Molecular marker analysis of hypoploid regenerants from cultures of barley × Canada wild rye

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Abstract: Canada wild rye (CWR, *Elymus canadensis* L., $2n = 4x = 28$) is a potential source of genes for disease resistance and environmental tolerance in barley (*Hordeum vulgare* L., $2n = 2x = 14$). Tissue cultures were initiated from immature inflorescences of CWR × 'Betzes' barley hybrids to promote CWR introgression into barley through possible tissue culture induced chromosome breakage and exchange. Among the plants regenerated, some were missing one ($2n = 20$) or part of one ($2n = 20$ + telo) chromosome. The objective of this study was to identify the missing chromosome or chromosome arm in these regenerants through the analysis of molecular (RFLP) markers that previously had been mapped in barley. Forty-six hypoploid regenerants that traced to 30 separate explants obtained from 10 interspecific hybrid plants were evaluated. DNA was digested with the restriction enzyme *Hin*dIII, Southern blotted, and probed with 39 genomic and cDNA barley clones that identified sequences polymorphic between barley and CWR. Eight of these probes identified band loss patterns that separated the regenerants into two groups. One group, all with barley cytoplasm, were missing a CWR chromosome homoeologous to barley chromosome 3; a second group, all with CWR cytoplasm, were missing a CWR chromosome homoelogous to barley chromosome 7. These results indicated that chromosome elimination in culture was not random. The two cytoplasm groups were further differentiated by probes that identified band shifts. These band shifts were caused by differences in DNA methylation.

Key words: *Hordeum vulgare*, aneuploidy, *Elymus canadensis*, tissue culture.

Résumé : L'élyme du Canada (*Elymus canadensis* L., $2n = 4x = 28$) constitue une source potentielle de gènes pouvant conférer la résistance à divers pathogènes et une tolérance à divers stress environnementaux chez l'orge (*Hordeum vulgare* L., $2n = 2x = 14$). Des cultures de tissus ont été initiées à partir d'inflorescences immatures d'hybrides provenant du croisement entre l'élyme du Canada et l'orge 'Betzes'. L'objectif ultime de ces travaux est de promouvoir l'introgression de segments du génome de l'élyme dans celui de l'orge via les bris chromosomiques et les échanges induits par la culture de tissus. Parmi les plantes régénérées, certaines se distinguaient par l'absence de tout un chromosome ($2n = 20$) ou d'une partie d'un chromosome ($2n = 20$ + 1 télo). L'objectif des travaux rapportés ici était d'identifier le chromosome ou la portion de chromosome absent chez ces individus grâce aux marqueurs RFLP caractérisés chez l'orge. Quarante-six individus hypoploïdes dérivés de 30 explants différents issus de 10 hybrides interspécifiques ont été examinés. L'ADN génomique a été digéré avec l'enzyme *Hin*dIII, transféré sur membrane et hybridé avec 39 sondes d'ADN génomique ou d'ADNc de l'orge qui révélaient des polymorphismes entre l'orge et l'élyme. Huit de ces sondes ont produit des motifs indiquant la perte de segments d'ADN chez ces individus et ont permis de diviser en deux groupes les individus hypoploïdes. Un groupe est formé d'individus qui portent tous le cytoplasme de l'orge et ayant perdu un chromosome de l'élyme qui est l'homéologue du chromosome 3 de l'orge. Un second groupe comprend des individus portant le cytoplasme de l'élyme et ayant perdu un chromosome de l'élyme homéologue au chromosome 7 de l'orge. Ces résultats indiquent que l'élimination chromosomique par suite de la culture de tissus n'a pas été aléatoire. Les deux groupes cytoplasmiques se distinguaient également par des polymorphismes de taille des fragments de restriction, lesquels résultaient de différences au niveau de la méthylation de l'ADN.

Mots clés : *Hordeum vulgare*, aneuploïdie, *Elymus canadensis*, culture de tissus.
[Traduit par la Rédaction]

Introduction

Wild species in the tertiary gene pool are a source of genes for cultivated crop improvement. Canada wild rye (CWR, *Elymus canadensis* L.) has several traits of interest for barley improvement, such as barley yellow dwarf virus resistance (Edwards et al. 1988; Griesbach et al. 1990), winter hardiness, and drought resistance (Park and Walton 1989).

Hybrids ($2n = 21$) between barley and CWR are sterile and chromosome pairing averages less than one bivalent per pollen mother cell (Mujeeb-Kazi and Rodriguez 1982). Therefore, a tissue culture project was initiated to induce chromosome doubling and rearrangement (Dahleen and

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Table 1. F_1 hybrid / explant Number, chromosome number, and cytoplasm source of hypoploid regenerants evaluated with RFLP markers: plants with the same F_1 hybrid and explant numbers were regenerated from the same callus culture.

Regenerant	Cytoplasm	F_1 hybrid / explant No.	Chromosome no.
1184	barley	D/1	20
1384[a]	barley	D/1	20
2172	barley	D/1	20
1857	barley	D/2	20
1797	barley	D/3	20 + telo
1985	barley	D/4	20 + telo
634	barley	D/5	20 + telo
1791[a]	barley	D/5	20 + telo
1929	barley	D/6	20
1128	barley	D/7	20 + telo
1227	barley	D/7	20
1334	barley	E/1	20 + telo
1448	barley	E/2	20
1097	barley	E/3	20
1697	barley	E/3	20
238	barley	E/4	20 + telo
1041	barley	E/5	20
1307	barley	G/1	20
1405	barley	G/2	20
1715[a]	barley	G/3	20
1645	barley	G/4	20
1499	barley	G/4	20
92	barley	G/5	20
1053	barley	G/6	20
1501	barley	G/7	20
1359	CWR	191/1	20
1668	CWR	193/1	20
1831	CWR	193/1	20
1962[a]	CWR	193/2	20
775	CWR	203/1	20 + telo
1586	CWR	203/2	20
1843	CWR	203/2	20
1131	CWR	204/1	20
1813	CWR	205/1	20
1169	CWR	206/1	20
1542[a]	CWR	206/1	20
1839	CWR	206/2	20
287	CWR	207/1	20
1466	CWR	207/1	20
1007	CWR	207/1	20
320	CWR	207/2	20
876	CWR	207/2	20
1111	CWR	207/2	20
1629	CWR	207/2	20 + telo
1743[a]	CWR	207/3	20
1745	CWR	207/3	20

[a]Regenerant that showed loss of additional CWR bands besides those from sequences that map to chromosomes 3 and 7 of barley.

Joppa 1992). Cytological analysis showed that 37 of 1864 regenerants were missing single chromosomes ($2n = 20$) and that an additional 9 plants had lost part of a chromosome ($2n = 20$ + telo). The remainder of the regenerants had chromosome numbers from 7 to 44.

The objective in this study of hypoploid regenerants was to identify the missing chromosome or chromosome arm through the analysis of restriction fragment length polymorphism (RFLP) markers that previously had been mapped in barley.

Materials and methods

Tissue culture of the hybrids and mitotic analysis of the regenerants were described previously by Dahleen and Joppa (1992).

Callus cultures were derived from immature inflorescences of CWR × barley hybrids and regeneration induced for 8–72 weeks, producing 1864 plants. Chromosome numbers of these plants ranged from 7 to 44. A subsample of 46 regenerants were analyzed in this study, and included 37 with $2n$ = 20 and 9 with $2n$ = 20 + telo. Twenty-one regenerants with CWR cytoplasm were derived from 7 different hybrids and 12 explants, and 25 regenerants with barley cytoplasm were derived from 3 different hybrids and 19 explants (Table 1).

DNA was extracted from 'Betzes' barley, CWR, and the hypoploid regenerants following the methods of Kleinhofs et al. (1993). DNA was digested with the restriction enzyme *Hin*dIII (New England Biolabs, Beverly, Mass.)[1] following the manufacturer's protocol. After separation of the fragments on 1% agarose gels and Southern transfer to nylon membranes (Hybond N^+, Amersham, Arlington Heights, Ill.), the DNA was hybridized with mapped genomic and cDNA clones from the North American Barley Genome Mapping Project to detect RFLPs (Kleinhofs et al. 1993; Table 2).

Methylation differences were studied by digesting DNA from two to four of the hybrid regenerants with CWR cytoplasm, from two to four hybrid regenerants with barley cytoplasm, and from the parents with *Msp*I and *Hpa*II. Both of these restriction enzymes cut the sequence CCGG but only *Msp*I will cut when the internal C is methylated. DNA from the paired samples was separated and transferred as before and probed with six marker clones that revealed band shifts (Table 2).

Results

Of the probes tested, 39 identified polymorphisms between the CWR and barley parents. These probes were then tested against 46 hypoploid ($2n$ = 20 or $2n$ = 20 + telo) regenerants derived from immature inflorescence cultures of 21-chromosome CWR × barley hybrids. Sixteen probes gave altered banding patterns when compared with the parents (Table 2). Altered banding patterns affected CWR sequences only and were of two types, either a loss of CWR sequences or a shift in CWR band size.

Eight of these probes showed CWR sequence losses (Fig. 1). Losses were not random, affecting sequences on barley chromosomes 3 and 7 only (Table 2). The pattern of CWR sequence loss differentiated the hypoploids into two distinct groups, which correlated with the female parent or cytoplasm of the original hybrid. Blot hybridization with four probes that identified sequences on barley chromosome 3 showed the loss of a CWR band in hypoploid regenerants with barley cytoplasm (Group 1). Seven of the regenerants in group one were telosomic. These regenerants retained the most distal marker on chromosome 3S (ABG065), indicating that these telosomics lacked a CWR chromosome arm homoeologous to the long arm of chromosome 3 in barley. Blot hybridization with four probes that identify sequences on barley chromosome 7 and are polymorphic between the barley and CWR parents showed the loss of a CWR band in hypoploid regenerants with CWR cytoplasm (Group 2; Fig. 1). Two of the regenerants in this group were telosomic. The markers tested gave good coverage of the long arm of barley chromosome 7 but the only marker for the short arm mapped near the centromere. It is likely that the two telosomics in this group (regenerants 775 and 1629) retained the arm of a CWR chromosome homoeologous to chromosome 7S in barley. Sequences that mapped to chromosomes 1, 2, 4, 5, and 6 did not show loss of bands.

Blot hybridization patterns of the other eight group one specific and group two specific probes that identify

Table 2. Mapped barley probes used to detect sequence losses or shifts in CWR × barley regenerants as compared with parental lines.

Probe No.	Chromosome location[a]	Mapped band loss or shift
ABC455	1C	None
ABG476	1C	Shift[b]
ABG701	1C	None
ABG704	1S	Shift[b]
ABG312	1S	None
ABG058	2S	Shift[b]
ABC451	2L	None
BG123	2L	None
ABG014	2L	None
ABC454	2S	Shift[b]
ABG460	3S	Loss
ABC174	3L	Shift[c]
ABG377	3L	Loss
ABG065	3S	Loss
ABG057	3S	Loss
ABG003	4S	None
ABG054	4L	None
ABG366	4L	None
ABG601	4L	Shift[b]
ABG715	4L	None
ABC321	4L	None
ABG059	5S	None
Hor2/Hor1	5S	Shift[b]
ABG053	5S	None
ABG055	5L	None
ABG257	5L	None
ABG494	5S	None
ABG702	5L	None
ABC160	5L	None
ABC164	5S	None
ABG062	6S	None
ABG711	6L	None
ABG466	6S	None
ABG379	6L	None
Nar7	6L	Shift
ABC302	7L	Loss
ABC309	7L	Loss
ABC155	7L	Loss
ABG395	7C	Loss

[a]C, centromeric; S, short arm; L, long arm.
[b]Probes hybridized to blots of DNA digested with *Msp*I and *Hpa*II.
[c]Parents monomorphic.

[1] Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

Fig. 1. Restriction fragment banding patterns for CWR, 'Betzes' barley, and 23 hypoploid ($2n$ = 20 or $2n$ = 20 + telo) tissue culture generated hybrids using probe ABC302. All DNA was digested with *Hin*dIII. Regenerants with CWR cytoplasm (E) show loss of a band from the CWR parent that is present in regenerants with barley (B) cytoplasm. Lane 19 lacked enough DNA to detect the CWR sequences. Lane 21 contains DNA from a regenerant that often showed multiple band loss.

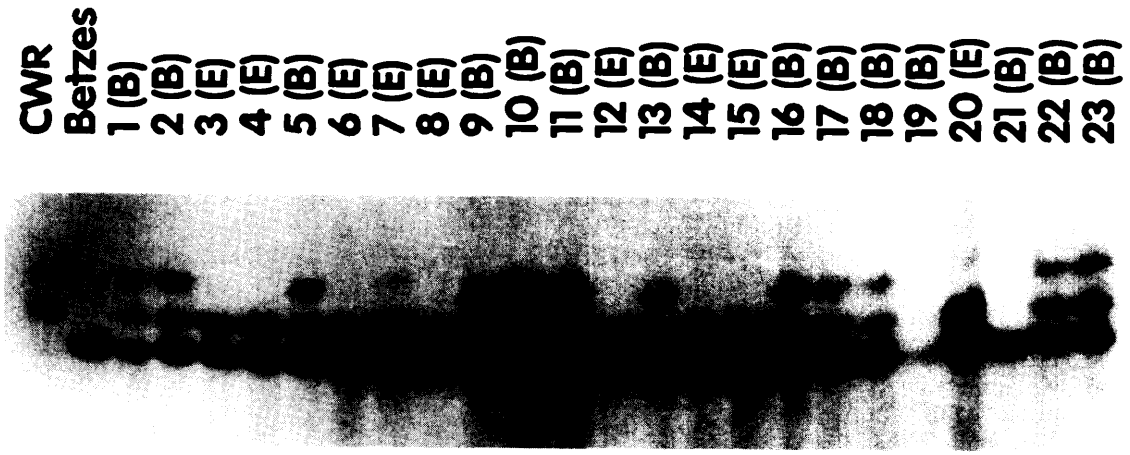
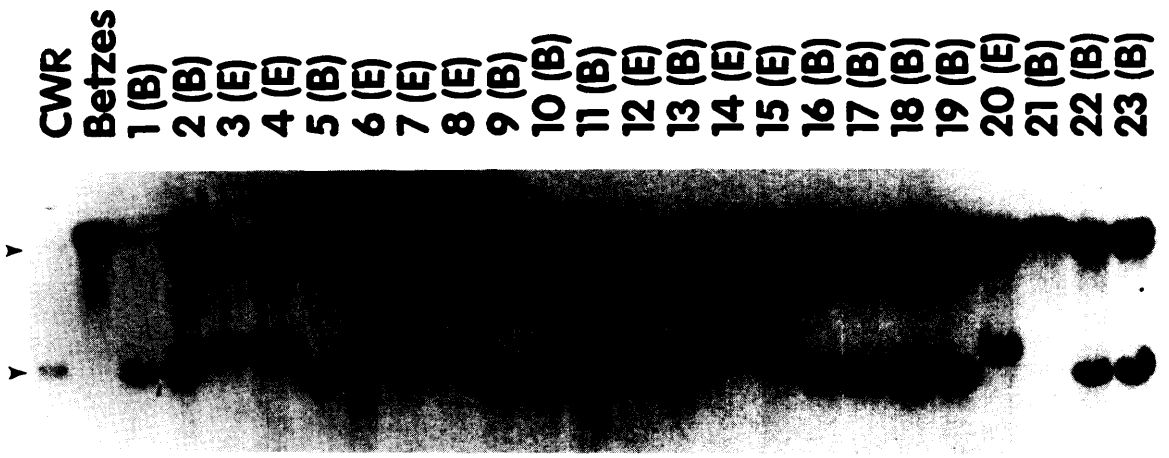


Fig. 2. Restriction fragment banding patterns for CWR, 'Betzes' barley, and 23 hypoploid ($2n$ = 20 or $2n$ = 20 + telo) tissue culture generated hybrids using probe ABG476. Arrows show CWR bands. All DNA was digested with *Hin*dIII. Regenerants with CWR cytoplasm (E) show a higher molecular weight band than regenerants with barley cytoplasm (B). Lane 21 contains DNA from a regenerant that often showed multiple band loss.



sequences mapping to six of the barley chromosomes revealed band shift polymorphisms (Fig. 2, Table 2). All of these band shifts involved sequences from the CWR parent. The banding patterns detected with these probes were correlated with the cytoplasm of the regenerants. For some probes, hypoploid regenerants with barley cytoplasm (Group 1) showed a shift to a higher molecular weight (HMW) band; with others, the hypoploid regenerants with CWR cytoplasm (Group 2) showed a HMW band.

Six hypoploid plants, three with CWR cytoplasm and three with 'Betzes' cytoplasm, were identified by unusual banding patterns (Table 1). These regenerants sometimes lacked some or all CWR bands, as in entry 21 in Figs. 1 and 2. With other probes, normal banding patterns were observed, including the band losses and shifts correlated with the cytoplasm described in the previous paragraph. Many of the probes that identified band loss in these six regenerants hybridized to a single HMW band, indicating possible loss or methylation of the restriction site recognized by *Hin*dIII.

The polymorphisms described above were observed when DNA from the regenerants was digested with *Hin*dIII. Because of the band shifts observed with eight of the probes and the methylation sensitivity of *Hin*dIII, possible methylation differences were suspected between the two cytoplasmic groups. Evaluation of from two to four members of the two groups using methylation sensitive and insensitive restriction enzymes and six of the probes that detected band shifts indicated that there were methylation differences between the groups. No differences were seen

between the two groups when their DNA was digested with *Msp*I (methylation insensitive). With three of the six probes, the DNA of hypoploids with CWR cytoplasm showed a different number of small bands than hypoploids with barley cytoplasm (Fig. 3) when both types were digested with *Hpa*II (methylation sensitive). This indicated that there were DNA methylation differences between the cytoplasm groups.

Discussion

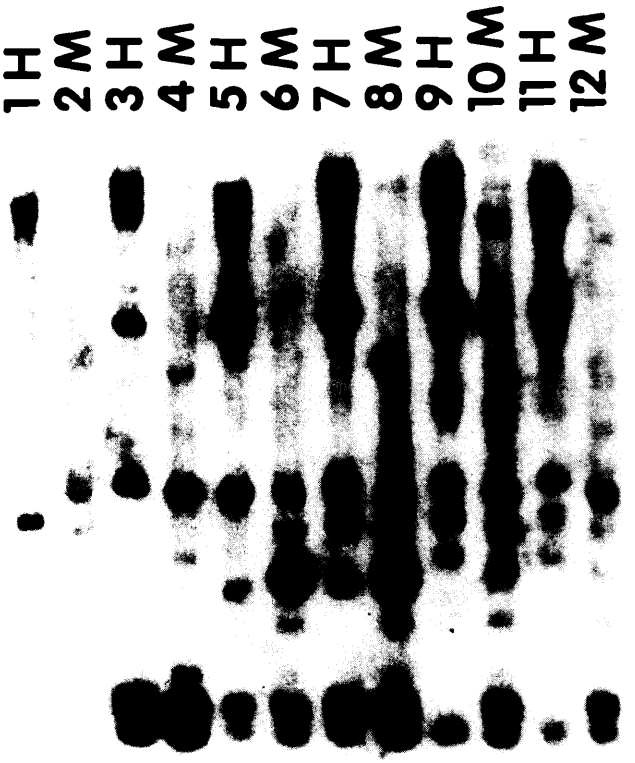
Aneuploidy is common in tissue cultures and in regenerated plants of many species and hybrids (Larkin and Scowcroft 1981; Bayliss 1980; Maddock 1986). In barley, while karyotypic changes are often found in nonregenerable callus (Singh 1986), regenerated plants usually have the normal euploid chromosome complement (Gaponenko et al. 1988). However, Orton (1980) examined barley × *Hordeum jubatum* hybrids and found aneuploidy and chromosome rearrangements in the regenerated plants. He hypothesized that a continuous mixture of the parental genomes was present in the callus cell population and was transmitted to regenerated plants. If this were the case in the barley × CWR cultures in this study, one would expect random chromosome loss in the hypoploid regenerants, assuming all karyotypes were equally capable of regeneration. This was not found. Instead, among the 46 hypoploid regenerants, only 2 of the 21 possible $2n = 20$ hypoploids were identified by molecular marker analysis, these missing either a CWR chromosome homoeologous to barley chromosome 3 or a CWR chromosome homoeologous to barley chromosome 7. The results indicate either (*i*) that these two chromosomes were the only ones lost in the callus cells, or (*ii*) that when a single chromosome was lost, the only hypoploid callus cells capable of regeneration were those missing one of these two chromosomes. These two possibilities could not be distinguished because callus cells were not evaluated cytologically.

Two primary types of variation in molecular marker banding pattern were observed in the hypoploids. Band loss, observed with eight of the probes, was as expected in plants missing one chromosome. The second type of observed variation involved shifts in RFLP patterns. Eight of the probes hybridized to sequences that were of different length in the two hypoploid cytoplasmic groups, indicating possible alterations in methylation patterns.

The results from the *Msp*I and *Hpa*II digests indicate a difference in the methylation pattern of DNA between hypoploid plants with CWR cytoplasm and hypoploid plants with barley cytoplasm. Methylation changes have been reported to occur in culture and in regenerated plants (Peschke and Phillips 1992; Kaeppler and Phillips 1993). Because the differences in banding patterns correlated with the cytoplasm of the regenerants, it is possible that nuclear–cytoplasmic interactions affected DNA methylation and regenerability of different hypoploid cell lines in culture.

Future studies may include in situ hybridization or genomic in situ hybridization to determine whether any chromosome rearrangement has occurred. Karyotyping using C-banding also may be used to identify the missing chromosomes cytologically.

Fig. 3. Restriction fragment banding patterns of paired *Hpa*II (H) and *Msp*I (M) digests of DNA from CWR (lanes 1 and 2), 'Betzes' (lanes 3 and 4), two $2n = 20$ regenerants with CWR cytoplasm (lanes 5–8), and two $2n = 20$ regenerants with barley cytoplasm (lanes 9–12). DNA was hybridized with probe ABG058. Regenerants show the same banding patterns when digested with *Msp*I but those with CWR cytoplasm show different patterns than those with barley cytoplasm when digested with *Hpa*II.



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