DvP population. Of especial interest are the 3 markers (cDNA34, cDNA 18, cDNA 21) which map close to QTL2 on LG1 (Muylle *et al.*, 2005). These markers show homology to a beta-glucanase, a chitinase and a beta-glucanase, respectively.

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Agro-ecological technology system for the production of white clover seed (*Trifolium repens L.*)

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ABSTRACT

The conventional technology of seed production in white clover relies upon sowing in a pure stand and on the application of specific herbicides against weeds. The present study demonstrates the possibility of achieving efficient seed yields by seeding white clover under a cover crop (*Lolium perenne* or *Festuca pratensis*), with no herbicide application, and where the first or the second forage harvest is used for animal grazing.

Key words: white clover, seed, cover crop, grazing

INTRODUCTION

Biological particularities specific to white clover (perennial, vegetative reproduction through stolons, high capacity of regeneration after mowing) influence seed production in this species. This may explain the large variation limits of seed production, between 100 and 1000 kg/ha (Breazu *et al.*, 1987).

Ecologic and technological conditions make important contributions to the vegetative and generative growth of white clover. These conditions influence directly the seed production components and its amount (Askarian *et al.*, 1993; Dragomir *et al.*, 2007; Newbould, 1986; Pasumarty *et al.*, 1993).

This paper presents the results of research concerning the achievement of an efficient technology for seed production, under agro-ecologic systems, also taking into consideration white clover's character of a pasture legume.

MATERIALS AND METHODS

Our research has been carried out during 2002-2004 at Banat's University of Agricultural Sciences and Veterinary Medicine Timisoara, on a cambic chernozem, slightly gleyied, clayey-loamy, slightly acid.

The experimental device was consisted of two parts:
- seed production, under conventional technology systems, by seeding white clover in pure stand, with three seeding rates (2 kg/ha, 4 kg/ha, 6 kg/ha) and controlling weeds through the application of herbicides (PIVOT 1.0 l/ha);

- seed production under agro-ecological technology systems, by seeding white clover in a cover crop (*Festuca pratensis*, *Lolium perenne*), with no application of herbicides.

The seeding was performed in the spring, with the following method: the white clover was seeded with the distance of 25 cm between rows (independent of the seeding rate), and the perennial grasses were seeded perpendicularly to the white clover rows, with the distance of 12.5 cm between rows, with a seeding rate of 10 kg/ha.

Before seeding, we have applied a uniform fertilization with $N_{50}P_{50}K_{50}$.

The biological material used was consisted of the following Romanian varieties: the variety Danitim of white clover, Marta of *Lolium perenne* and Tampa of *Festuca pratensis*.

During all years of production, seed harvesting was performed at the second mowing.

RESULTS AND DISCUSSION

Under the conventional production system of white clover seeds, carried out in Romania, the application of herbicides represents the most important technological factor for yield increase (Table 1).

Table 1. Herbicide and seeding rate influence upon seed production in white clover, seeded in pure stand (average for the production years, 2002-2004).

Application of herbicides	Seeding rate for white clover	Percentage of weed species (%)	Number of white clover plants per m^2	Number of capitula per m^2	Seed yield	
					Kg/ha	%
Without herbicides	2 kg/ha	46	67	257	93	100
	4 kg/ha	38	72	275	105[*]	113
	6 kg/ha	25	105	288	116[**]	124
With herbicides (Pivot 1.0 l/ha)	2 kg/ha	15	82	308	146	157
	4 kg/ha	12	112	376	166[**]	178
	6 kg/ha	7	143	392	137	147
	DL 5% 8.4	1% 15.5	0.1% 33.7			

So, depending on white clover density, achieved through various seeding rates, the amount of weed species has ranged between 25 and 46%, and in the case of PIVOT application (1.0 l/ha), the percentage of weeds has decreased to between 7 and 15%. We may also observe a remarkable increase in the number of white clover plants and in the number of capitula/m^2, achieved through the increase of seeding rate and also through the application of herbicides.

Seed production, in the case of the conventional technology, was strongly influenced by the effect of herbicide application and by the seeding rate of white clover. So, the biggest yield, of 116 kg/ha, in the variants with no herbicide application, was achieved by seeding white clover with a rate of 6 kg/ha, and in the variants with herbicide application, the maximal yield was 166 kg/ha, with the rate of 4 kg/ha.

The results achieved in the associated crop of white clover, seeded in mixture with perennial grasses, made evident the possibility to produce seeds in this legume and under such technology

system, because the yield level is similar to that obtained under conventional technologies (Table 2). In the case of this technological system, perennial grasses have limited the amount represented by weed species up to a level of 20%, obtaining a seed yield of 138 kg/ha, when the white clover was seeded with a rate of 6 kg/ha and in association with *Lolium perenne* (10 kg/ha).

The calculations of economic efficiency prove that both technologies used for the production of white clover seeds will be profitable (Table 3).

On the whole, if the economic profitability indices are about 20% bigger under the conventional technology than under the proposed technology (agro-ecologic), total expenses are lower in the agro-ecologic technology. The seed cost is very similar for both technologies: 2.29 €/kg, in the conventional technology and 2.43 €/kg, in the agro-ecologic technology.

Table 2. Influence of gramineous species and seeding rate upon seed production in white clover, seeded in association with perennial grasses (average for the production years, 2002-2004) (Agro-ecologic technology).

Grass species	Seeding rate for white clover	Percentage of weed species (%)	Number of white clover plants per m^2	Number of capitula per m^2	Seed yield	
					Kg/ha	%
Festuca pratensis (10 kg/ha)	2 kg/ha	36	73	212	98	100
	4 kg/ha	32	82	227	95	97
	6 kg/ha	24	80	213	122[***]	124
Lolium perenne (10 kg/ha)	2 kg/ha	34	80	216	124	126
	4 kg/ha	29	78	245	126	128
	6 kg/ha	20	93	269	138[**]	141
	DL 5% 7	1% 11.8	0.1% 19.3			

Table 3. Profitability calculation for seed production technology in white clover.

Specification	Conventional technology (Ct)	Agro-ecologic technology (Aet)	Differences (Aet-Ct)
Total expenses (€/ha)	380	335	-45
Total income (€/ha)	1037	862	-175
Profit (€/ha)	657	527	-130
Unitary cost (€/kg)	2.29	2.43	+0.14

CONCLUSIONS

- Seed production in white clover may be achieved through the application of conventional technologies (pure stand and application of herbicides), and also of agro-ecologic

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technologies (crop associated with a grass cover crop, without herbicides), because the yield difference is only 10-20% bigger in the conventional one;

- Within the system of sustainable agriculture, it is advisable to apply agro-ecologic technologies, in which the white clover is considered as a pasture plant;
- The profitability is similar in the two technological systems applied.

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Influence of habitat on genetic diversity of *F. pratensis* and *L. multiflorum* ecotype populations from permanent grassland

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ABSTRACT

Permanent grassland harbours genetic resources of many forage plant species. Although these resources are often used for broadening gene pools in breeding programs, little is known about the importance of grassland sites for conservation of genetic diversity. In order to evaluate genetic diversity present in permanent grassland and to identify valuable sites for collection and conservation strategies, 12 ecotype populations collected at different habitats and four cultivars each of *Festuca pratensis* Huds. and *Lolium multiflorum* Lam. were analysed by means of SSR markers. Analysis of molecular variance revealed a lower within population variation for *F. pratensis* than for *L. multiflorum*. *F. pratensis* ecotype populations were clearly separated from cultivars and clustered to three groups according to geographic regions. No population differentiation was observed for *L. multiflorum* suggesting the existence of a single gene pool. In contrast, *F. pratensis* populations were structured according to management and environmental factors prevailing at collection sites.

Key words: genetic structure, geographic region, Italian ryegrass, management, meadow fescue, SSR markers

INTRODUCTION

Permanent meadows and pastures are reservoirs of genetic diversity of which well adapted ecotype populations have traditionally been collected as base material for breeding purposes since they are known to be useful for the improvement of specific morphological traits (Boller *et al.*, 2005; Fjellheim *et al.*, 2007). The intensification of agriculture during the last decades however has lead to a replacement of permanent grassland by more productive leys and to a loss of diversity. To stop genetic erosion and to ensure long-term supply of valuable germplasm for breeding purposes, appropriate collection and conservation strategies should be defined. An important prerequisite for the identification of valuable collection and conservation sites is the detailed knowledge of genetic diversity of ecotype populations from well characterised habitats. In order to evaluate the influence of habitat and management on genetic diversity and genetic structure, two important forage grass species with contrasting abundance have been chosen; widespread *L. multiflorum* and less abundant *F. pratensis*.

MATERIALS AND METHODS

At different habitats across Switzerland, twelve ecotype populations per species were collected. Additionally, four cultivars per species were included. Twenty-three individual

plants per population were investigated with 24 SSR markers. Genetic variation within and among the populations was determined by analysis of molecular variance, the relationships among the populations by means of cluster analysis and the influence of environmental factors on genetic structure by redundancy analysis.

RESULTS AND DISCUSSION

Analysis of molecular variance revealed differences between the two species as to the extent of variation within and among populations. In *F. pratensis*, 92.6% of the total variance occurred within populations and 4.8% between populations. Less variation was found between *L. multiflorum* populations with only 2.3% of the total variance. According to cluster analysis, *F. pratensis* cultivars and ecotype populations were clearly separated from each other. Ecotype populations were further subdivided into three groups corresponding to the geographical regions (Northern foothills of the Alps, western and eastern part of Switzerland) they were sampled from. A significant influence of altitude, longitude, latitude and different management practices on genetic structure was confirmed by redundancy analysis. The results of this study indicate that habitat and management affect *F. pratensis* but not *L. multiflorum* ecotype populations (Peter *et al.*, 2008). Reasons may be that *F. pratensis* occurs in locally rather isolated areas and in more contrasting climates than *L. multiflorum*. Despite the clear grouping according to geographical regions, no population of a specific habitat was found to be significantly more diverse and the level of heterozygosity was consistent across all populations. For maintenance and long-term conservation of genetic diversity, *F. pratensis* habitats of contrasting management regimes in different geographical regions should be considered. The extremely low population differentiation of widespread *L. multiflorum* suggests in contrast the existence of a single gene pool, possibly influenced by human-mediated gene flow between cultivars and ecotype populations in surrounding leys.

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New genomic resources for orchardgrass

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ABSTRACT

One of the initial requirements of utilizing genomic approaches in plant improvement is the availability of DNA sequence information. Toward the goal of generating sequence information for forage and pasture grasses, we are developing an EST library from orchardgrass, or cocksfoot (*Dactylis glomerata*). Tissues collected from orchardgrass included water and salt stressed shoot and roots, etiolated seedlings, and low temperature acclimated crowns. Library construction, sequencing, and bioinformatics are carried out through cooperation with the University of Illinois W.M. Keck Center for Comparative and Functional Genomics. The resulting EST library will be normalized, tagged for identification by tissue, and sequenced from both the 5' and 3' ends in order to include the 3'untranslated regions (UTR). SSRs identified from this library containing contigs/singletons will be aligned to rice chromosomes to determine predicted locations of the SSR markers. PCR primers designed from 3'UTR regions from other species contained high degrees of polymorphism and the same is expected from this orchardgrass library. Additionally, the sequence data from the library will be used to identify SNPs. The USDA ARS Forage and Range Research Laboratory has an extensive orchardgrass improvement program and our objective is to identify phenotypic associations and apply the markers to a marker-assisted selection program for increased salt tolerance and winter hardiness. Sequence information and resulting primers will be presented.

INTRODUCTION

Orchardgrass is a major forage grass for irrigated areas in the western U.S. However, it lacks tolerance to many abiotic stresses including salinity, drought, and winter injury. Breeding efforts are ongoing at FRRL to improve these traits in orchardgrass. In conjunction, with the applied breeding efforts we are developing a program to genetically characterize and determine underlying genetic mechanisms for these traits in orchardgrass. The objective of this study was the creation and characterization of an EST library for use in an orchardgrass molecular breeding program.

MATERIALS AND METHODS

Plant tissue came from clones representing the orchardgrass cultivars Ambasador, Paiute, and Potomac. EST libraries are being constructed in conjunction with the Roy J. Carver Biotechnology Center, University of Illinois.

RESULTS AND DISCUSSION

Three tissues were harvested 1) cold-acclimated (7 d at 4°C, 2 d at 0°C, and then 6 hours at -4°C), 2) etiolated seedlings (germinated in germination pouches at room temperature and dark conditions for 10 d), and 3) salt/drought stressed roots and shoots (seedlings grown for 8 wk with 40% ET_0 irrigation rate and EC 6 irrigation water). Pilot studies on 200 clones showed approximately 5% redundancy. Sequencing is limited to clones at least 600bp in length, and is 50% completed.

Techniques for marker-free transgenic alfalfa

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ABSTRACT

The presence of selectable marker genes (SMGs) in transgenic plants is often criticized for putative environmental and health risks. Techniques to avoid the presence of SMGs in transgenic plants are available, but have not yet been implemented in forage crops. We are assessing the efficiency of co-transformation and markerless transformation in obtaining marker-free transgenic alfalfa. Co-transformation with two T-DNAs, each carried by a different culture of *Agrobacterium tumefaciens*, was accomplished. Fifteen putative co-transformed plants were regenerated from two transformation experiments. Two of these plants were crossed to a non transgenic plant, and their progenies were examined by PCR for transmission of both T-DNAs. Segregation of the two T-DNAs was observed in one progeny. Markerless transformation was performed with a SMG (*Npt*II) and a reporter gene (*GUS*) to estimate the percentage of transgenic somatic embryos that can be obtained by regeneration without selection. In two replicated experiments, from 0 to 1.7% of the embryos were transgenic. Though feasible, both marker-free approaches need to be improved for efficiency before they can be routinely adopted.

Key words: co-transformation, genetic engineering, markerless transformation, *Medicago sativa*, selectable markers

INTRODUCTION

Selectable marker genes (SMGs) are often linked to the useful genes to efficiently produce transgenic plants. However, they are generally useless outside of the lab and their presence in transgenic crops has been a matter of concern. Techniques to avoid the presence of SMGs in transgenic plants are available. Marker-free transgenic plants can be obtained in two ways: by introducing the useful gene without an SMG and identifying the transgenics among the regenerated plants using molecular techniques (de Vetten *et al.*, 2003; Popelka *et al.*, 2003); or by removing SMGs after the selection of transgenic cells/plants. The latter task can be accomplished in two ways: co-transformation (reviewed in Ebinuma *et al.*, 2001) or post-transformation excision of SMG sequences.

Co-transformation aims at introducing the useful gene and the SMG at different chromosomal sites. To accomplish this objective each gene is inserted in a separate transformation vector, or in the same vector but in separate transferred DNA (T-DNA) regions. Plants are then regenerated under selection pressure, and consequently they will contain the SMG; among the plants obtained, those also containing the useful gene are identified by molecular analyses. If the two genes are not concatenated, their separation is accomplished by selfing or crossing and selecting the plants containing only the useful gene among the progenies.

This work is aimed at assessing the efficiency of co-transformation and markerless transformation (transformation with the useful gene only) to obtain marker-free transgenic alfalfa. We are using three easily detectable genes, *Npt*II, *hem*L and *GUS*, to facilitate this task.

MATERIALS AND METHODS

Alfalfa (genotype Regen SY1, Bingham, 1991) co-transformation was performed with two binary vectors (pPZP-*NptII* and pPZP-*hem*L, Figure 1), each carried by a different culture of *Agrobacterium tumefaciens,* strain LBA4404. The *hem*L gene of *Synechococcus elongatus,* that confers resistance to gabaculine (Gough *et al.*, 2003; Rosellini *et al.*, 2007), was used as selectable marker gene, whereas the kanamycin resistance *Npt*II gene played the part of the useful gene. Two co-transformation experiments were performed as described in Rosellini *et al.* (2007). Somatic embryos regenerated on gabaculine selection were used as starting material for a second regeneration cycle with kanamycin selection. Kanamycin resistant somatic embryos from the second regeneration cycle were converted into plants. PCR screening was used for a preliminary confirmation of the presence of both genes. Two co-transformed plants (C9 and C12) were crossed to an unrelated male parent (genotype CLA) and the T$_1$ progenies were screened for segregation of the two genes by PCR amplification with coding sequence-specific primers.

Two markerless transformations (each with two replications) were performed, using two constructs: pPZP-*Npt*II and pCAMBIA-2301 (Figure 1).

Two regeneration cycles were performed in each experiment: all the embryos obtained in the first cycle without selection were used for a second cycle with kanamycin selection. The kanamycin resistant calluses from the pCAMBIA-2301 transformations were also tested for *GUS* expression (GUS staining kit, Sigma).

Genomic DNA from the putative transgenic plants was extracted with the GenElute miniprep kit (Sigma). Real Time PCR (qPCR) was performed with the MX3000P machine (Stratagene) using the Brilliant SYBR Master Mix (Stratagene). Ten, 5, 2, 1 and 0.5 T-DNA copy equivalents per genome were realized by dilutions of the binary vector pPZP-*hem*L-*Npt*II (Rosellini *et al.*, 2007). Twenty-five ng of genomic DNA template from each of 15 transgenic events were used as template.
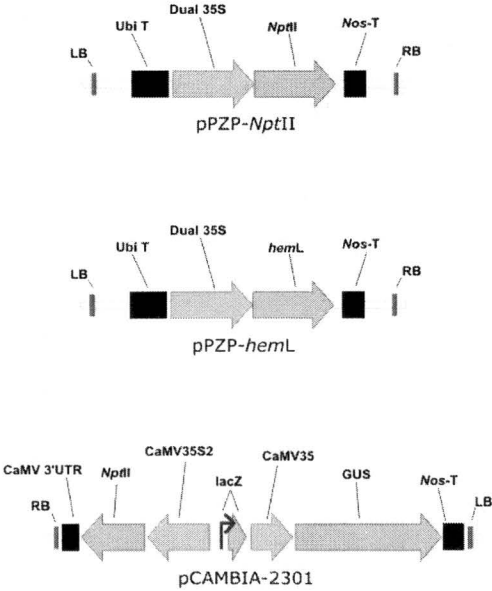
Fig. 1. Schematic representation of the T-DNAs used for co-transformationa and markerless transformation.

Primers for the *Npt*II and *hem*L coding sequences were designed with the Primer3 software. Southern blot analysis was performed following classical methods.

RESULTS

Co-transformation. A total of 15 co-transformed plants were regenerated in two experiments; the estimated co-transformation efficiencies were 9.3 and 4.3% (Table 1).

Segregation of *Npt*II in the T_1 progeny of the transgenic plant C12 fitted a 1:1 ratio (26 PCR-positive, 30 PCR-negative) indicating a single insertion event, whereas two copies were likely present in plant C9 (40 PCR-positive, 10 PCR-negative progeny plants). Independent segregation of *hem*L and *Npt*II was observed in the C9 progeny, demonstrating that marker-free plants can be obtained (Figure 2). On the contrary, co-segregation of the two genes was observed in the C12 progeny (not shown) suggesting co-integration of the two T-DNAs.

Table 1. Results of two experiments of co-transformations using the two constructs pPZP-Npt*II and* pPZP-hem*L.*

Experiment	Leaf explants	Embryos I cycle *	Embryos II cycle **	Co-transformation %
1	197	107	10	9.3
2	207	117	5	4.3

* Gabaculine selection; ** Kanamycin selection
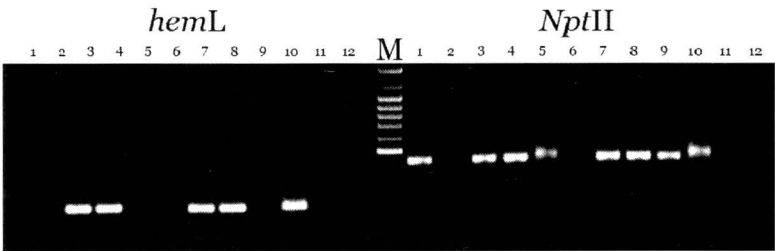
Fig. 2. PCR assessment of segregation of the Npt*II and the* hem*L genes in the cross progeny of co-transformed plant C9 crossed with a non transgenic pollen donor.*

Markerless transformation. Thirteen and 1 putative transgenic (kanamycin resistant) events were obtained from the two pPZP-*Npt*II experiments (Table 2); 0 and 5 events from the pCAMBIA-2301 transformations were kanamycin resistant; 3 of the 5 were also *GUS* positive; the corresponding efficiencies, as expressed by the percentage of transgenic (kanamycin resistant) somatic embryos among those regenerated without selection, ranged from 0 to 1.7%.

Table 2. Markerless transformation: results of two replicated experiments.

Construct	Replica-tion	Leaf explants	Embryos I cycle *	Embryos II cycle**	GUS positive embryos	Transgenic/ total embryos(%)
pPZP-*Npt*II	1	125	778	13	-	1.7
	2	275	927	1	-	0.1
pCAMBIA-2301	1	116	664	0	0	0
	2	129	326	5	3	0.9-1.5

* No selection; ** Kanamycin selection; -: non applicable;

qPCR copy number estimation. A good agreement between T-DNA copy number as estimated by the qPCR and Southern techniques (Table 3) was found. Plant *Npt*II -7 contains 6-7 copies according to qPCR and 4 according to Southern experiments: it might be a case of co-integration of several copies of the T-DNA at the same site that are not resolved by restriction of the DNA.

Table 3. Transgene copy number estimation by qPCR and Southern hybridizations in some of the transgenic plants.

Plants		*Npt*II gene		*hem*L gene	
		qPCR	Southern	qPCR	Southern
co-trasformed	C9	2.1	2	1.32	1
	C12	1.2	1	2.18	1-2
	C27	0.8	1	0.82	1
	C30	0.8	1	1.02	1
	C72	0.9	1	1.13	1
	C98	1.2	0	1.37	1
markerless	*Npt*II-2	1.7	2	-	-
	*Npt*II -3	1.4	2	-	-
	*Npt*II -4	1.0	1	-	-
	*Npt*II -5	1.2	nt	-	-
	*Npt*II -6	1.5	nt	-	-
	*Npt*II -7	7.6	4	-	-
	*Npt*II -8	0.7	nt	-	-
	*Npt*II -9	1.1	nt	-	-
	*Npt*II -11	1.0	nt	-	-
Non-transgenic control		0	0	0	0

nt: not tested; -: not applicable

DISCUSSION

We demonstrated that marker-free transgenic alfalfa can be obtained by both co-transformation and markerless transformation. The average co-transformation percentage was 6.8%, which translates into one plant with the useful gene in 15 plants with the SMG. This value is lower than those reported by the literature for this co-transformation approach (10-19%, reviewed by Ebinuma *et al.*, 2001). In addition, part of the co-transformed plants would likely display co-segregation of the two constructs, as a consequence of co-integration (reviewed by Ebinuma *et al.*, 2001). In fact, 1 out of 2 plants tested for segregation in this work showed co-segregation of the two transgenes.

Markerless transformation allowed us to obtain from 0 to 13 transgenic events per experiment. Based on the percentages of transgenic somatic embryos, the number of plants that should be generated and screened to obtain one transgenic plant would be 60 or more. This efficiency is in line with that realized in species that are easier to transform than alfalfa, like potato (de Vetten *et al.*, 2003) and tobacco (Jia *et al.*, 2007), and makes the practicability of markerless transformation questionable. In order to be routinely applicable, both techniques need to be improved for efficiency, possibly by using a more virulent *Agrobacterium* strain.

Finally, we showed that qPCR can help to screen for single copy insertions in transgenic alfalfa, permitting to reduce the recourse to the Southern hybridization technique.

ACKNOWLEDGEMENTS

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Inheritance of crown rust resistance in perennial ryegrass

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ABSTRACT

Three perennial ryegrass plants were selected for a genetic study based on low disease reaction to different single pustule isolates of crown rust. Each of the three resistant plants was crossed with a highly susceptible plant of the variety Aurora. In the two of the three F1 populations the percentage of resistant plants varied from 22 - 89% depending on the rust isolate used. This suggests that in the corresponding parental genotypes, resistance to different *P. coronata* pathotypes is controlled by several genes. Evaluation of the third F1 population revealed that the resistance in this specific cross is controlled by one single dominant gene. Segregation ratios of 1:1 (susceptible: resistant) was observed in all of the three isolates tested. Moreover, 93% of the plants were resistant or susceptible, respectively, to all of the three isolates tested.

Key words: *Lolium perenne* L., *Puccinia coronata*, virulence

INTRODUCTION

Effective resistance in ryegrass cultivars depends on the virulence of the regional population of crown rust (*P. coronata* Corda f. sp. *lolii* Brown). However, a high number of different races or virulence phenotypes were found in the European crown rust population (Schubiger *et al.* 2007). The objectives of this study were (i) to determine the inheritance of crown rust resistance in three perennial ryegrass plants which showed resistance to different isolates of crown rust and (ii) to identify crown rust isolates with identical avirulence / virulence gene products.

MATERIALS AND METHODS

Three perennial ryegrass plants were selected for a genetic study based on low disease reaction to different single pustule isolates of crown rust. M6830/05 (from cultivar Orval) was resistant to 100, M6894/05 (Vincent) to 88 and M6776/05 (Carrera) to 17 isolates out of 106 isolates tested. Each of the three resistant plants was crossed with a highly susceptible plant of the variety Aurora (A10, A11, M6973/04). The F1 populations were evaluated for resistance to 3 to 11 different single pustule isolates of crown rust using a detached leaf assay (Schubiger *et al.*, 2007). Each of these isolates were avirulent to the respective resistant parent and virulent to the Aurora plants. The Chi square statistic was used to test for goodness of fit to expected segregation ratios. A P value greater than 0.05 suggests that the progenies follow the tested ratio.

RESULTS AND DISCUSSION

In the three F1 populations the percentage of resistant plants varied from 22 to 89% depending on the rust isolate used.

Cross M6830/05 x Aurora A11: The F1 progeny showed a high frequency of resistant plants (Table 1). This suggests that M6830/05 possesses several independently segregating major genes. Segregation ratios of 7:1 (resistant: susceptible plants) and 3:1 suggests that 3 and 2 genes, respectively, are involved in resistance to the corresponding isolate. Each of these genes can confer resistance to the isolate individually. A 1:3 ratio (one isolate) suggests that resistance to this crown rust isolate is controlled by two genes.

The genotype M6830/05 is supposed to have several resistance genes, which are independent of each other.

Cross M6894/05 x Aurora A10: In most F1 progenies the observed frequencies of resistant and susceptible plants followed the expected ratio of 1:1 (Table 2). This segregation ratio suggests that resistance to the corresponding isolate is controlled by a single dominant gene. However, there is evidence that the isolates tested are avirulent to different resistance genes. Three isolates showed a segregation ratio of 1:3 as discussed above.

Cross M6776/05 x Aurora M6973/04: The observed frequency of resistant and susceptible F1 progenies corresponds to a 1:1 segregation ratio in all of the three isolates tested (Table 3). Moreover 93% of the progenies were either resistant or susceptible to all of the three isolates tested. This reveals that the resistance in this cross is controlled by one single dominant gene. All three isolates have the same avirulence gene product.

Table 1. Response of F1 progenies to inoculation with different isolates of Puccinia coronata: resistant parent M6830/05 from cultivar Orval and susceptible parent A11 from cultivar Aurora.

Isolate	origin	collec-ted on	number of plants		ratio tested	Chi-square	P value
			r	s			
12.02	Zurich	LP	97	15	7:1	0.08	< 0.78
12.08	Zurich	LP	99	12	7:1	0.29	< 0.59
502.03	Svalöf	LP	86	26	3:1	0.19	< 0.66
517.08	Gumpenstein	LP	67	45	1:1	4.32	< 0.04
531.01	Merelbeke	LI	76	36	3:1	3.05	< 0.08
532.02	Merelbeke	LP	92	20	3:1	3.05	< 0.08
560.02	Loughall	LP	30	81	1:3	0.24	< 0.62
567.01	Hohenheim	LP	96	16	7:1	0.33	< 0.57

Table 2. Response of F1 progenies to inoculation with different isolates of Puccinia coronata: resistant parent M6894/05 from cultivar Vincent and susceptible parent A10 from cultivar Aurora.

Isolate	origin	collec-ted on	number of plants		ratio tested	Chi-square	P value
			r	s			
05.02	Zurich	LP	35	21	1:1	3.50	< 0.06
502.03	Svalöf	LP	22	34	1:1	2.57	< 0.11
512.01	Radzikow	LP	23	33	1:1	1.79	< 0.18
529.01	Les Rosiers	LP	12	42	1:3	0.22	< 0.64
530.05	Pulling	LP	25	31	1:1	0.64	< 0.42
531.01	Merelbeke	LI	27	29	1:1	0.07	< 0.79
548.12	Luesewitz	LP	25	29	1:1	0.30	< 0.59
550.01	Orchies	LP	25	31	1:1	0.64	< 0.42
550.07	Orchies	LP	25	31	1:1	0.64	< 0.42
554.03	Perugia	LP	17	37	1:3	1.21	< 0.27
560.02	Loughall	LP	13	42	1:3	0.05	< 0.82

Table 3. Response of F1 progenies to inoculation with different isolates of Puccinia coronata: resistant parent M6776/05 from cultivar Carrera and susceptible parent M6973/04 from cultivar Aurora.

Isolate	origin	collec-ted on	number of plants		ratio tested	Chi-square	P
			r	s			
12.08	Zurich	LP	51	60	1:1	0.73	< 0.39
512.01	Radzikow	LP	56	56	1:1	0.00	< 1.00
550.07	Orchies	LP	53	57	1:1	0.15	< 0.70

CONCLUSION

The results suggest that in two of the three parental genotypes, resistance to crown rust isolates is controlled by several genes. In genotype M6776/05, which was resistant to only a small number of isolates, the resistance is controlled by one single dominant gene.

The F1 progenies offer great opportunities for molecular studies. Selected plants of the F1 generation were again crossed with a susceptible Aurora plant to produce F2 seeds. Evaluation of the F2 populations is now underway.

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Investigation of some wild annual vetch (*Vicia* L)

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ABSTRACT

Experiments have shown that by preserving the characteristics specific to the cenopopulations the tested species (*Vicia villosa* Roth, *V. angustifolia* L., *V. hirsuta* Gray) readily adapt to different years' diverse ecological conditions. *V. villosa* was distinguished for its largest biodiversity. Averaged data suggested that according to crude protein content in plant aboveground mass, stem, leaf and inflorescence ratio in plant mass all the tested spontaneous species were more valuable than the cultivated *V. sativa* species. For detailed tests and application in plant breeding, cenopopulations were selected according to earliness of flowering, productivity, crude protein content, and disease resistance.

Key words: *Vicia villosa*, *V. angustifolia*, *V. hirsuta*, *V. sativa*, diseases, pests, agonomic parameters

INTRODUCTION

Vetch (*Vicia*) genus belongs to Fabaceae family. About 200 species are known, of which about 15 grow in Lithuania. Annual vetches, *V. sativa*, *V. angustifolia*, *V. villosa*, *V. hirsuta*, are typical plants of agrophytocenoses. *V. hirsuta* and *V. angustifolia* are attributed to weed group. *V. sativa* and *V. villosa* are domesticated. *V. villosa* is semi-natural, grows in phytocenoses as a weed.

Investigations of vetch composition and parameters as well as biochemical composition are being done (Trusov *et al.*, 2004), medicinal properties are studied, caryological tests and agrotechnical experiments are conducted (Stupakov *et al.*, 1989), plant breeding is performed, vetch root system and interaction of Rhizobium bacteria are investigated (Pandey *et al.*, 2004).

The objective of the present study was to investigate and estimate adaptive capacity of *V. angustifolia* L., *V. villosa* Roth, *V. hirsuta* Grey under the conditions of changing climate seeking to conserve the germplasm and select promising forms suitable for practical use.

MATERIALS AND METHODS

The plant material was *V. angustifolia, V. hirsuta, V. villosa,* species of *Vicia* genus, grown *ex situ* (2001-2004). *V. sativa* was grown for the sake of comparison. *Ex situ* spring vetches (*V. angustifolia*, V. *hirsuta, V. sativa)* were sown in the second half of April, winter vetch (*V. villosa*) was sown in the first half of September.

Morphological plant assessment was based on measurements of stem height, branching, number of branches, number of pods, seed number per pod, 1000 seed weight, fresh weight per plant. Crude protein content was determined in air-dried mass (Kjeldahl method), content of carotenoids in green material (colorimetry method).

RESULTS AND DISCUSSION

V. angustifolia. This vetch is thought to be the pioneer of the genus (Sinskaja, 1969). It is characterised by a great phenological and morphological diversity. According to the length of the growing season, the groups differed not only in the beginning of flowering but also in other parameters (Table 1). Late forms were characterised by taller plants, greater mass and higher 1000 seed weight and crude protein content and clearly expressed leaf polymorphism. Leaves accounted for 52 per cent of the total plant mass of *V. angustifolia* (Figure 2). Leaves had 23.7 per cent of protein. Content of carotenoids in green material was 0,5mg/g.

V. sativa (sown vetch). This species is found on the whole territory of Lithuania (Grigas, 1971). It was formed through a range of transitional forms from *V. angustifolia* (Sinskaja, 1969). It is the most widespread species of cultivated (domesticated) vetch. Originally, it was a food crop known in Europe since the Neolithic times and later became a forage and green manure crop (Sinskaja, 1969).

In our experiments, V.*sativa* variety was grown as a control. The seed ripening period of this variety coincided with that of *V.villosa* early accessions (Table 1). The stem height was equal to that of medium early *V. hirsuta*, and plant mass was lower than that of *V. villosa*. However, it was several times higher than that of *V. angustifolia* and *V. hirsuta*. According to pod productivity this vetch lagged behind *V. villosa* and *V. hirsuta* and was distinguished by the seed number per pod. The number of seeds is genetically determined and is only weakly affected by the environmental conditions. It is a relatively stable parameter exhibiting the relatedness of these two species. According to the ratio of stems, leaves and inflorescences in the total plant mass these two species are also very close (Figure 2).

Vicia hirsuta. During the experimental years, the first plants of early accessions started flowering at the end of June (Table 1), and of late forms one week later. The parameters of this species cenopopulations were characterised by a relatively small diversity. Populations whose height varied from 70.2 cm to 91.2 cm were dominant. Leaves accounted for 45.6 per cent of the plant air-dried mass (Figure 2). Leaves contained 26.6 per cent of crude protein, i.e. similar to *V. villosa* (sand vetch) and surpassed other species (Figure 1).

The variation in protein content was relatively narrow. *V. hirsuta* was noted for a relatively weak response to diverse ecological conditions of different years. *V. hirsuta* had the biggest content of carotenoids in green material (0.59mg/g). Due to these characteristics and high crude protein content, *V. hirsuta* is a valuable crop for plant breeding and further genetic tests.

Vicia villosa. Winter vetch is an archeophyte that arrived in Lithuania together with winter cereals (Gudžinskas, 1999). All the cenopopulations tested were very diverse both in terms of phenological and morphometrical parameters. For all parameters, the plants of very early cenopopulations lagged behind the other groups (Table 1). The number of seeds per pod was found to be the least variable. Leaf to stem ratio (Figure 2) in the total plant mass was similar. The total value of plant mass was increased by inflorescences that contained the largest amount of crude protein (Figure 1). Content of carotenoids in green material was the smallest one (0.44mg/g).

Table 1. Earliness groups of Vicia angustifolia, V. hirsuta, V. villosa.

Earliness / Species	Length of growing period (days)	Stem height (cm)	Green mass of one plant (g)	Number of pods per plant	Number of seeds per pod
V. angustifolia					
Ultra early	75-80	35.1±5.4	2.3±0.7	7.1±0.9	7.6±1.1
Early	81-85	42.6±7.2	3.2±1.1	9.4±1.2	8.0±1.1
Average early	86-95	48.7±9.6	3.6±1.6	13.2±1.9	7.5±1.6
Late	< 96	54.3±6.4	4.0±0.8	11.7±1.2	5.8±0.9
Average		4.5±12.1	3.3±1.8	11.6±2.2	7.4±2.1
V. hirsuta					
Early	65-75	70.2±5.8	1.1±0.2	54.1±8.2	2.0±0.00
Average early	76-90	86.4±7.9	1.9±0.3	56.5±11.9	2.0±0.01
Late	<91	91.2±5.3	2.1±0.2	57.0±7.4	1.9±0.01
Average		84.4±14.4	1.7±0.5	56.8±138	2.0±0.01
V. villosa					
Ultra early	100-105	78.4±13.4	21.5±5.6	14.6±4,1	4.4±0.3
Early	106-110	101.5±12.1	28.8±4.2	23.5±8.6	4.7±0.5
Average early	111-120	121.8±18.2	32.2±7.6	29.0±11.8	4.5±0.8
Late	121-130	135.2±16.5	34.4±5.4	33.8±4.9	4.5±0.5
Ultra late	<131	154.2±17.2	33.2±6.2	36.2±6.3	4.3±0.3
Average		124.4±28.2	32.6±9.9	30.5±16.3	4.5±0.9
V. sativa					
‚Tverai'	105	84.0±5.9	17.5±2.4	16.5±3.2	5.2±0.5

In different years, winter vetch cenopopulations varied for all biometrical parameters. This suggests that this species is polymorphic and can adapt well to changing ecological conditions (Table 1).
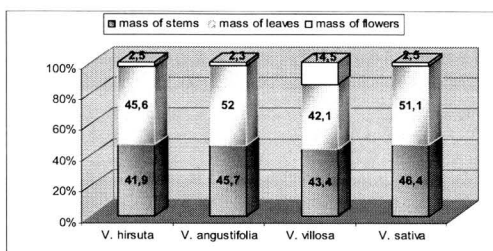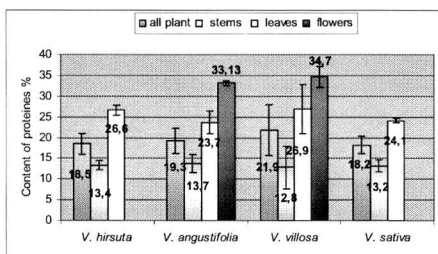
Fig. 1. Protein content of dry vetch mass.   Fig. 2. Stems, leaves and flowers proportion in vetch.

V. villosa exhibited the longest growing season and was heavily affected by diseases and pests. The end of the growing season was influenced by the spread of diseases. In 2001 and 2003 in the second half of the growing season when the weather was rather hot and wet, powdery mildew caused by *Erysiphe communis* Grev. f. *vicea* Jacz. was identified. About 50 per cent of cenopopulations were affected. Disease resistance of different cenopopulations was diverse. Nine cenopopulations resistant to powdery mildew and seven populations resistant to *Ascochyta* blight were identified.

CONCLUSIONS

All species had populations with valuable characteristics (total plant mass, stem to leaf ratio, length of the growing season, disease resistance) that can be used in breeding. A special attention should be drawn to *V. villosa*, as a protein-rich plant producing abundant green material. *V. villosa* provides a good soil cover and is used as a weed control means, and as a soil amendment.

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New microsatellite loci for red clover (*Trifolium pratense* L.) and study its polymorphism

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ABSTRACT

A microsatellite map of red clover was constructed quite recently by Sato *et al.* (2005). Kölliker *et al.* (2006) defined other microsatellite loci. The objective of this study was obtaining new SSR markers to its other application for genetic diversity study eventually QTL analysis. Resources of SSR oligonucleotides was white clover (*Trifolium repens*). In last years was found in the same way 13 SSR markers of it 11 were polymorphic (Jungmannova, Repkova, 2005). Products were amplificated mainly in Czech cultivars therefore markers were not suitable for genetic diversity evaluation among cultivars of core collection. In the same way 16 other novel SSR markers were found. Allelic variability was assessed on 11 parents' genotypes.

Key words: genetic diversity, polymorphism, red clover, SSR

INTRODUCTION

Several different types of DNA marker are currently available for genetic analysis and new marker types are being developed continuously (Manninen, 2000). Microsatellites (SSRs) occur frequently in most eukaryote genomes and can be very informative, multiallelic and reproducible (Vos *et al.*, 1995).

Traditional methods to develop SSRs are based on isolating and sequencing genomic libraries, which contain putative SSR tracts (Adams *et al.*). A novel source for generating SSRs is provided by screening EST databases available online (Kota *et al.*, 2001). Jungmannova B., Repkova J. (2005) used SSRs from other biological species.

MATERIAL AND METHODS

For genetic analysis (study polymorphism) was used parental generation of three red clover (*Trifolium pratense*) cultivars with different characteristics: Kvarta CZE, Fresco CZE and Rezista CZE.

The DNA of the clover plants was extracted from leaves harvested from 15 plants per sample. Commercial Kit (Sigma) was used. Resource of microsatellite primers was a map from white clover. Same way of obtaining new primers also choose Jungmannova, Repkova (2005). To amplification products 16 novel SSR marker were used. The PCR was performed in a 20 μl volume containing 1x DyNAzyme buffer, 1,5 mM $MgCl_2$, 0,2 mM of each dNTP, 1,2 μM of each primer, 2U Polymerase and 50 ng DNA template. PCR was performed. Thermocycler was programmed according to Herrmann (2006). PCR products were visualised by ethidium bromide staining after electrophoresis on 3% agarose gels running in TBE.

Similarity estimates were used to construct dendrogram by using the unweighted pair-group method with arithmetic averages (UPGMA). The products of SSR amplification were recorded with regard to the presence (1) or absence (0) of bands to obtain a similarity matrix. Genetic similarity (GS) was calculated according to Nei and Li (1979).

RESULTS

From 16 pairs of primers 10 SSR were choosen on the basis polymorphism and PIC values. 11 parents' genotypes were evaluated by these SSRs. The number of alleles per locus ranged from 3-10 with an average of 6.2 and the PIC values ranged from 0.42 to 0.80. The highest PIC was calculated for TRSSRATS055 and TRSSRATS0054. For the other primers PIC was low then 0.60. Samples were divided to clusters according to origin except exchange F6 to K6.

DISCUSSION AND CONCLUSION

These experiments verified that SSRs are available for other utilization (e.g. to study genetic diversity). Alluded SSR markers from *Trifolium repens* are applicable for analyses of *Trifolium pratense*. The most suitable markers are TRSSRATS055 and TRSSRATS054 with the highest PIC value. Combination of primers almost differentiated referenced origins of *Trifolium pratense*.

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Improvement of the quality of grass-clover mixtures for forage production

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INTRODUCTION

The last years, there is an evident interest of dairy farmers, government and breeding sector for fodder crops of high quality adapted to durable production and even for the production of 'functional foods'. Clover and grass/clover mixture for forage production present many interesting advantages in this sense. Traits such as protein stability, water-soluble carbohydrates content and fatty acid compositions are determining factors of forage quality. They influence the intake and the profit of the animal, the composition of urine, of milk and meat and finally the consumer's health.

A high content of polyunsaturated fatty acids (PUFA) in the fodder leads to higher PUFA and conjugated linoleic acid (CLA) contents in the meat and milk. Omega-3 PUFA (linolenic acid) and CLA in the human diet prevent the occurrence of cancer and heart diseases. Beside linseed oil cake, grass and grassland products are important vegetal sources of linolenic acid (Chilliard *et al.*, 2001; Dewhurst *et al.*, 2001). However, not all these grass PUFA are found back in the milk and meat due to the action of the plant lipases and, as a consequence, to biohydrogenation in the rumen. The association of clovers with grasses in the fodder leads to a higher Omega-3 content in the milk than pure grass forage (Dewhurst *et al.*, 2003). Opposite to a higher supply (grass and clover have a comparable fat content), red clover, as described for protein, would also protect grass fats against hydrolysis (lipolysis) (Van Ranst *et al.*, 2006). These effects are present during wilting, ensiling and possibly in the rumen. Red clover presents a high polyphenol oxidase (PPO) activity and high polyphenol content (PPO substrate) while this activity is much lower for grasses (Jones *et al.*, 1995; Winters *et al.*, 2003). There are indications that PPO activity would play an important role in the inhibition of lipolysis in silage (Jones *et al.*, 1995; Lee *et al.*, 2004). The inhibition capacity of PPO is possibly depending on the agricultural practise (such as wilting, crushing after cut). As PPO is an aerobic enzyme activated when the plant is stressed, the effect should take place before ensiling and not within the silage. White clover presents no or very low PPO activity (Jones *et al.*, 1995), however a gain of linolenic acid in the duodenum is also observed with white clover silage (Van Ranst, 2005). It is suggested that saponines, present in relatively high concentration in white clover (Sakamoto *et al.*, 1992), could be responsible for the inhibition of the lipase (Van Ranst, 2005). Saponines have already been studied for their anti-nutritional function in pig farming (to inhibit the fat metabolism) and in human medicine (against overweight) (Balkema-Boomstra, 2004).

In the rumen, the grass protein is rapidly transformed in ammonia and often insufficiently to microbial protein due to a bad protein/energy balance. Better protein stability (slower break down) in combination with high water-soluble carbohydrates content (energy) induces a better profit of the nitrogen in the cow and lower nitrogen leaching in the environment.

A good insight on the some aspects of the interactions between grass and clovers for fodder production and conservation is missing. For traits such as protein stability, fatty acid composition and interacting polyphenols, there are no fast screening techniques that can be applied in practical grass and clover breeding programs. The classical methods (chemical analysis, *in vitro* determination, *in situ* incubation in the rumen …) are usually too long and work intensive for routine applications. Faster and simpler evaluation methods exist like chromatography, Near Infrared Reflectance Spectroscopy (NIRS) or regression curves based on chemical analysis. For protein stability, more simple estimation methods are based on the washable fraction, solubility in buffers, in vitro breakability with enzymes or NIRS (De Boever *et al.*, 1998).

GENERAL AIM OF THE PROJECT

Our project started in 2007 aims to acquire the knowledge and techniques to allow the breeding of grass and clover for the farm production of forage of high quality. Fodder grasses with high linoleic acid and water-soluble carbohydrate contents and high protein stability and clovers with high protein en fat protection factors are seeked for. The study includes ryegrasses (*Lolium perenne* and *L. multiflorum*), timothy (*Phleum pratense*), orchard grass (*Dactylis glomerata*), *Festuca pratensis*, *F. arundinacea*, red clover (*Trifolium pratense*) and white clover (*T. repens*).

SPECIFIC OBJECTIVES OF THE PROJECT

The first specific objective of the project is to develop screening techniques to allow handling large numbers of plants for the evaluation of different quality aspects. Techniques for the analysis of fatty acid composition and protein stability and for the determination enzyme activity (PPO, lipase) and metabolite content interacting with protein and fat breakdown (saponins, polyphenol) are being developed for grass and/or clover. NIRS, gas chromatography and chemical analysis are being used. The results from the different techniques will be compared, as well as the analysis of fresh material versus dried material. Regression curves integrating other chemically analysed components will be calculated.

The second objective is to evaluate the existing variation and the heritability of the traits within breeding populations and between varieties using the most appropriate method developed previously. The variation will be studied on plots and individual plants. The heritability study will be performed by divergent selections. Correlations with other traits will be studied.

The last objective is to evaluate the effect of wilting, ensiling and crushing on the fodder quality of grass-clover in relation with the protective effect of clover on unsaturated fatty acids. Therefore, the lipase inhibiting activity of clover on crude fodder mixture will be studied in a more practical situation. *In silo* and *in vitro* study with different managements (timing and percentage) of the grass/clover mixture will be performed.

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Complete evaluation of the Czech core collection of *Trifolium pratense*, including morphological, molecular and phytopathological data

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ABSTRACT

During the year 2006, the Czech national core collection of red clover (*Trifolium pratense* L.) was established based on morphological data obtained from a set of fifty seven tetraploid accessions (varieties and newly bred varieties) and 130 diploid accessions (varieties, newly bred varieties and wild forms collected in the nature) of the world collection. The core collection, established in the year 2005, includes seventy six origins. In 2007, 68 available items were evaluated in detail and new molecular and phytopathological data were obtained. The whole core collection was evaluated for resistance to important fungal and viral pathogens. Tested plants were inoculated by spore-suspension of *Fusarium* spp. fungi and also by *Bean yellow mosaic virus* (BYMV) inoculum. The correlation between the reactions to both pathogens tested was determined. The obtained resistance data were used to complete the core collection accessions description.

Microsatellites (SSRs) were used for the study of genetic diversity of 68 core collections accessions which were available.

Key words: cluster analyses, core collection, correlations, genetic diversity, morphological traits, phytopathological data, ploidy level, red clover

INTRODUCTION

The study of genetic resources and their evaluation has a long tradition in the Czech Republic (Vacek, 1963; Pelikán *et al.*, 2005). During the last 50 years about 2000 accessions have been evaluated, described and stored in gene banks, but many characters have not been evaluated yet at the plant individuals. The importance of wild species/forms is increasing as input materials for breeding programmes and for use in organic farming and nature protection.

Genetic resources of fodder crops such as red and white clover and the possibilities of their use have recently been studied (Boller *et al.*, 2003; Drobná, 2004; Drobná & Žáková, 2001). The study of variability in the collections has been mostly based on morphological, phenological and agronomical characteristics of individual genotypes. By the application of the multidimensional statistics the varieties could be differentiated or aggregated according to evaluated traits/characters (Užík & Žofajová, 1997). Kouamé & Quesenberry (1993) used the

cluster analysis for the classification of the red clover collection as the base for creating core collection.

Molecular markers are used to study genetic diversity. Simple sequence repeats (SSRs) are short tandem repeat units of between 1 and 6 bp in length (Tautz, 1989). The variation in the number of repeats present in these loci determines differences in length of the amplified fragments (Manifesto *et al.*, 2001). Microsatellite loci proved to be highly polymorphic and useful as genetic markers in many plant species (Smith, 1997).

Red clover is mainly damaged very seriously by fungi of the genus *Fusarium*, the pathogens of *Fusarium* dry rot of root collar and root, and *Bean yellow mosaic virus* (BYMV). The effect of these pathogens also became evident in the decrease of persistence of host plants after infection. With the respect to the present status of forage crops and complex biology, the mass application of direct methods of control cannot be expected in the near future. Effective control measures will include, as in the other countries, a combination of suitable technology of cultivation and the use of resistant varieties. For this reason the continuous study as well as the development of breeding materials with desirable characteristics is very important. The main aim of phytopathological evaluation was to find the level of resistance to *Fusarium* spp. and BYMV of all the core collection.

The aim of the study was to describe 68 Czech red clover core collection accessions by morphological, molecular and phytopatological data in detail.

MATERIAL AND METHODS

Morphological and yield analysis

Forty four diploid and 24 tetraploid accessions of red clover (*Trifolium pratense*) core collection consisting of varieties, newly bred varieties and wild forms collected in the nature, were included into detailed evaluation of morphological and yield characters. Thirty plants of each origin were cultivated in the spacing of 50 x 50 cm on the field during the years 2004-2005. Ten plants of each origin were evaluated both in the field and in the laboratory for fifty characters. All the evaluated traits were transformed into the nine-point scale according the Czech national descriptors list. For the evaluated quantitative characters the point estimations of the mean value were made.

Phytopathological evaluation

The level of resistance of 68 accessions was tested by artificial techniques under laboratory and greenhouse conditions. Young plants were inoculated by the pathogens inoculums (Nedělník, 1986; Pokorný, 1989). The inoculum of *Fusarium* spp. fungi was prepared as a mixture of various isolates, which were collected from *Trifolium pratense* plants cropped in the Czech Republic. The BYMV inoculum was prepared on the same principles. The accessions of core collection were evaluated for ADI (average degree of infection) of *Fusarium* spp. and IPP (infected plants percentage) of BYMV.

Molecular analysis

The DNA of the clover plants was extracted from leaves harvested from 13-15 plants per origin. Commercial GenElute[TM] Plant Genomic DNA Miniprep Kit (Sigma) was used. DNA concentrations were determined using Lightwave II (WPA) and all samples were diluted to a concentration of 20 ng/µl and stored at -80°C.

The PCR was performed in a 20 µl volume containing 1x DyNAzyme buffer (10 mM Tris-HCl, pH 8,8, 1,5 mM $MgCl_2$, 50 mM KCl and 0,1 % Triton X-100), 1,5 mM $MgCl_2$, 0,2 mM of each dNTP, 1,2 µM of each primer, 2U of DyNAzyme[TM] II DNA Polymerase (Finnzymes, Espoo, Finland) and 50 ng DNA template. PCR was performed in TC-512 (Techne). The thermocycler was programmed according to Herrmann (2006), with an initial

denaturation of 4 min at 95°C, followed by 30 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C, then followed further 10 same cycles with annealing temperature 53°C and 10 min at 72°C as final extension. PCR products were visualised by ethidium bromide staining after electrophoresis on 3% agarose gels running in TBE. To amplification of the products 8 novel SSR markers from *Trifolium repens* were used. The number of alleles per locus ranged from 3-8 with an average of 4,4. PIC values ranged from 0,40 to 0,86.

For the morphological and molecular data, a cluster analyses for the sixty eight core collection accessions were performed using the software SYN-TAX 2000 (PODANI, 1994). The UPGMA method was used as algorithm for clustering and Euclidean distance as the measure of distance for morphological data and Jaccard coefficient for molecular data. Both matrices of dissimilarity coefficients were compared by Mantel test performed in the software POPTOOLS (www1). 999 iterations were used and cophenetic correlations for both matrices were calculated.

RESULTS

No clear difference between diploid and tetraploid accessions within the core collection was found by cluster analysis of morphological and molecular data, may be due to the fact that 4n accessions were derived from 2n natural accessions.

The cophenetic correlation coefficient of the dendrogram was 0,76 and 0.86 based on morphological and molecular data, respectively. Mantel test revealed a probability of $P=0,29$, which was statistically not significant ($P>0,25$). Correlation of both matrices found within Mantel test was not significant (r=-0,045).

The core collection accessions showed a certain level of susceptibility to *Fusarium* spp. and BYMV infection. The results indicated that evaluated pathogens (especially *Fusarium* spp.) are very important in *Trifolium pratense* cropping and it is necessary to breed for resistance to them. The correlation between resistance of core collection accessions to *Fusarium* spp. and BYMV pathogens was determined, but was not significant ($P>0,197$).

DISCUSSION

In our study, no significant correlation was detected between the observed patterns of morphological and molecular variation. The same results were obtained by many other authors in populations of some crops (e.g. Bruschi *et al.*, 2003; Martinez *et al.*, 2003; Greene *et al.*, 2004). Royo & Itoiz (2004) stressed there can be several reasons for low congruence between morphological and molecular classifications, but the most important ones are that the genetic regulation of one or another marker is different and that the morphological expression is conditioned by the state of the plant, agricultural practices and by environmental conditions.

In the last 15 to 20 years much literature deals with root-rot disease of red clover. The primary causal agents are fungi in the genus *Fusarium*. Species most often mentioned are *F. oxysporum, F. solani, F. avenaceum*. Control of root rot is difficult, that is why resistant material is necessary for successful red clover growing. In case of BYMV the situation is very similar. For this reason breeding programme for resistance to these pathogens was started in the Research Institute for Fodder plants in Troubsko in nineties (Pokorny *et al.*, 2003).

In conclusion, 68 accessions were described by morphological, molecular and phytopathological data. The cluster analyses differ accessions into clusters, correlation between molecular and morphological data was not significant. Sixty eight core collection accessions showed a certain level of susceptibility to *Fusarium* spp. and BYMV infection. All the obtained data can be used by red clover breeders and growers.

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Identification and characterization of genes involved in self-incompatibility (SI) in *Lolium perenne*

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ABSTRACT

Self-incompatibility (SI) in *Lolium perenne* is controlled gametophytically by two multiallelic and independent loci, *S* and *Z*, which have been mapped to linkage groups 1 and 2 respectively. None of the gene products of *S* and *Z* have yet been identified. Suppression subtracted cDNA libraries were developed from in-vitro pollinated stigma subtracted with unpollinated stigma to identify SI related genes. Previous comparative mapping work had identified regions on rice chromosomes 5 and 4 with synteny to *L. perenne S* and *Z* loci, respectively. Through a BLAST search against rice sequences, candidate clones having rice homologies on rice chromosomes 4 and 5 were tested for expression pattern by RT-PCR and some showed a tissue specific expression pattern, only in pollinated stigma, implying their roles in SI response. Calcium-dependent protein kinase domains were identified in two candidate genes. The Ca^{2+} antagonist verapamil was shown to inhibit or delay SI response.

Key words: Ca^{2+}, comparative genetics, protein kinase, suppression subtracted hybridization

INTRODUCTION

Self-incompatibility (SI) circumvents the tendency towards self-fertilization and inbreeding. Some inbreeding will occur in breeding programmes that aim to fix genes controlling desirable agronomic traits and in such cases the limitation of *S* and *Z* allele diversity may reduce seed set. Perennial ryegrass (*Lolium perenne*) is one of the most important pasture-grass species for the temperate zone. Self-incompatibility in *L. perenne* is controlled gametophytically by at least two multiallelic and independent loci, *S* and *Z*. A pollen grain is incompatible when both its *S* and *Z* alleles are matched in the pistil. The *S* locus has been mapped to linkage groups (LG) 1 and the *Z* locus has been mapped to LG2 in accordance with the Triticeae consensus map, but none of the gene products of *S* and *Z* have yet been identified (Baumann *et al.*, 2000). The objective of this work is to identify putative SI genes and the genes involved in the downstream SI reactions in perennial ryegrass by constructing and screening subtracted libraries with pollinated- and unpollinated- stigmas.

MATERIAL AND METHODS

Lolium perenne plants, genotyped for *S* and *Z*, were from the core mapping family for the International Lolium Genome Initiative (ILGI) (Jones *et al.*, 2002). The PCR based cDNA

subtraction was carried out using unpollinated stigmas as driver and in-vitro pollinated stigmas as tester. Sequences of candidate clones were BLAST for rice homology. Those in the regions for *S* and *Z* loci as identified in previous comparative mapping work were analyzed by RT-PCR with various tissues. Full length cDNAs of interesting candidate genes were generated. Inhibitor studies were carried out for gene function analysis.

RESULTS

Through a BLAST search against rice sequences, thirteen candidates with rice homologies on rice chromosomes 4 and 5 were tested for their expression pattern during SI response on a pollination time-series by RT-PCR. Seven candidates, including some protein kinases, showed a tissue specific expression pattern, only in pollinated stigmas (Figure 1). Ca^{2+} dependent protein kinase domains were identified in full length sequences for C3 and C94. Their functions were investigated through inhibitor studies and the Ca^{2+} antagonist verapamil was shown to inhibit or delay SI response (Figure 2).

Stigma		Pollinated stigma after		Leaf		Root		Name	Homology to rice
Mature	Immature	20 min	1h	♀	♂	♀	♂		
								C130	SCP-like extracellular protein
								C135	beta-expansin 2 precursor
								C136	pollen-specific kinase partner protein
								C151	transposon protein
								C3	CBL-interacting serine/threonine-protein kinase 15
								C10	expressed protein
								C94	Calcium-dependent protein kinase, isoform 2
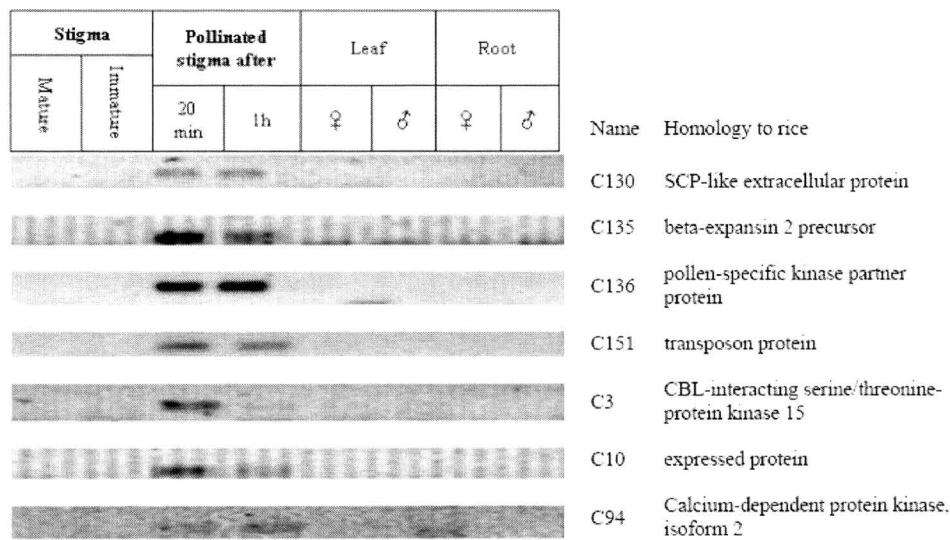
Fig. 1. Candidates in flanking physical regions of S & Z were tested on different materials and tissues. Some protein kinases showed a tissue specific expression pattern, only in pollinated stigmas.
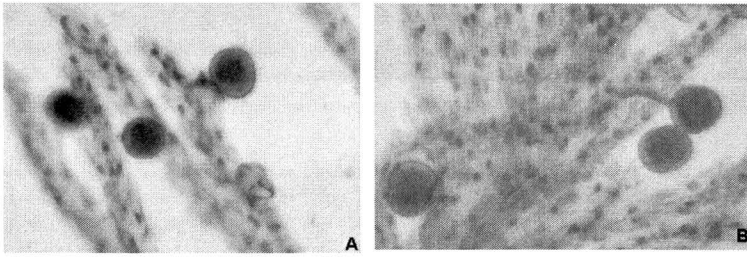
Fig. 2. A Ca^{2+} antagonist was shown to inhibit or delay the SI response. Unpollinated mature stigmas were placed on: A: agar plate without Ca^{2+} antagonist; B: agar plate containing 1mM (±) verapamil hydrochloride. Pollen tube growth was detected in self-pollinated stigmas treated with verapamil.

DISCUSSION

SSH combined with comparative genetics provides a useful approach for the identification of SI related genes in *L. perenne*. A role of Ca^{2+} in the mediation of SI response was identified. Potential involvement of protein kinases in the SI reaction was also detected.

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