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Doctoral Dissertation

Common Bunt in Winter Wheat - Identification and Integration of Genetic Resources for Resistance Breeding

submitted by

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Affidavit

I hereby declare that I have authored this dissertation independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included. Any contribution from colleagues is explicitly stated in the authorship statement of the published papers.

I further declare that this dissertation has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Neusiedl am See, 22 September 2023 Magdalena LUNZER *(manu propria)*

Der unermesslich reichen, stets sich erneuernden Natur gegenüber wird der Mensch, soweit er auch in der wissenschaftlichen Erkenntnis fortgeschritten sein mag, immer das sich wundernde Kind bleiben und muss sich stets auf neue Überraschungen gefasst machen.

Max Planck (1858-1947), deutscher Physiker und Nobelpreisträger

Supervisory team

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Preface

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Contents

List of publications

Publications that comprise the main part of this cumulative dissertation

My maiden name was Ehn, so *M. Ehn* and *M. Lunzer* denote the same person.

1. Ehn M, Michel S, Morales L, Gordon T, Dallinger, HG and Buerstmayr, H (2022) Genome-wide association mapping identifies common bunt (*Tilletia caries*) resistance loci in bread wheat (*Triticum aestivum*) accessions of the USDA National Small Grains Collection. Theor. Appl. Genet. 135: 3103-3115. DOI: 10.1007/s00122-022-04171-3 [published with open access]
   Declaration of authorship:

   - **M. Ehn collected and analyzed the data and wrote the original draft**
   - S. Michel supervised the study and edited the original draft
   - L. Morales supervised the study and edited the original draft
   - T. Gordon conceptualized the study and provided additional data
   - H.G. Dallinger reviewed and edited the original draft
   - H. Buerstmayr conceptualized and supervised the study and edited the original draft

2. Lunzer M, Buerstmayr M, Grausgruber H, Müllner AE, Fallbacher I and Buerstmayr H (2023) Wheat (*Triticum aestivum*) chromosome 6D harbours the broad spectrum common bunt resistance gene *Bt11*. Theor. Appl. Genet. 136: 207. DOI: 10.1007/s00122-023-04452-5 [published with open access]
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   - **M. Lunzer collected data in 2020, 2021 and 2022, carried out data analysis and wrote the original draft**
   - M. Buerstmayr supervised linkage map construction and edited the original draft
   - H. Grausgruber reviewed and edited the original draft and acquired funding
   - A.E. Müllner provided data from 2015 and 2016
   - I. Fallbacher carried out data collection in 2019
   - H. Buerstmayr conceptualized and supervised the study, generated the populations and edited the original draft

3. Lunzer M, Dumalasová V, Pfatritsch K, Buerstmayr H and Grausgruber H (2023) Common bunt in organic wheat: unravelling infection characteristics relevant for resistance breeding. [submitted]
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   - **M. Lunzer conducted laboratory analysis and prepared the initial manuscript draft**
   - V. Dumalasová carried out field trials and collected phenotypic data
   - K. Pfatrisch carried out field trials and collected phenotypic data
   - H. Buerstmayr supervised marker-assisted selection and edited the original draft
   - H. Grausgruber organised funding, curated the data, conceptualized and supervised the study and edited the original draft

Additional contribution

1. Lunzer M, Michel S, Buerstmayr M, Grausgruber H and Buerstmayr H (2023) How long does it take to develop high performing and common bunt resistant winter wheat lines using organics-compliant methods? In: Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs (Ed.), Proceedings of the 73rd Annual Conference 2022, New plant breeding techniques: myths, facts and reality, 21-23 November, Raumberg-Gumpenstein, pp. 27-28.; ISBN: 978-3-900397-07-4, DOI: 10.5281/zenodo.7875597 [published with open access]
   Declaration of authorship:

   - **M. Lunzer carried out field trials, marker-assisted selection and data analysis and wrote the original draft**
   - S. Michel generated genomic-estimated breeding values
   - M. Buerstmayr supervised marker-assisted selection
   - H. Grausgruber acquired funding and edited the original draft
   - H. Buerstmayr conceptualized and supervised the study, generated the population and edited the original draft

Abstract

Common bunt caused by the fungi *Tilletia caries* and *Tilletia laevis* was once among the most devastating wheat diseases, capable of destroying large portions of the harvests. Its typical symptoms, the so-called 'bunt balls' replacing wheat grains, became a rare phenomenon during the second half of the 20th century, though. This was achieved by the development of synthetic fungicides that could be applied as seed dressings and prevented infestations with many diseases. Common bunt has re-emerged recently on areas devoted to organic farming, where the use of synthetic fungicides is not allowed. As more and more land is being managed organically, though, the need for bunt-resistant cultivars has become urgent. Since resistance to common bunt has long been absent from the list of wheat breeding goals, this thesis aims to help compensate the lack of research and breeding activities accumulated during the last decades. New genetic sources for common bunt resistance were identified in the wheat gene pool and unlocked for their use in resistance breeding. In a genome-wide association study, bread wheat accessions with resistance against two bunt diseases as well as markers corresponding to the resistance-conferring loci were determined. Through genetic mapping in bi-parental populations, the major bunt resistance factor *Bt11* was identified on wheat chromosome 6D. Marker-assisted selection of resistance loci was successfully applied in multi-parent breeding lines with high genetic variation, leading to the development of common bunt resistant and agronomically adapted material. The newly identified genetic sources and selection through molecular markers enable breeders to develop cultivars with stable resistance through combination of multiple genetic loci in a shorter time and broaden the pool of resistance factors available for breeding programs.

Kurzfassung

Gewöhnlicher Steinbrand, verursacht von den Pilzen *Tilletia caries* und *Tilletia laevis*, war einst eine zerstörerische Weizenkrankheit, der große Teile der Ernte zum Opfer fielen. Seine typischen Symptome, die sogenannten 'Brandbutten' an Stelle der Weizenkörner, wurden jedoch während der zweiten Hälfte des 20. Jahrhunderts selten. Verantwortlich dafür war die Entwicklung von synthetischen Fungiziden, die als Beize appliziert wurden und Infektionen mit zahlreichen Krankheiten verhinderten. Der Gewöhnliche Steinbrand ist in den letzten Jahren auf biologisch bewirtschafteten Flächen, auf denen die Anwendung solcher Fungizide verboten ist, wieder zum Problem geworden. Durch den zunehmenden Biolandbau entstand so ein dringender Bedarf an steinbrandresistenten Weizensorten. Da Steinbrandresistenz lange kein Züchtungsziel in Weizen war, soll diese Arbeit zur Kompensation des in den letzten Jahrzehnten akkumulierten Mangels an Forschungs- und Züchtungsaktivitäten beitragen. Neue genetische Ressourcen für Steinbrandresistenz wurden im Weizen-Genpool identifiziert und für die Anwendung in der Resistenzzüchtung erschlossen. Mittels genomweiter Assoziationskartierung wurden Akzessionen mit Resistenz gegenüber zwei Steinbrand-Krankheiten sowie genetische Marker für die Resistenzorte bestimmt. Genetische Kartierung in bi-parentalen Populationen führte zur Lokalisierung eines Hauptfaktors für Steinbrandresistenz, *Bt11*, auf dem Weizenchromosom 6D. In multi-parentalen Züchtungslinien mit hoher genetischer Variabilität wurde marker-gestützte Selektion für Resistenzorte erfolgreich zur Entwicklung von steinbrandresistentem und gleichzeitig agronomisch angepasstem Material angewendet. Die neu identifizierten Resistenzquellen sowie die Selektion anhand molekularer Marker ermöglichen durch die Kombination mehrerer genetischer Faktoren die raschere Züchtung von Sorten mit dauerhafter Resistenz und erweitern den Resistenzkatalog, der für Züchtungsprogramme zur Verfügung steht.

Popularized summary

For almost as long as humans have been growing wheat, farmers have been battling crop losses and reductions in grain quality caused by a disease called common bunt. This fungal disease spreads through spores present in the soil, but mostly on contaminated seeds. Even though the fungus invades young seedlings already shortly after sowing, disease symptoms only become visible the following summer when the wheat ears develop. Instead of healthy grains, the ears of diseased plants contain the so-called 'bunt balls' - grain-shaped accumulations of fungal spores in a thin shell. The dark brown spore powder inside has a distinctive smell reminiscent of rotten fish or herring brine.
Due to the widespread nature of common bunt in the past, extensive research was conducted, leading to the development of treatments to prevent infestations. Since the 1950s, these chemicals have been so successful that hardly anyone has paid much attention to the disease. In recent decades, however, more and more farmers have turned to organic farming, where chemical treatments are forbidden. This has led to a resurgence of common bunt in organic wheat and particularly in organic seed production. The most economically and environmentally efficient solution to this are resistant varieties. Because of time-consuming processes involved in research and development, such wheat varieties are scarce at the moment.
In response to this challenge, my colleagues and I embarked on a search for new sources of resistance and strategies to incorporate them into breeding programs. We discovered exotic wheat variants that are resistant not only to common bunt but also to the closely related dwarf bunt disease. Having determined the genetic basis of their resistance, we can now use these variants to create new breeding materials through crossbreeding with high-performing varieties. To maintain resistance in wheat varieties over the long term, it is important to exploit a wide range of resistance sources. So far, only a few major factors for common bunt resistance have been explored for breeding. This work unlocks a new factor conferring reliable and widespread effectiveness which has not been utilized before.
To develop wheat varieties endowed with these resistance factors, it is essential to pinpoint their locations on the wheat genome. Additionally, genetic markers are needed to identify plants carrying the resistance factor, as resources in breeding programs will only be dedicated to such resistant plants. Given the unpredictability of the traits that will become important in wheat breeding in the coming years, maintaining high genetic diversity in plant material is crucial, extending beyond just common bunt resistance factors. Therefore, this work shows approaches for applying genetic markers in highly diverse populations, allowing for the production of material that is both common bunt resistant and well-suited for agricultural use in just a few years.
These findings pave the way for accelerated development of new varieties so that farmers soon have access to a wider range of resistant cultivars, ensuring the successful production of organic wheat in the future.

Populärwissenschaftliche Zusammenfassung

Beinahe so lange wie Weizen vom Menschen kultiviert wird kämpfen Landwirte bereits gegen Ernteausfälle und Einbußen in der Kornqualität, die durch Befall mit Gewöhnlichem Steinbrand verursacht werden. Diese Pilzkrankheit verbreitet sich über Sporen, die im Boden, meist aber an verunreinigtem Saatgut vorhanden sind. Obwohl der Pilz bereits kurz nach der Aussaat in die jungen Keimlinge einwächst, werden die Krankheitssymptome jedoch erst im darauffolgenden Sommer sichtbar, wenn sich Weizenähren entwickeln. Anstatt gesunder Körner enthalten die Ähren kranker Pflanzen sogenannte Brandbutten, das sind kornförmige Ansammlungen von Pilzsporen in einer dünnen Hülle. Das darin enthaltene dunkelbraune Sporenpulver hat einen charakteristischen Geruch nach faulem Fisch oder Heringslake.
Weil der Gewöhnliche Steinbrand früher so weit verbreitet war, wurde viel dazu geforscht und schließlich Beizmittel entwickelt, mit denen Befall verhindert werden kann. Seit den 1950er-Jahren sind diese Chemikalien so erfolgreich im Einsatz, dass sich kaum noch jemand mit Steinbrand beschäftigt hat. Seit mehreren Jahrzehnten setzen jedoch immer mehr Landwirte auf biologische Produktion. Für sie sind chemische Beizmittel verboten, wodurch es plötzlich wieder verstärkte Probleme mit Weizensteinbrand im Biolandbau und vor allem auch in der Produktion von Bio-Saatgut gibt. Die wirtschaftlichste und nachhaltigste Lösung dafür sind resistente Weizensorten, die aber aufgrund langwieriger Prozesse in Forschung und Entwicklung erst in sehr geringer Zahl vorhanden sind.
Deshalb habe ich mich in dieser Arbeit auf die Suche nach neuen Resistenzquellen und Wegen zu deren Integration in Züchtungsprogramme begeben. Gemeinsam mit meinen Co-Autoren konnte ich exotische Weizenvarianten finden, die sowohl gegen den Gewöhnlichen Steinbrand aber auch gegen den nahe verwandten Zwergsteinbrand resistent sind. Nachdem wir die genetische Grundlage für ihre Resistenz bestimmt haben, können diese Varianten in weiteren Projekten für Kreuzungen mit leistungsstarken Sorten verwendet werden, um neues Züchtungsmaterial zu erzeugen. Damit Widerstandsfähigkeit in Weizensorten lange erhalten bleibt, ist es wichtig, eine möglichst große Bandbreite an Resistenzquellen zu verwenden. Bisher sind nur ein paar wenige Hauptfaktoren für Steinbrandresistenz für die Züchtung erschlossen worden. Durch die vorliegende Arbeit wird der Katalog um einen Faktor mit zuverlässiger und überregionaler Wirkung erweitert, der bisher nicht genutzt werden konnte.
Um Weizensorten mit Resistenzfaktoren zu entwickeln, müssen die Positionen dieser Faktoren auf dem Weizengenom bekannt sein. Außerdem braucht es genetische Marker, mit denen Pflanzen identifiziert werden können, die den Resistenzfaktor in sich tragen, denn nur in diese wird in einem Züchtungsprogramm weiter investiert. Da es schwer vorherzusagen ist, welche Eigenschaften in den kommenden Jahren in der Weizenzüchtung von Bedeutung sein werden, ist eine hohe genetische Diversität des Pflanzenmaterials essentiell, nicht nur hinsichtlich der Steinbrand-Resistenzfaktoren. Deshalb wird in dieser Arbeit auch gezeigt, wie man in Populationen mit hoher Diversität genetische Marker anwenden und so in nur wenigen Jahren Material erzeugen kann, das steinbrandresistent aber gleichzeitig auch schon gut angepasst für die landwirtschaftliche Verwendung ist.
Die Ergebnisse beschleunigen die Entwicklung neuer Sorten, damit Landwirten rasch eine größere Palette an resistentem Weizen zur Verfügung steht und so auch in Zukunft erfolgreich Bio-Weizen produziert werden kann.

1 Introductory overview

When it comes to common bunt in wheat, there are still many unanswered questions and questionable answers. Scientists face the challenge of having to bridge the gap between research from the mid-1900s and modern-day techniques and demands. This section aims to introduce the characteristics which distinguish working on common bunt in wheat from research on other diseases. Wherever possible, a comprehensive account from historic approaches and findings to modern-day methodology and knowledge is provided.

1.1 The common bunt pathogen - in the past and today

The seed-borne origin of bunt infections in wheat was discovered in 1755 by the French Mathieu Tillet (Tillet, 1937), by profession a botanist, agronomist, metallurgist and administrator but most importantly, a curious investigator. Until a fungal pathogen was identified as the causal agent of bunted wheat, another 52 years passed. Bénédict Prévost from Geneva in Switzerland was born in the year of Tillet's dissertation on bunt infections. In 1807, he published his *Mémoire sur la cause immédiate de la carie ou charbon des blés [...] et sur les préservatifs de la carie* in Paris in which he describes the observations leading him to the conclusion that bunt was caused by a fungus (Prévost, 1807). Kühn (1859) contributed another important piece to the solution of the puzzle on bunt in wheat: he showed that infection occurred through penetration of the seedling by fungal hyphae. The following subsections summarize what researchers have discovered since these pioneers laid the foundations starting more than 200 years ago.

(a) Bunt balls in a ripe wheat ear.

(b) Bunt balls (left) and healthy wheat grains (right).

Figure 1: Teliospore accumulation in so-called *bunt balls* which replace healthy wheat grains. In figure (a) glume and lemma have been removed on a wheat ear in the field at the time of ripening to reveal the bunt balls. (Picture by Hermann Buerstmayr) In figure (b), bunt balls and grains have been extracted from ears. (Author's picture)

1.1.1 Pathogen biology and infection characteristics

Two members of the *Tilletiaceae* family cause common bunt: *Tilletia caries* (D.C.) Tul. & C. Tul. (syn. *T. tritici* (Bjerk.) G. Winter) and *Tilletia laevis* J.G. Kühn (syn. *T. foetida* (Wallr.) Liro). Morphologically, these species differ only in terms of their teliospore surfaces. While spores of *Tilletia caries* appear reticulate under light microscopy, those of *Tilletia laevis* have a smooth surface. Distinguishing between them can nevertheless be difficult, as the two species are able to hybridize (Holton, 1942) and all kinds of intermediate spore morphology types have been observed (Fischer and Holton, 1957). Apart from teliospore surface structure, hardly any differences have been reported between *T. caries* and *T. laevis*. They have the same environmental requirements in terms of conditions for successful infection with a rather wide temperature range (Goates and Hoffmann, 1987) and almost no stimulation by light. This constitutes also an important difference to the closely related pathogen causing dwarf bunt in wheat: *Tilletia controversa* spores germinate in a narrower temperature range and need low levels of light for growth stimulation (Gassner and Niemann, 1954). Hansen (1959) described that common bunt spores are able to develop infectuous hyphae at 3 °C and 15 °C. The optimum temperature for infection varies between studies: Holton and Heald (1941) reported in their literature review that 6-10 °C are optimal, while Johnsson (1992) observed highest infection levels at 6-7 °C in his field studies and Faris (1924) obtained maximum bunt infestation at 5 °C or 10 °C depending on the cultivar. But not only soil temperature is important for successful common bunt infections - also soil moisture plays a role (Purdy and Kendrick, 1957). At higher temperatures of 15-25 °C, water content in the soil is critical for spore germination which is largely inhibited when moisture is below 11 %. In addition, optimal environmental conditions for infections vary between different races of common bunt (Kendrick and Purdy, 1962).
However, if soil temperature and moisture are conducive, bunt spores produce infection hyphae which invade young wheat seedlings. In the experiments conducted by Hansen (1959), fungal mycelium was detected in the coleoptiles four days after inoculation at both low (3 °C) and high (15 °C) temperatures. The coleoptile was also the only place where hyphae grew intracellularly while they spread through all other tissues intercellularly. For successful symptom development later on, it is essential that fungal mycelium reaches the host plants apical meristem before internode elongation (Swinburne, 1963). Hansen (1959) observed this stage 50 days after inoculation, which roughly corresponds to the five-leaf-stage according to Swinburne (1963). As the critical period for common bunt infection are the initial stages of host plant development, everything that delays plant growth and prolongs this period fosters successful colonization by the pathogen. As an example, increased incidence occurs at higher seeding depths of 7 cm compared to 4 cm. Once the fungi have reached the growing point, they are carried on by plant growth and finally reach the ears (Swinburne, 1963). At ear development, hyphae are present in all parts of the wheat flowers and spores start to develop in the ovaries. Anther development and flowering are inhibited by bunt infection and in fully bunted florets, no pollination occurs. Through production of teliospores inside the pericarp, bunt sori develop instead of grains (figure 1). The number of cell layers in the pericarp is strongly reduced compared to healthy kernels (Hansen, 1959), leading to a thin shell around the sori (also called "bunt balls") that easily breaks and releases the spores.
These spores emit the distinct odour typical for common bunt which reminds many people of decaying fish or herring brine and lead to the designation of the disease as "stinking smut" or "Stinkbrand" in German. The compound causing this odour was determined to be trimethylamine by Hanna et al. (1932). Interestingly, they only found trimethylamine in spores from *Tilletia laevis* but

(a) Partially infected grain (b) Partially infected ear

Figure 2: Partial common bunt infections. (a) Accumulation of teliospores becomes visible as a black perimeter or (b) black bunt balls among healthy white grains in ears that have been cut open to reveal the inside of spikelets. (Author's pictures)

not in those from *T. caries* in their first report. In a follow-up note to the publication, W.F. Hanna added that the compound had also been found in strains of *Tilletia tritici* (Hanna, 1932) which is supported by the nowadays common view that bunt spores generally emit the typical fishy smell. Apart from fully bunted kernels, also partial infections are possible. These have been reported already more than a hundred years ago by Faull (1907) and Appel (1909) who describe the occurrence of both partially bunted ears and partially bunted grains. The same partial infections in ears and grains are illustrated in figure 2 and were observed by other scientists during the following decades (Sampson, 1927; Güssow and Conners, 1929). Gieseke (1929) found the highest frequency of partial infections in cultivars with some levels of resistance while this phenomenon was rare in genotypes with high bunt incidence (above 70 %). Detailed descriptions of how infection characteristics differ between partially and fully bunted kernels are provided by Gassner (1938) and Hansen (1959). The latter concluded that, contrary to fully bunted grains, in partially bunted ones successful pollination has taken place, leading to a viable and germinable embryo. The fact that partial infections have mainly been observed in genotypes which are not highly susceptible to common bunt might be due to some resistance mechanisms which impair fungal growth (Sampson, 1927; Gieseke, 1929). Gassner (1938) conducted field trials with partially bunted kernels and observed that the spore patches inside otherwise healthy fruit bodies were indeed able to infect the seedlings developed from these kernels. This observation highlights the importance of such partial infections as only partially bunted grains and ears go unnoticed rather easily compared to fully diseased forms and can also not be sorted out from healthy grains by mechanical processes or washing (Gassner, 1938). Faull (1907) already noted in his report: "This fact [the occurrence of partially bunted grains] alone may account for the prevalence of smut in the fields of many wheat-growers who treat their seed before sowing" (page 13). However, since these few experiments conducted in the first decades of the 20$^{\text{th}}$ century, this phenomenon has not been investigated further according to available literature. Publication 3 continues the investigations by presenting results on partially infected ears in a winter wheat diversity panel across two years.

1.1.2 Historical and economic importance

Bunt as a disease of wheat might have been known ever since wheat cultivation started. It was mentioned in writings dating back to ancient Rome but for a long time its name was used interchangeably with other wheat diseases like rusts, smut or mildew (Woolman and Humphrey, 1924). A few authors have reviewed historical accounts dealing with the occurrence of bunt in wheat in Europe. Woolman and Humphrey (1924) found a work dating back to 1637 by Richard Remnant which mentions bunt as the cause of heavy losses in wheat production in England. Bunt infections continued to endanger wheat yields all across Europe and later on also in North America throughout the centuries. In an account from 1763, the troubles caused by bunt of wheat, potential causes and some recommendations for countermeasures are described in a German journal (Benevenuti, 1763). In a similar period, numerous reports and publications about common bunt were published in Spain (Martínez Moreno et al., 2020). The situation was also severe in France. In 1739, a large share of wheat harvests in the Electorate of Chattellarault was destroyed by the disease. Between 1730 and 1750, farmers even approached newspapers and asked for information on bunt of wheat and for advice on how to control it. In fact, the contrasting opinions on the nature of bunt lead to a prize set out by the Academy of Arts and Sciences of Bordeaux for the person who handed in the best dissertation on the cause and cure for what was called "the blackening of wheat" (Zundel, 1939). This prize was won by Tillet in 1755 (Tillet, 1937), meaning that his experiments were not only fuelled by pure curiosity and interest but also by an urgent need for information about common bunt because of the heavy losses caused by it in the midst of the 18$^{\text{th}}$ century. In the 1841 edition of the german encyclopedia 'Brockhaus', more than one third of the whole record dedicated to *Weizen* (wheat) dealt with bunt of wheat and its symptoms, even including a drawing of bunt spores and bunted wheat grains (Brockhaus, 1841). According to reports mentioned in Martínez Moreno et al. (2020), common bunt continued to threaten wheat production in Spain until the first decades of the 20$^{\text{th}}$ century, but accounts of serious losses became less frequent. Based on publications from the early 1900s, the situation was similar in the wheat belt of North America. Shares as high as 30-40 % of the harvest destroyed by bunt were not uncommon in Western Canada before 1900. Common bunt was even regarded as potentially limiting possibilities for wheat production in that area (Güssow and Conners, 1929).

Faull (1907) states: "Indeed, it has been estimated that for the last few years six per cent of the crop of Western Canada that has been officially inspected has been rejected on account of smut, and this represents a part only of the loss from this cause" (page 7). This aspect of the multiple ways bunted wheat causes losses to farmers was also highlighted by Cherewick (1953). He pointed out that apart from the actual yield reduction through bunt balls instead of grains, additional losses occur from the discounts obtained when marketing contaminated grain. Adverse economic effects additionally occur due to increased costs for seed treatments or other control measures, destroyed harvest equipment if bunt dust explosions cause fires and through negative health effects on humans as spores can induce allergic reactions (Holton and Heald, 1941). Even though bunt apparently was a well-known problem in the first half of the last century in North America, detailed data about the extent of and losses by bunt infections were scarce (Cherewick, 1953), probably due to the difficulties of measuring the economic effects of all these different types of losses (Holton and Heald, 1941). This can largely still be held true to date in many wheat producing areas.

In 1914, fires caused by threshing bunted crops and the resulting harm to humans and damages to machines caused losses of over half a million dollars in Eastern Washington (Cardiff et al., 1914). The

so-called "smut explosions" became such a serious problem that threshing machines were equipped with special amendments to prevent these incidents (Holton and Heald, 1941). Estimates for Manitoba, Canada from 1916 to 1937 assumed overall losses due to smut and bunt to amount to around $650.000 (Craigie, 1939). Naturally infected samples inspected by Heald (1921) at Pullman, Washington, showed maximum incidence levels of more than 80 % which can be almost directly translated into yield loss. In Illinois, U.S., losses due to bunt were estimated to be $2.275.000 only from yield reduction, an additional 100.000$ were lost due to dockage on the market price for grain bearing bunt spores (Güssow and Conners, 1929). In view of these numbers, it is easily understandable that D.E. Stephens and H.M. Woolman concluded in 1922 that "Bunt, or the stinking smut of wheat, has undoubtedly been the cause of a greater aggregate loss to the world than any other crop pest. [...] bunt has steadily taken its toll in all localities where wheat has been grown throughout the centuries that this cereal has been the world's principal bread crop" (page 5) (Stephens and Woolman, 1922).

The situation did not improve greatly during the following years, since between 1945 and 1951, 44.6 % - 71.3 % of all wheat samples examined in the Canadian provinces Alberta, Saskatchewan and Manitoba bore spores of *Tilletia* (Cherewick, 1953). In the Pacific Northwest of the United States, high infection levels were still reported in the 1950s: in 1952 and 1954, 28 % and 22 % of the wheat harvests were graded as "smutty", respectively (Purdy and Kendrick, 1957). Although most reports of direct or indirect losses due to bunt infections until the 1950s originate from North America, Holton and Heald (1941) assembled and impressive collection of such reports from all over the world. From these data, they deduce that annual losses caused by *Tilletia* infections amount to millions of dollars.

During the first decades of the 20$^{\text{th}}$ century, the first chemical treatments that provided appropriate protection first against seed-borne and later on also against soil-borne bunt infections were developed (Darnell-Smith, 1917; Riehm, 1913; Holton and Purdy, 1954, 1955; Purdy, 1955). These technical advances led to an almost complete eradication of common bunt in many parts of the major wheat growing areas in the course of the century (Güssow and Conners, 1929; Cherewick, 1953; Hoffmann and Waldher, 1981; Hoffman, 1982).

However, probably due to incomplete protection by seed dressings, contaminations by bunt spores with concentrations above 500 spores per gram of seed were regularly observed in Sweden between 1967 and 1987. Between 0 % and 8 % of all inspected samples per year bore such high spore loads (Johnsson, 1991). After a long period of insignificance to wheat production, common bunt reappeared in organic farming. Weinhappel and Girsch (2003) reported that infections with *Tilletia tritici* frequently caused problems with yield and quality of organic wheat harvests in Austria. Voit et al. (2012) described common and dwarf bunt as the most serious diseases in organic farming leading to considerable financial losses. During the 2010s, common bunt incidences in Germany were sometimes so high that the harvested grain was rendered unfit even for use as animal feed (Voit et al., 2017). In a study investigating 14 farm warehouses as well as two central warehouses in Budapest and Jászapáti in Hungary, spores of *Tilletia caries* and *T. laevis* were detected in several of the samples collected on farms and all samples collected in the Budapest warehouse (Halász et al., 2014). Despite these rather alarming accounts of common bunt outbreaks in Europe during the past decades, I could not find data or reports on the economic impact of such events for this region. In the United States and Canada, where the share of the wheat acreage under organic cultivation is much smaller than in countries like Austria or Germany (Research Institute of Organic Agriculture (FiBL), 2023), estimates of yield losses due to common bunt amounted to 2,215,441 bushels (30,294.3 tons)

and 519,051 bushels (14,126.2 tons) for 2021 and 2022, respectively (Wheat Disease Loss Calculator by the Crop Protection Network, available via `https://loss.cropprotectionnetwork.org/crops/wheat-diseases` [accessed 2023-08-25]).

1.1.3 Current research activities

During the past few years, bunt research has also entered the 'omics' era. Recent studies are focusing on insights into changes in the host plant metabolome after infections, on molecular methods for detecting infections at early stages and on genomic analysis of the bunt fungi.
A major problem with common and also dwarf bunt is that infection occurs in the seedling stage but can only be visually recognized months later when wheat plants start heading. Therefore, efforts have been made for developing methods that allow the detection of fungal DNA in samples of young wheat plants. Valente et al. (2023) published a protocol for using TaqMan Real Time PCR on samples of young durum wheat plants prior to the tillering stage. The assay was specific for identification of *T. laevis* in the test samples and also robust since it could be used on crude extracts without need for further purification. With a limit of detection as low as 10 fg of fungal DNA in the wheat sample, this method can be regarded as very sensitive and poses a good option for early screening of wheat plants for infection with *T. laevis*. Similar to this study in durum wheat, a method to quantify DNA from *Tilletia* species in young hexaploid wheat plants was developed in France. The quantitative PCR (qPCR) test was was conduted on samples collected in the two- to three-leaf-stage when wheat seedlings were seven to eight weeks old. It yielded results which correlated well (Pearson's correlation coefficient of $r = 0.89$) with visual scores obtained at maturity and therefore could also be applied for early screening of bunt infections (Cadot et al., 2021). Another method serving the same purpose was proposed by Ren et al. (2021) who used three different microscopy techniques to observe and analyze changes in wheat tissues caused by infection with *T. laevis*. With scanning electron microscopy, they were able to detect fungal hyphae in roots and leaves already directly after infection in the one-leaf stage. Apart from proposing a method for early detection of infection, this study also provided detailed insights into the establishment and proliferation of the common bunt pathogen in its host. Taken into account that infestation with bunt does not become evident when observing wheat plants with the naked eye for a rather long time, it is striking that Ren et al. (2021) already detected cell deformations due to fungal hyphae in the two-leaf stage. Soon afterwards, mesophyll cells started to rupture and the plasma membranes of cells broke. When plants started tillering, the alterations caused by the fungus were already so severe that chloroplasts in leaf cells were scattered inside the cells or even emerged from them. Macroscopically, fungal teliospores can only be observed in the bunt balls where they accumulate. With microscopy techniques, both hyphae and teliospores were found in all kinds of plant tissues, though. From roots and stems up to the glumes, awns and anthers, fungal mycelium and spores were detected (Ren et al., 2021).
In an attempt to improve identification of bunt contaminations in grain samples, Mei et al. (2023) developed a method to detect the compound causing the fishy smell emitted by bunt spores, trimethylamine. They determined the content of this metabolite in their samples using a headspace solid phase microextraction method with gas chromatography-mass spectrometry. Levels of detection down to 0.05 % kernels infected with *T. laevis* or 0.02 mg/kg of trimethylamine were achieved. The trimethylamine-containing teliospores have also been targets for researchers trying to find ways of reliably discriminate *Tilletia caries* and *T. laevis* from the causal agent of dwarf bunt, *T. controversa*. This is important since dwarf bunt is a quarantine pathogen in several countries. In a

study using 67 samples of the three bunt species, Forster et al. (2022) were able to show that teliospores of *T. controversa* could be distinguished from those of common bunt with an accuracy of 98.51 % using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Applying two different methods for clustering their data, they found that integrating both lead to the most reliable results but still no discrimination between samples of *T. caries* and *T. laevis* was possible. Similar results were obtained by Sedaghatjoo et al. (2021) who developed a loop-mediated isothermal amplification (LAMP) assay for *T. controversa* detection. For this purpose, they compared publicly available genome sequences of six species of *Tilletia* and identified regions of the DNA unique to dwarf bunt isolates. The LAMP assay built on such a specific DNA region had a limit of detection of 5 pg of genomic DNA and showed 100 % sensitivity and 97.7 % specificity when tested in five German laboratories.
When analyzing the genome sequences of 21 *Tilletia* isolates, Sedaghatjoo et al. (2021) observed a clear separation of *T. controversa* from *T. caries* and *T. laevis*, but the two common bunt pathogens could not be resolved into distinct clades. A more detailed insight into bunt genomics is provided by Sedaghatjoo et al. (2022). Using 16 different genome sequences of the three *Tilletia* species, they also got one phylogenetic group for *T. controversa* which was distinct from the common bunt pathogens, but *T. caries* and *T. laevis* appeared as a single monophyletic group. These results and the high genomic identity between *T. caries* and *T. laevis* hint at a potential conspecificity of the two despite their differences in spore morphology - they might represent two morphotypes of the same species. All genome assemblies used or developed in this study were between 29.4 and 31.9 Mbp in size. The number of single nucleotide polymorphisms (SNPs) in these genomes was generally low when comparing it to genomes of other species in the division of the Basidiomycota. Based on this observation, Sedaghatjoo et al. (2022) hypothesized that the divergence of dwarf bunt and common bunt might have occurred rather recently. In addition, the Tilletiales present a special order in the class of the Exobasidiomycetes and the subdivision of the Ustilaginomycotina because they lack most of the genes whose function has been determined in species used as model fungi. This might be due to fundamental differences in terms of life cycle and host-pathogen interactions of the Tilletiales compared to other orders in that subdivision of fungi (Sedaghatjoo et al., 2022).

1.2 Resistance against common bunt in wheat

The wheat gene pool naturally contains genetic resources conferring resistance against common bunt. While most commercially available wheat cultivars are susceptible to bunt pathogens (Dumalasová, 2021; Liatukas and Ruzgas, 2008), less adapted germplasm like landraces often show good resistance. Among more than 10,700 wheat landraces which were screened for common bunt resistance, hotspots for resistant germplasm were identified in a region stretching from Balkan to the Middle East, from Serbia to Iran. The largest numbers of resistant landraces were found in Kosovo and Iran (Bakhtaran region). Overall, common bunt resistance is a rather rare phenomenon among wheat accessions, though, as only 5.5 % of lines in the test panel showed less than 5 % infection compared to susceptible checks (Bonman et al., 2006). Even though 5.5 % is a relatively small share, it still represents a pool comprising several hundreds of different resistance sources. However, detailed investigations and characterizations of the genetic architecture have been conducted for only few of these potential donors. A range of genotypes showing differential reactions to a set of bunt isolates has been assembled in the so-called bunt differential set. Due to its importance for bunt research, this set of wheat accessions shall be described in more detail.

1.2.1 The bunt differential set

The center for bunt research during most of the 20th century was North America. In Canada and the United States, scientists were investigating and experimenting with different *Tilletia* races. During the first decades of that century, as Rodenhiser and Holton (1937) criticize, the methodology applied by the researchers was adapted to local conditions, rendering the results unfit to be generalized to a broader scale. This lack of standardization in the host plants used and the classification of pathogen races was tackled in their publication by the introduction of a set of six winter and four spring wheat varieties that should serve as differential hosts (Rodenhiser and Holton, 1937). With these ten wheat lines, they compiled the first version of the bunt differential set that is widely used today. In fact, two of the winter wheat varieties proposed in this initial set, 'Ridit' (CI 6730) and 'Hohenheimer' (CI 11458), are still included in the current set as differentials for the resistance factors *Bt3* and *Bt5*, respectively (Goates, 2012).

For decades, the classification system with the wheat differentials proposed by Rodenhiser and Holton (1937) was widely used in the United States. However, correct discrimination between races and assignment of classifiers remained a matter of discussion which hampered standard testing of wheat varieties for bunt resistance (Kendrick, 1961). To improve the situation at least for common bunt, a reclassification for races of *T. caries* and *T. laevis* was proposed by Kendrick (1961) based on a modified set of differential lines. He kept three varieties from the original set (among them 'Ridit' and 'Hohenheimer'), included two cultivars ('Oro' and 'Omar') that had been used in a previous race testing study and added two new wheat accessions so that his differential set included seven winter wheat genotypes. Of these newly added accessions, Selection 50077 (CI 13561) is still used today as the differential line for *Bt7* (Goates, 2012). Based on the seven differentials, Kendrick (1961) identified 17 distinct races among the known isolates of bunt, eliminating some existing duplicates. None of the differential cultivars was resistant to all of these races although 'Omar' was intermediately infected (24 %) by only a single race.

As some of the differentials presented by Kendrick (1961) were not single-gene differentials but assumed to carry combinations of different resistance factors, Hoffmann et al. (1967) applied some further modifications to the set of differentials and introduced two new single-gene genotypes. They also used a different wheat accession to test for the Hohenheimer resistance, which is today designated *Bt5* but was then called *Ho*. While this alternative Hohenheimer-line was not passed on to the modern differential set, one of the single-gene lines was: 'Turkey' (CI 1558) is today used as the differential line for the *Bt4* resistance factor (Goates, 2012). Hoffmann et al. (1967) also changed the previous classification system of the reaction of the differentials to bunt races from a system with three classes (*R* for resistant with 0-10 % infection, *I* for intermediate with 11-40 % infection and *S* for susceptible with more than 40 % infection (Kendrick, 1961)) to a binary system. As their view was that the classification should be pathogen-focused, they used only the two classes *A* for avirulent reactions (up to 10 % infection) and *V* for virulent reactions (everything above 10 % infection) of the different bunt races on individual differentials (Hoffmann et al., 1967). This classification still persists to date (Goates, 2012).

A few years after this modification of the differential set by Hoffmann et al. (1967), another update and extension were suggested. Hoffmann and Metzger (1976) introduced a set of ten winter wheat differentials and used the *Bt*-designation for individual resistance factors which is still applied today. Their set therefore comprised differential lines for *Bt1* to *Bt10* out of which *Bt1* to *Bt5* and *Bt7* were adopted from the previously mentioned collections (Hoffmann and Metzger, 1976). Designations of

differentials were done following the pathogen-focused approach proposed by Hoffmann et al. (1967) with the virulence/avirulence pattern of bunt races tested on a line determining which resistance factor was harboured by this line. Before the introduction of the *Bt*-names, letter designations derived from the cultivar names in which these factors were identified were used. These were available for resistances derived from varieties 'Martin' (M_1=*Bt1* and M_2=*Bt7*), 'Hussar' (H=*Bt2*), 'Turkey' (T=*Bt4*) and 'Rio' (R=*Bt6*) (Briggs, 1926; Briggs and Holton, 1950; Schaller et al., 1960). The change from these letters to *Bt*-designations was initiated by Robert J. Metzger in 1970. He assembled a comprehensive list of the new *Bt*-symbols, previous designations, genetic material containing the respective resistance factor and chromosomal locations as far as they had been assigned (Metzger, 1970).

Interestingly, while Kendrick (1961) still describes 'Hohenheimer' (*Bt5*) and 'Ridit' (*Bt3*) as having unknown resistance components, these two differentials are listed as carrying single bunt resistance genes in Hoffmann and Metzger (1976). In fact, all ten lines in the differential set presented in the latter publication are described as being monogenic - a postulation that is frequently questioned in discussions among bunt researchers today and has already been contradicted by some studies (Chen et al., 2016; Wang et al., 2019; Muellner et al., 2020).

Apart from extensions to the differential set, also the number of identified bunt races steadily increased as isolates with distinct virulence patterns were detected for both common and dwarf bunt or produced by hybridization of different *Tilletia* species (Hoffmann and Metzger, 1976). Already 39 different races, some of which showed virulence to eight resistance genes in parallel, are listed in Hoffmann and Metzger (1976). The most promising resistance gene according to their virulence tests was *Bt8* since the differential line 'Yayla 305' (PI 178210) was the only one on which all races showed avirulent reactions.

Taking into account that the designations of bunt resistance factors were done based on phenotypic observations of virulence reactions of pathogenic races (pathogen-focused) and not on the genetic architecture of individual wheat genotypes (host-focused), it seems reasonable that the differential wheat lines used as representatives for the different resistance factors could change over time while the *Bt*-designation still stayed the same. Such changes definitely occurred during the two decades between the publication by Hoffmann and Metzger (1976) and the comprehensive overview over bunt and smut diseases of wheat published by Wilcoxon and Saari (1996). Metzger and Hoffman (1978) for example already used altered differentials for *Bt1* and *Bt7* compared to Hoffmann and Metzger (1976). In the chapter on common and dwarf bunt in "Bunt and Smut Diseases of Wheat" (Wilcoxon and Saari, 1996), Goates (1996) first provided the list of bunt differential lines for resistance factors *Bt1* to *Bt15* that is still used nowadays. In addition to some changes in the genotypes representing individual *Bt*-factors compared to the historic set, this list also contains an extension of the previous ten differentials by five more lines (*Bt11*-*Bt15*). While lines for *Bt1* to *Bt13* are hexaploid wheats, the remaining two are durum wheats. Blair Goates compiled this set based on wheat lines which were postulated to be monogenic for individual *Bt*-factors according to historical publications and added lines developed by Robert J. Metzger to harbour only one resistance factor (Goates, 1996).

Apart from the numbered *Bt*-designations, two more *Bt*-factors have been postulated. On the one hand, bunt resistance conferred through a translocation from *Agropyron intermedium* into wheat was identified in the cultivar 'Zarya'. This gene was designated *BtZ* (Varenitsa et al., 1987; Mozgovoi et al., 1987; Goates, 1996). On the other hand, in the latest publication on the bunt differential set, Goates (2012) adds a resistance factor derived from wheat accession PI 173437 as *Btp* to the bunt

differentials. The *Bt*-designation was assigned to this new factor by Robert J. Metzger. As Goates (2012) describes, race testing of bunt isolates received less attention than before after the two researchers J.A. Hoffmann and R.J. Metzger retired during the 1980s. However, new isolates continued to be discovered and were added to the previously existing set of 39 bunt races. In total, Goates (2012) tested eleven isolates of *Tilletia caries* or *T. laevis* and 31 isolates of *T. controversa* for their reaction to differential lines *Bt1-Bt15* and *Btp*.
This has been to date the last publication dealing extensively with race testing on the bunt differential set. The author notes that "An advantage of using these differentials is that they are morphologically distinguishable from each other for the most part [...]". Even though this might be true, over the long time this set has been in use at different research stations globally, impurities of seed samples and admixtures between samples have frequently occurred and are causing problems in the comparability of results obtained from the differentials. This was a matter intensively discussed at the XXII International Workshop on Bunt and Smut Diseases held at the BOKU Campus in Tulln, Austria from June 13-15, 2023. The troubles caused by impure differential lines yielding doubtful results were addressed in a first move by Joshi et al. (2023) who characterized head types of differential lines and compared their performance across environments. Nevertheless, to re-establish reliability and reproducibility of tests on the differential set and enable future race identification and designation of bunt isolates it would be necessary to develop and distribute a revised differential set containing only pure, phenotypically unambigous wheat lines. In addition, improved results might be achieved by exchanging some of the highly unadapted and exotic landraces currently contained in the set by genotypes which are known to harbour the same resistance factors but are more adapted and can be grown under modern agricultural practices without e.g. extensive lodging. Efforts to realize such a new differential set are currently discussed in the bunt community and might become effective in the near future.

1.2.2 Qualitative vs. quantitative resistance

It was first proposed by Schaller et al. (1960) that the gene-for-gene concept introduced by Flor (1956) for the combination of flax and flax rust can also be applied for wheat and bunt of wheat. For a long time, resistance to bunt was then regarded to be conferred by major genes and therefore to be of exclusively qualitative nature (Hoffmann and Metzger, 1976; Goates, 1996; Gaudet and Menzies, 2012). In line with this concept, resistance or susceptibility of the wheat differentials used for race designation was assumed to be governed by single major genes as described in the previous chapter. As early as 1933, though, genetic factors conferring a resistance showing no dominance in the inheritance patterns were observed in a cross between varieties 'Hope' and 'Jenkins' (Smith, 1933). 'Hope' was a spring wheat cultivar which showed resistance to bunt when sown during March or April but was strongly infected when sown in late October or early November (Smith, 1932). Despite the wide-spread view of common bunt as a trait with qualitative genetic architecture, to date more quantitative resistance loci than major genes have been mapped and made available for breeding purposes as outlined in the following paragraphs.

Identification of resistance loci through genetic mapping
The first of the major *Bt*-genes to be localized on the wheat chromosome was *Bt10*. Markers to the gene had already been developed by Demeke et al. (1996) and Laroche et al. (2000), but despite this easier way to select for *Bt10* in breeding programs, it was still unclear where its chromosomal location was. This was finally revealed through a study by Menzies et al. (2006) who mapped the

gene to the short arm of chromosome 6D using microsatellite markers.
However, a different type of resistance to bunt governed by minor effect genes had already been described a lot earlier in offspring from a cross with the resistant cultivar 'Redman'. The resistant parent 'Redman' was found to possess one major resistance conferring gene as well as two minor genes, which were nevertheless able of conferring high levels of resistance when acting together (McKenzie, 1964). In experiments in both open fields and controlled evironments with hard red spring wheat lines a type of non-race specific resistance against common bunt was identified which was only expressed under field conditions. One of the cultivars exhibiting high levels of field resistance had 'Redman' in its pedigree which potentially caused its specific resistance reaction (Gaudet and Puchalski, 1989). Fofana et al. (2008) attempted to find chromosomal locations for this non race-specific resistance to common bunt in Canadian wheat cultivars to make it available to marker-assisted selection. They also mention that pyramiding both race-specific and non race-specific genes in breeding lines would pose a very good way to develop cultivars with durable resistance. In this first study conducting mapping of quantitative trait loci (QTL) conferring common bunt resistance in wheat, Fofana et al. (2008) detected three QTL on chromosomes 1B and 7A which explained between 3 % and 21 % of the phenotypic variation with the largest effect QTL located on 1B. This chromosome was also identified as the location of the main resistance conferring locus in the broadly bunt resistant cultivar 'Blizzard' registered in the U.S. during the 1990s (Wang et al., 2009). In another mapping study investigating the resistance mechanisms in the wheat cultivar 'Trintella', Dumalasová et al. (2012) also found a major QTL on chromosome 1B which explained approximately 30 % of the total phenotypic variation in common bunt incidence. Resistance of 'Trintella' was additionally conferred by three minor QTL on chromosomes 5B, 7A and 7B which explained 4 % and 5 % of the phenotypic variation, respectively. Dumalasová et al. (2012) compared their results to those achieved by Wang et al. (2009) in their study on 'Blizzard' and concluded, that the much lower resistance level observed in 'Trintella' compared to 'Blizzard' was possibly due to a less effective allele that was present in the 'Trintella' but not in 'Blizzard' since they shared the major resistance locus on chromosome 1B. This hypothesis was contradicted by the results of Muellner et al. (2021) who also conducted a genetic mapping study in a population with 'Blizzard' as the resistant parent. Due to the developments in marker technology since the first mapping studies, Muellner et al. (2021) were able to construct linkage maps with much higher marker density and better mapping resolution. This enabled them to identify additional QTL in the genome of 'Blizzard' apart from the 1B locus which most likely are responsible for the high and stable resistance this cultivar has shown in several studies until today (Huber and Buerstmayr, 2006; Wang et al., 2009; Muellner et al., 2021; Rabl et al., 2023). In Canadian breeding programs using the cultivar 'McKenzie' to introgress bunt resistance into breeding lines, it was observed that recovery of the parental type after conducting crosses was challenging in some instances. From this observation, Knox et al. (2013) deduced that multiple genes were responsible for the resistant phenotype of this cultivar and conducted a mapping study using 613 microsatellite markers. They were able to identify a locus on chromosome 7B which lead to a reduction in infection levels of up to 15 % but concluded from their phenotypic scorings that additional, undetected QTL were potentially involved in resistance of 'McKenzie'.
The non race-specific nature and higher durability of quantitative resistances renders these loci interesting alternatives or additions to qualitative resistance genes which, especially when present in high frequency in registered cultivars, may be subject to resistance breakdowns. *Bt10* was and is heavily used in North American wheat breeding programs, meaning that a loss of this resistance due

to e.g. a new, widespread race of bunt would pose a great risk to wheat production in this area. This prompted attempts to identify new, quantitative sources of common bunt resistance. In a mapping population resulting from a cross between a moderately and a highly resistant cultivar, bunt resistance QTL on five different chromosomes (1B, 4B, 4D, 6D and 7D) were detected out of which two co-located with or were in neighbouring positions to QTL influencing plant height. This link between common bunt resistance and reduced plant height at the same or similar genetic positions provided a trait especially interesting for wheat breeding programs. While the 6D locus was likely to correspond to *Bt10*, all the other loci had minor effects but when present together conferred resistance similar to the levels achieved by *Bt10*. This mapping population was also the first one showing epistatic interactions between common bunt resistance loci (Singh et al., 2016).
Epistasis was again observed in another study from Canada conducted by Bokore et al. (2019) who used a mapping population with the moderately resistant variety 'Lillian' as the source for common bunt resistance. Three relatively stable minor effect QTL on chromosomes 3D, 5A and 7A were identified in this population, explaining a maximum of 5.4 %, 7.4 % and 9.9 % of the total phenotypic variation for common bunt, respectively. The loci on 3D and 7A showed epistatic interactions, yielding an additional positive effect bunt incidence when the two QTL were present together (Bokore et al., 2019).
As already indicated by the results of several of the studies described above, the 1B chromosome seems to play an important role in common bunt resistance in different genetic backgrounds. This observation was further supported by the findings of Zou et al. (2017) who mapped a moderate effect QTL explaining 18.7 % of the phenotypic variance in common bunt infection to 1B. Another minor effect QTL was found on chromosome 3A which explained 7.9 % of the total variance.
Bt10, the first bunt resistance gene to be localized on the wheat chromosome, was mapped to the short arm of 6D (Menzies et al., 2006). However, the second resistance gene that got mapped, *Bt9*, was also located on this chromosome, but on the distal end of the long arm of 6D. It was shown to be distinct from *Bt10* and SSR markers linked to *Bt9* unlocked it for use in marker-assisted selection (Steffan et al., 2017b). Only two years later, the position of this highly effective gene providing resistance against many races of both common and dwarf bunt (Hoffmann and Metzger, 1976; Goates, 2012; Goates and Bockelman, 2012) was refined by Wang et al. (2019). They genotyped their plant material with the 90K SNP iSelect Platform (Wang et al., 2014), achieving good mapping resolution. Apart from the major resistance locus on 6DL, a second QTL on the long arm of chromosome 7A was present in the mapping population. Despite the high number of markers employed in linkage map construction, the marker density at the distal end of chromosome 6DL was still rather low, leading to a relatively large interval for the main resistance locus (Wang et al., 2019). The same problem was encountered in the mapping populations examined in publication 2 of this thesis and is described in detail there.
The third resistance gene comprised in the bunt differential set that was mapped and unlocked for breeding purposes was *Bt12*. Using the Illumina 15K SNP chip, Muellner et al. (2020) located *Bt12* on the short arm of wheat chromosome 7D. They experienced similar problems with scarcity of polymorphic markers as described in Wang et al. (2019) and publication 2 of this thesis, even though they used crosses between a landrace and genetically very distant modern cultivars. Apart from the major resistance locus corresponding to *Bt12*, an additional minor effect locus was mapped to chromosome 4B. While the 7D QTL was associated with both common and dwarf bunt, this minor locus conferred only common bunt resistance. Still, these findings supported the doubts about the

monogenic nature of several bunt differentials already expressed by Chen et al. (2016).

Identification of markers associated with bunt resistance through genome-wide association studies (GWAS)

Apart from QTL mapping studies relying on the construction of linkage maps for specific mapping populations, another approach to identify regions on the wheat genome associated with bunt resistance has been successfully applied in recent years. Through genome-wide association studies focused on marker-trait associations (MTAs) in genetically more diverse panels, more chromosomal locations potentially conferring bunt resistance have been added to the collection of genetic regions that breeders could exploit in their programs. Since GWA studies are usually carried out on diversity panels, an additional aim, apart from the search for MTAs is often the identification of new genetic resources and plant material showing full or high disease resistance.

In a panel of 125 synthetic hexaploid wheats developed through crosses between tetraploid durum wheats and the wheat D-genome donor, *Aegilops tauschii*, MTAs with common bunt infections were found for SNPs on seven different chromosomes. Six markers were identified to decrease common bunt incidence significantly compared to genotypes which harboured the contrasting allele at the respective loci. The favourable alleles for all MTAs together explained 19 % of the total phenotypic variance observed on the synthetic wheats (Bhatta et al., 2018).

The genetic architecture of winter wheat lines from Nebraska in terms of common bunt resistance was investigated through a GWA study on a panel of 330 lines. Variation in common bunt incidence levels in this panel was high with only few genotypes classified as "very resistant" or "resistant". A large number of SNPs on 14 different chromosomes were significantly associated with common bunt resistance across test locations. Those markers with the strongest association and largest allele effects were located on chromosomes 1A, 1B, 3A, 4A, 5B, 6A, 6B and 7B. Before any of the regions showing significant MTAs can be targeted through marker-assisted selection, though, the markers need to be validated in different genetic backgrounds (Mourad et al., 2018).

Another study by Gordon et al. (2020) worked on MTAs for dwarf bunt resistance in a diversity panel originating from the National Small Grains Collection (NSGC) of the United States Department of Agriculture (USDA). In the panel comprising 246 bread wheat accessions, significant MTAs were found on chromosome 6D within a region flanked by markers for *Bt10*. Among the few GWA studies published on bunt resistance, this is the only one that preselected lines entering the panel based on historical scores for dwarf bunt resistance available in the Germplasm Resources Information Network (GRIN) to reduce confounding effects resulting from population structure. The merits of such a pre-selection avoiding effects potentially complicating analyses became evident when GWA mapping for common bunt resistance was performed on the same panel (see publication 1 in this thesis). The differences between phenotypic scores and genetic architecture detected in these two studies conducted on the same panel but investigating different diseases also pointed out that, contrary to the widely acknowledged assumption (Metzger and Hoffman, 1978; Goates, 1996, 2012), common and dwarf bunt are not always governed by the same genes.

1.3 Common bunt control - resistance breeding approaches

This section outlines the development of resistance breeding against common bunt from the first variety tests and crosses to modern breeding goals and selection schemes focused on organic farming. Since resistance breeding efforts largely came to a halt following the development of chemical

treatments preventing bunt infections, the subject of seed dressings with fungicides is also briefly touched upon.

1.3.1 Early breeding efforts and strategies

In their extensive literature review, Woolman and Humphrey (1924) describe how resistance breeding against common bunt started - researchers were testing existing varieties for their reactions to bunt in the field. They did not yet know what was causing resistant reactions, but through their experiments they gained knowledge about the geographic origin and agronomic properties of cultivars that were not susceptible to bunt infections. In Hohenheim, Germany, Otto von Kirchner and Carl Freiherr von Tubeuf were trying to find solutions to the problem of bunted wheat through systematic variety testing starting in 1903. They screened various types of small grain cereals from bread and durum wheat to einkorn, emmer and spelt. Of the examined winter wheat genotypes, only very few showed resistance against bunt, among them 'Hohenheimer No. 77' and 'Cimbal's Fürst Hatzfeld' (von Kirchner, 1916).
One of the early reports on attempts to breed bunt resistant varieties is that of Pye (1909) from Australia. In his experiments with durum and bread wheat, he found three cultivars ('Medeah', 'Florence' and 'Genoa') which showed some levels of resistance to artificial bunt infections. The durum variety 'Medeah' was especially interesting for him and he crossed it with other varieties that he describes as "high-typed wheats, possessing to some degree the power to resist bunt" (page 373). When examining the offspring of these crosses, he came to a conclusion that would continue to bother breeders throughout the following century and beyond: producing lines with satisfying levels of resistance is rather easy; but producing lines that are not only resistant but also fulfill all requirements in terms of yield and quality and thereby satisfy farmers and people along the processing chain is hard.
Already in the first half of the 20$^{\text{th}}$ century, researchers agreed that resistant varieties are the cheapest, most efficient and easiest way of preventing bunt infections (Briggs, 1930; Cherewick, 1953). One of the first suggestions for setting up a breeding program for bunt resistance in wheat can be found in the work of Gieseke (1929). He outlines that the development of resistant offspring does not necessarily depend on a cross involving a highly resistant parental line but is also possible through crossing two moderately susceptible varieties. For efficient resistance breeding, he suggests to advance offspring from a cross until the third filial generation F_3 to fix desired allele combinations in the lines. F_4 lines should then be tested both for agronomic properties and reactions to bunt infections. Artificial infection should be carried out several times to draw reliable conclusions about the resistance levels of individual lines. Should the agronomic characteristics determined in the parallel tests not satisfy the requirments, one would at least have achieved valuable pre-breeding material which could be improved further by following this breeding scheme. To achieve stable resistance, it is essential to conduct artificial infections with a mixture of the most virulent bunt races (Gieseke, 1929). Similar recommendations can be found in a publication by Fittschen (1939) who states that the first two trials with artificial infection should be carried out with a composite of highly virulent races. Even during a time when seed treatments to prevent bunt infections were already quite common, Gieseke (1929) underlines the necessity of developing bunt resistant wheat varieties since remote areas may take long in adopting the treatment procedures. Also serious damages to wheat harvests reported from the United States despite the wide-spread use of seed treatments were obviously a concern in Europe.

In 1930, a strategy still common today was first proposed for use in bunt resistance breeding by Fred N. Briggs. He introduced the back-cross method where after an initial cross between a resistant variety and a susceptible one with very desirable agronomic properties, offspring in generation F_1 is crossed again to the susceptible parent. In the following two generations, lines are tested for their resistance to bunt infections and only the resistant lines are kept. In the third generation, the breeder is able to select for homozygous resistant lines looking most alike to the susceptible parent which he or she should cross again to this more elite variety. Following this scheme, lines possessing resistance factors fixed in homozygous allelic state in the genome combined with desirable characteristics of the elite, bunt susceptible parent can be developed (Briggs, 1930).
Through such efforts by scientists and breeders, several bunt resistant varieties were available by the mid-1900s. In Canada, farmers could grow three different cultivars out of which one, 'Renown', was resistant to all bunt races known at the time (Cherewick, 1953). However, when chemical treatments for seed disinfection appeared on the market, resistance breeding was given less attention (Hoffmann and Waldher, 1981).

1.3.2 Control through seed treatments with fungicides

Before 1900, copper sulphate and formaldehyde were the most frequently used chemicals applied as seed treatments. However, these substances had to be applied as wet treatments and caused considerable damage to the grains (Cherewick, 1953). Major improvements were achieved with the invention of treatments containing copper carbonate as these could be applied by dusting the seeds and did not harm the grain even if it was stored in treated conditions for some time (Darnell-Smith, 1917). Around 1930, disinfectants with organomercury (chlorophenol mercury) as the active ingredient were developed (Riehm, 1913). These could be applied in different ways depending on the commercial product and the type of mercuric compound. Even though shortly afterwards several new types of seed treatments with chemicals other than mercury were introduced, the organic mercury disinfectants still remained highly popular despite their toxicity to humans and animals and therefore quite challenging application. Their popularity most likely resulted from their broad effectiveness against a range of pathogens and the fact that they did not impair seed vigour if applied in the right dosage (Cherewick, 1953). It was not until the 1950s that one of the alternative chemicals, hexachlorobenzene, had its break-through and became widely used. This was due to the fact that hexachlorobenzenes were the first treatments that were shown to be effective against soil-borne bunt infections while previous disinfectants only provided protection against seed-borne spores (Holton and Purdy, 1954, 1955; Purdy, 1955). During the following decades, seed treatment became a standard procedure and efforts continued to broaden the range of available chemicals controlling both seed-borne and soil-borne bunt (Hoffmann and Waldher, 1981). With respect to the high degree of bunt susceptiblity among wheat cultivars commonly grown in the United States (Hoffmann and Metzger, 1976), though, scientists still advocated for the continued and consistent use of highly effective fungicides (Hoffmann and Waldher, 1981).

1.3.3 Organic farming regulations demand new approaches

Things started to change slowly when the demand for organically produced food grew during the 1960s and 1970s. Until the late 1980s, standards for certification of organic products had been established which strictly excluded the use of synthetic fertilizers and pesticides (Kuepper, 2010), including seed treatments to prevent bunt infections. It took approximately another two decades

until the challenges for wheat production resulting from these restrictions became evident. According to the Web of Science (`www.webofscience.com`), only 247 research items related to the search terms "common bunt" and "wheat" and including keywords like "Tilletia caries/tritici" or "Tilletia laevis/foetida" were published in the almost 80 years between 1921 and 1999. From the beginning of the new millenium, though, this number increased and from January 1, 2000 until August 2023, 333 publications matching the search terms are available in the database. As outlined in the previous sections, these works contributed to an improved understanding of the genetics and biology of the bunt-wheat interaction. However, the renewed scientific interest in bunt diseases and the parallel rise in the popularity of organic agriculture (Willer et al., 2023a) also lead to the development of new breeding approaches focused on organic systems. In Austria, standardized procedures for testing value for cultivation and use (VCU) of organic winter wheat were first available in 2001. Breeding companies who want to develop varieties for the organic market are faced with the challenge that testing sites, procedures and facilities dedicated to breeding goals specific to organics are needed, leading to a high demand for resources (Löschenberger et al., 2008). In consequence, researchers and breeders have come up with strategies for minimizing additionally needed resources by combining existing infrastructure for conventional or low-input breeding with newly established organic testing. Löschenberger et al. (2008) describe a winter wheat breeding scheme relying on low input test sites for the first five generations. Only after the most important selection step is done in generation F_4, organic test sites are included. The authors highlight that by following such selection strategies focused on traits important in organic farming, genotypes were selected for variety registration trials which would have been discarded under conventional selection because of the different performance requirements. In a similar approach, Baenzinger et al. (2011) propose to combine selection for organic and conventional purposes in early generations with a focus on highly heritable traits. Later on, selected lines should be split up and separate approaches for conventional and organic breeding programs should be followed.

Detailed knowledge about the performance of parental genotypes used to conduct crosses under organic conditions is essential (Löschenberger et al., 2008). Since hotspots for bunt resistant wheat accessions lie in the Balkan region, Turkey and countries of the Near and Middle East (Bonman et al., 2006), resistance donors are often landraces or genotypes generally not adapted to growing conditions in the major wheat producing areas of the world. The integration of such exotic accessions in commercial breeding programs is tedious and time-consuming since many unwanted traits are introgressed into breeding lines together with the desired bunt resistance. This disadvantage can be alleviated and genetic diversity in breeding programs increased through pre-breeding activities which are mostly conducted at research institutions. From initial crosses between e.g. wild progenitors (Valkoun, 2001; Moore, 2015) or landraces and non-adapted cultivars (Publications 2 and 3 and additional contribution in this thesis), offspring which is more suitable as starting material for a breeding program is generated. Also the lines comprised in the bunt differential set pose interesting donors for bunt resistance, but most of these are also exotic genotypes with many unfavourable characteristics as described in chapter 1.2.1. Pre-breeding material based on bunt differentials has already been generated in projects like those by Oncica and Saulescu (2007), Muellner et al. (2020) and publication 2 in this thesis.

Another tool applied in resistance breeding against bunt rather recently is genomic selection. While this method is well-established and widely used for other traits, it hasn't been deployed for bunt research yet. Two pioneering works explored the potential of genomic predictions to select for bunt

resistance so far: Semagn et al. (2022) used genome-wide marker data on a spring wheat diversity panel with 203 lines as well as on two recombinant inbred line (RIL) spring wheat populations to derive predictions about seven different disease-related traits, among them common bunt infections. They compared three cross-validation schemes and three different prediction models and achieved mean prediction accuracies of up to 0.87 for bunt incidence. Best performances were obtained from a model taking genotype-environment interactions into account.
The second work available according to my literature research is one conducted by Krause and Krause (2023) who were investigating genomic selection for dwarf bunt resistance. Their work was based on a winter wheat diversity panel comprising 384 accessions for which genotypic data was obtained from two different genotyping platforms yielding 1519 and 3330 SNPs, respectively. Prediction accuracies varied depending on the genotyping platform used to generate SNP data with a clear advantage of the larger data set resulting from the wheat 90K iSelect array (Wang et al., 2014). With these data, a mean prediction accuracy for dwarf bunt infections of around 0.65 was achieved, indicating high potential for the application of genomic selection in bunt resistance breeding (Krause and Krause, 2023).

1.4 Significance of wheat in organic farming

In this chapter, I would like to shed some light on the importance of wheat as a staple crop with a focus on the organic sector. Highlighting the extent of farmland and the amount of wheat yields potentially affected by bunt infections should underline the necessity of research and breeding for resistance against this disease. Since my home country, Austria, is among the countries with the largest organic farming area relative to the total arable land worldwide, I will start with a global view on the topic and then go into detail about the Austrian situation.

1.4.1 ...worldwide

Of all countries worldwide, 74 have regulations on organic agriculture included in their legislation (Willer et al., 2023e). The absolute acreage of organic agricultural land and the share it takes of the total arable land have been continuously growing over the past 20 years (Willer et al., 2023a). In the ten years between 2011 and 2021, the amount of organically farmed land worldwide increased by 108 % and reached 76.4 million hectares in 2021 (Willer et al., 2023d). Of that acreage, 19.3 % are dedicated to arable land crops. While the largest share of the total organic area worldwide is covered by permanent grassland, in Europe, Northern America and Asia the biggest part is actually covered by arable crops (Willer et al., 2023b). When it comes to cereals, 0.7 % of the global area is farmed organically. Among the organic cereals, wheat has a share of more than one third with 31.33 % (Schlatterer et al., 2023). Worldwide, 0.8 % of the whole wheat yield comes from organic fields with the largest proportion of this area located in Europe (1.1 of a total of 1.7 million hectares). Compared to the whole cereals sector, organic wheat production has been growing less since 2004 (Research Institute of Organic Agriculture (FiBL), 2023).
The European Union (EU) imports a lot of organic wheat, amounting to a share of 1.3 % of the total imports in 2022. However, import volumes had decreased by 41.4 % in 2021 compared to 2020 (Willer et al., 2023c). This was mainly due to diminished wheat supplies from Ukraine as a consequence of the conflict between Ukraine and Russia (Sahota, 2023). In 2022, though, Ukraine reached the third rank among the most important suppliers of organic products to the EU since imports of soybeans, wheat and maize increased. Organic wheat imports from Ukraine rose by 85.3 %

while organic wheat imports to the EU in total continued their decreasing trend with −36.6 % in 2022 (European Commission, 2023).

1.4.2 ...in Austria

Wheat was grown on 247,424 hectares in Austria in 2023. Of this total area, 17.6 % were farmed organically. Compared to the total wheat acreage, which increased by only 1 % compared to 2022, the organic wheat area grew more and gained 5 %. Taking into account the last five years from 2018 to 2023, a clear trend towards organic wheat production becomes evident in Austria: while the conventional wheat acreage decreased by 8.7 %, the organic acreage grew by 24.7 % (Agrarmarkt Austria, 2023a).
Compared to other countries globally, Austria had the highest share of organic production (16.9 %) for cereals worldwide in 2021, taking first rank before Estonia and Sweden (Research Institute of Organic Agriculture (FiBL), 2023). In the list of countries with the largest shares of organics compared to the total farming area, Austria was listed third with 26.5 % after Liechtenstein and Samoa in 2021.
This high percentage of organically managed farmland is also reflected in the market statistics for Austria: the organic market comprised 11.6 % of the total retail sales in 2021. A larger organic market could only be found in Denmark (Schlatterer et al., 2023).
However, it should be noted that yields of organic wheat fields show a large gap compared to conventional yields. In the period from 2017 to 2022, organic yields only reached an average of 62.7 % of the conventional harvests (Agrarmarkt Austria, 2023b). This corresponds well to results published by Döring and Neuhoff (2021) who investigated the limits of wheat yields under organic conditions if fertilization depended on biological nitrogen fixation through leguminous crops. According to their calculations, the ratio of yields between organic and conventional was 0.62 for high reference wheat yields of 7.5 tons per hectare and a share of 33 % legumes in the crop rotation. In view of these limitations in terms of yield, efforts should be made to eliminate any further yield reductions due to pathogens in organic wheat production as far as possible. Results from the seed certification testings carried out by the Austrian Agency for Health and Food Safety (AGES) for fall-planted wheat show that in the seasons 2018/2019 to 2021/2022, an average of 5.8 % of all samples contained between eleven and 300 bunt spores per grain. For these samples, seed treatment is mandatory to make them marketable, necessitating the investment of additional financial resources for the producer. In addition, an average of 24.6 % of all samples contained one to ten bunt spores per grain. At these contamination levels, the producer is only notified of the detection of bunt in the sample, but no further action is required. However, under optimal environmental conditions, such small numbers of spores per grain may already lead to infections in the field. In general, the fact that almost a quarter of all samples tested for seed certification contained at least low levels of *Tilletia* spores indicates that bunt is not a rare but rather a fairly common occurrence in organic wheat fields in Austria.

1.5 The organic toolbox for common bunt management

The advantages of bunt-resistant cultivars and efforts for their development have already been discussed. In addition, farmers can apply several other measures to keep common bunt incidence in their wheat fields at bay which are, contrary to the use of synthetic fungicides, compliant with the requirements for organic agriculture. Combining some of the strategies described in this chapter with

the use of resistant varieties would be especially beneficial as it would lower disease pressure and therefore render frequent breakdowns of introgressed resistances more unlikely.

1.5.1 Hygiene and planting recommendations

Seventy years ago, Cherewick (1953) wrote in his *Account of Smut Diseases of Cultivated Plants in Canada* on page 15: "However, as is true of many other plant diseases, man himself is the worst offender in disseminating the smut fungi. He transports smutted seed from district to district, from country to country, and from continent to continent, thus enabling the pathogens of smut diseases to become established wherever their host plants are cultivated." Fortunately, humans as the main disseminators of bunt fungi have improved their behaviour at least to a certain extent in the meanwhile, but there is still a lot of potential for further amendments.
Using only clean, bunt-free seed for sowing has been a common recommendation to avoid infections with *Tilletia* for a long time. Faull (1907) suggested that wheat growers should use seeds harvested from dedicated breeding plots maintained at their farms. Seeds from these breeding plots should be hand selected and therefore provide high quality of the material. As this method did not receive widespread implementation, farmers are still often faced with the question of how to determine whether their seed lots are free of bunt spores or not. In Austria, seed samples can be analysed for contamination with bunt spores at AGES. This is mandatory for seed certification but can be used by farmers for any type of seed sample (`https://www.ages.at/en/plant/seeds-and-seedlings/seed-and-seedling-testing`). If samples contain between eleven and 300 bunt spores per grain, they need to be treated with registered chemicals for seed dressings in order to be admitted as seed material. Marketing seeds with more than 300 spores per grain is prohibited even if seed is treated (AGES Österreichische Agentur für Gesundheit und Ernährungssicherheit, 2022).
As indicated in the opening quote of this paragraph, bunt spores are often distributed by human activities - a way of contamination that could be avoided e.g. by thorough cleaning of equipment. Almost a century ago, Güssow and Conners (1929) noted: "Undoubteldy, the practice in vogue of moving threshing machines from one farm to another is responsible to a considerable extent for the spread of smut diseases and their introduction into farms previously quite free from smut" (page 4). They even propose a method for cleaning threshers and a system which should enable farmers to check whether the machines used at their fields have been properly treated. During the past century, the practice of moving threshing machines between farms has not ceased from being "in vogue", as Güssow and Conners (1929) called it, but has rather become the norm. If a field infested by common bunt is harvested, all equipment gets contaminated by spores disseminated from bunt balls which break open during threshing. In case the tools and machines are not properly cleaned before moving to the next field, healthy grain harvested there will get contaminated, too. The farmers will in consequence face dockages when trying to market the grain or will experience problems with bunt infections if grain is used as farm-saved seed for sowing in the next season. If heavily infested fields are harvested, infections cannot only be spread by a lack of field hygiene but also directly through wind dissemination to neighbouring fields while threshing. According to Woolman and Humphrey (1924), this possibility was already described in a report by Otto Appel from 1913 who observed that bunt infections were caused by spores emitted by a threshing machine that were transported by wind to an adjacent field. A picture taken by Andreas Sarg in 2021 (Figure 3) proves that such events are still happening in our time.

Figure 3: Photo of a threshing machine harvesting wheat infected by common bunt. As bunt balls break open during the threshing process, spores are blown out of the machine and distributed by wind. (Picture by Andreas Sarg, 2021)

The amount of spores transmitted through insufficient seed and field hygiene which can provoke infections and spread of the disease is very small. For a susceptible cultivar, 0.01 % of bunt spores in the whole grain load (weight of bunt spores being 0.01 % of the total grain weight) is enough to contaminate the harvest with more than 300 spores per grain. In this calculation, the loss of spores occurring during harvest is already taken into account. The infection level necessary to achieve such a ratio of spore weight to grain weight is as low as 0.12 % or 2.2 infected ears in a plot of 11 m^2. It can result from contaminations as low as 21 spores per grain (Waldow and Jahn, 2007). This indicates that if cultivars lacking bunt resistance factors are grown, already small spore loads are sufficient to provoke infections and in consequence a buildup of inoculum if the contaminated seed is sown.

Planting recommendations

Concerning agricultural practices, everything that promotes rapid plant development during germination and early seedling stages is suitable to reduce chances of successful bunt infection. Seeds should be sown at a uniform and shallow depth (Faull, 1907). Experiments by Hecke (1909) first showed that lower soil temperatures at the time of sowing promote bunt infections while sowing at higher temperatures leads to lower incidence levels. His experiments were conducted with spring wheat, but literature reviewed by Woolman and Humphrey (1924) and Güssow and Conners (1929) from the first half of the 20th century indicates that the same applies for autumn sown wheat. Johnsson (1992) also concluded from data on field experiments conducted in Sweden from 1940 to 1988 that temperatures in the first eleven days after sowing were negatively correlated with the number of bunted ears in experimental plots. Earlier sowing dates in autumn when temperatures are usually higher are therefore likely to correlate with lower common bunt infection levels. However, caution needs to be exercised when farmers decide for earlier sowing dates as this could promote other diseases like wheat dwarf virus (Buerstmayr and Buerstmayr, 2023).

Apart from temperature, another aspect of environmental conditions influencing bunt infections is soil moisture. In general, lower moisture contents favor infections, while very moist soils are not conducive (Güssow and Conners, 1929). As mentioned in section 1.1.1, Purdy and Kendrick (1957) found out that the moisture content suitable for bunt infections also depends on soil temperature. At the optimal infection temperatures of 5-10°, ideal conditions for the pathogen are present if soil moisture ranges between field capacity and the permanent wilting point (Kendrick and Purdy, 1959). Decisions about agricultural practices like those just listed are often made based on factors other than considerations about bunt infections. In addition, potential unwanted effects of measures leading to unfavourable conditions for bunt infections on incidence levels of other diseases have to be kept in mind. Holton and Heald (1941) summarized the situation like this: "For the most part, bunt control by the application of cultural practices is not popular, mainly because these practices usually modify long-established farming methods. Therefore this method of controlling bunt probably is not resorted to except in extreme circumstances where seed treatment and resistant varieties are not satisfactory" (page 171).

1.5.2 Crop rotation - survival of bunt spores in the soil

At the moment, there is quite a lot of disagreement among both researchers and practicioners whether common bunt should be regarded as an exclusively seed-borne disease or if soil-borne infections can also play a role. I hypothesize that most of the historic accounts and findings on common bunt are not widely known in this community, as soil-borne infections have been quite frequently reported and investigated in the past.

In his studies of the bunt disease at the Dookie Agricultural College in Australia, Pye (1909) occasionally observed infected ears in untreated plots which resulted from artificially inoculated trials conducted at the site two years before. He attributes these observations to bunt balls detached from the infected plants or to other means how the spores from these previous trials were introduced into the soil. In the United States, even whole regions were known for problems with soil-borne common bunt spores. Infections resulting from infested soil were apparently not causing troubles outside the Pacific Northwest during the first decades of the 20^{th} century in the U.S. (Flor, 1933). The reason for this phenomenon was revealed by Heald and George (1918). They identified spore showers disseminated during harvests as the cause of these soil-borne infections. From these bunt dust clouds, spores would settle on the soil and, due to predominantely dry weather conditions, survive until wheat was sown in autumn. These spores were then able to infect the emerging seedlings (Heald and George, 1918). Consequently, C.S. Holton and L.H. Purdy performed dedicated studies to examine measures for preventing soil-borne common bunt infections in this region as most chemical treatments were only effective against seed-borne spores. Common bunt incidence levels in plots sown with untreated seed samples in uninoculated, naturally infested soil ranged between 8 % and 20 % in a single year in their study, showing considerable infection pressure from spore-laden soil (Holton and Purdy, 1954).

As soon as the problems with soil-borne infections especially in the U.S. could be kept at bay by the use of hexachlorobenzenes (Holton and Purdy, 1955), scientific and practical interest in this phenomenon decreased. Only with the onset of the new millenium, people started to re-recognize the importance of the topic and investigations about the duration of spore viability in the soil were conducted. This information is crucial to determine how long the pause between two rotations of wheat or other susceptible small grain cereals should be to avoid soil-borne infections. Borgen (2000)

conducted a field trial under farm conditions using source areas to provoke soil-borne infections. These source areas were cultivated with artificially infected wheat which was harvested following standard procedures. Plant residues and spores set free at threshing were ploughed into the soil in early autumn. In the two years following the cultivation of bunted wheat, different crops mimicking standard crop rotations were grown. Starting in the third year after the introduction of bunt spores into the soil, the source areas were used for field tests with untreated, bunt-free seed in three more consecutive years. Anders Borgen observed bunt infections in all test years in at least some of the test fields, indicating that at least a certain amount of bunt spores was still viable in the fifth year after their introduction into the soil. Incidence in the fifth year after contamination of the source area ranged between 0.04 % and 0.75 %. In line with the calculations of Waldow and Jahn (2007), Borgen (2000) concluded that these infection levels might be low but are still too high to be neglected in commercial wheat production and would lead to problems in marketing and usage of the grain as seed material. Another interesting aspect observed in these trials was that infection levels were higher in the second year after an infected crop was grown compared to the first year, directly after the infected crop. The author attributed this to the fact that spores may have been transferred to deeper soil layers below 25 cm by ploughing after the infested crop was harvested and brought back up only after harvest of the crop in the next year. Therefore, the spores would rest close to the surface in the layer were grain is sown in the second year after the inoculum was introduced into the soil and be able to cause higher infections then.

Different crops, brassica cover crops and manure applications in crop rotations have already been examined for their potential to reduce bunt infections. Trefoil-grass was less potent in reducing the amount of viable spores in the soil than a crop rotation containing rye or triticale and pea between two sowings of wheat. The main reason for this is that tillage is reduced to a minimum in years of grass cover in the rotation compared to more frequent tilling in rotations containing cereals and legumes. In addition, the application of manure decreased infectious spore potential in the soil while using mustard as a cover crop did not yield significant reductions (Voit et al., 2017). In Austria, a research project entitled *CARIES* was conducted from 2012 to 2016 to investigate infection characteristics of common bunt (Diethart et al., 2017). First results from this project were presented by Diethart et al. (2015) who could demonstrate soil-borne infections. They compared artificial introduction of spores on the surface with treatments where spores were buried in the soil in depths up to 7 cm and observed higher infection levels with the latter treatment.

Based on the results presented in this section, crop rotations in areas with high potential for bunt infections should be as wide as possible and include regular tilling. If common bunt infections have occurred, no wheat should be grown for at least five years to avoid soil-borne infestation in following rotations. When planning crop rotations, farmers should not resort to sowing other small grain cereals like spelt, durum wheat, barley, triticale or even einkorn and emmer directly after bread wheat as these can also become infected with common bunt (von Kirchner, 1916; Gaudet and Menzies, 2012; Diethart et al., 2017).

1.5.3 Alternative seed treatments

In an attempt to control bunt infections and treat wheat seeds by means other than synthetic fungicides, several different substances and biological agents have been tested during the past decades. However, full control and protection similar to seed dressings with systemic fungicides was not achieved by any alternative treatment. If multiple treatments are combined, if infection pressure

is very low or if farmers want to exert extra caution when using clean, certified seeds, such applications can, however, still be of value for the prevention of bunt infections.
In a series of field trials over three years biologically based products and formulations containing different microorganisms were tested for their efficiency in controlling seed-borne common bunt infections. Out of the tested applications, milk powder applied directly to the seeds gave the best control while compost mixed into the soil even promoted infections. One of the bacterial strains applied as seed treatments, *Pseudomonas chlororaphidis* strain MA 342, was able to fully prevent bunt infections when applied together with milk powder and did not decrease germination rates of the seeds (Borgen and Davanlou, 2000). Meanwhile, this bacterium has been formulated into biological seed dressing products called *Cerall*® and *Cedomon*®. These products support plant growth and decrease bunt infections by competing with the pathogen for space and nutrients and by stimulating immune responses in the plants (Intrachem Bio Deutschland GmbH & CoKG, 2023). In experiments by Wiik (2021), *Cerall*® was able to reduce bunt infections while at the same time slightly increasing seed vigour.
The effect of milk powder as seed treatment was also investigated by El-Naimi et al. (2000). They examined skimmed milk powder, hucket, which is a type of local skimmed milk, and also wheat flour, all of which they applied in a concentration of 160 g per kilogram of seeds. These treatments reduced bunt infections caused by *Tilletia tritici* and/or *Tilletia laevis* to levels between zero and 46.1 %, depending on the test year. The authors speculate that the bunt control achieved by the biological substances could be due to them increasing the antagonistic effects of microorganisms already present in the soil or metabolites which are toxic to the pathogens. As effects of the seed treatments on seed health and yield were not examined and no estimates of financial resources needed for such treatments are given, these alternative control methods would need further evaluation before being applied outside test environments (El-Naimi et al., 2000). Biological substances are indeed able to exert adverse effects on seed vigour and germination rates. In experiments using essential clove oil in different formulations as submersion or spray treatments, reduced germination rates and seedling emergence after submersion of samples in pure essential clove oil in a concentration of 0.3 % for ten minutes have been observed. In general, treatments applied as submersions were more effective in reducing infections with *T. laevis* than spray treatments. However, the positive effect of clove oil treatments on incidence levels could also be due to a washing effect and not directly related to protection of the seedlings by the active ingredients (Valente et al., 2023).
Common bunt as a fungal pathogen could also be controlled by other fungi acting as antagonists. Both *in vitro* and field tests with *Cylindrocarpon olidum* were conducted by Yolageldi and Turhan (2005) and showed highly different results. While the antagonist was able to completely inhibit germination of pathogen spores under controlled conditions, it only reduced infection rates by around 50 % in the field. Experiments by Goates and Mercier (2011) with the biofumigant fungus *Muscodor albus* were characterized by generally low bunt infection levels in the susceptible, untreated controls. However, the volatiles emitted by the antagonistic fungus reduced incidence in both test years and eliminated it in those trials which showed low initial disease pressure. Slightly better results were achieved when applying *Muscodor albus* as in-furrow treatments compared to seed treatments (Goates and Mercier, 2011). Both antagonistic fungi have not been evaluated further concerning their effects on seed vigour, yield levels or other possible interactions. These studies can therefore be seen as indicators of potential applications of these biological control agents, but do not yet provide extensive data which could be directly translated into practical applications.

Another option for preventing common bunt infections in organic farming is the plant fortification product *Tillecur*® which contains ground plant ingredients. *Tillecur*® improves seedling health and promotes vigorous growth. Its effectiveness in protecting wheat plants against bunt infections has been shown in field experiments. Waldow and Jahn (2007) applied the product in trials artificially inoculated with different numbers of common bunt spores per seed and observed strong reductions in infection levels or complete control at all inoculum dosages compared to control plots. In all tests, *Tillecur*® provided better control of bunt infections than treatments of seed samples with hot water (52° for 10 min) (Waldow and Jahn, 2007). Wiik (2021) observed incidence levels between 0.3 % and 0.1 % after *Tillecur*® treatment. Similarly high levels of protection were achieved with mustard powder and vinegar treatments in his tests. However, it has to be noted that infection levels in the control plots were overall low in these experiments with incidence in untreated seed not exceeding 15 %. Vinegar treatments can be seen as a modified form of acetic acid treatments. The latter were applied to inoculated seed samples by fumigation in concentrations of 2 g and 4 g per kg. While this acetic acid fumigation was able to reduce common bunt infections compared to the untreated samples, no full control could be achieved with either concentration (Sholberg et al., 2006).

Among the many options available for preventing infections with common bunt in winter wheat, none is as easy to implement and as effective as the cultivation of resistant varieties. Since the area of organic wheat cultivation is steadily increasing and the number of registered cultivars fully resistant to bunt is still small, as many resources as possible should be allocated to resistance breeding efforts. In order to prevent frequent breakdowns of resistance factors present in commercial cultivars, the genetic diversity for bunt resistance available to breeders needs to be broadened. Academic research can enable and support progress in protecting (organic) wheat cultivation against bunt infections by unlocking new resistance sources and developing highly resistant pre-breeding material. How this thesis contributes to these tasks is described in the following sections.

2 Research aims

The goals of this thesis were to extend knowledge on common bunt infections in winter wheat and to foster the breeding of disease-resistant cultivars. These goals were pursued by addressing the following questions:

1. Which wheat accessions are sources of common bunt resistance loci and can broaden the genetic diversity available for resistance breeding? (Publications I and II)

2. How can novel resistance factors be identified and efficiently integrated in applied breeding programs? (Publications II and III)

3. For optimizing common bunt phenotyping, which infection characteristics need to be considered in field testing? (Publication III)

3 Publication 1

Genome-wide association mapping identifies common bunt (*Tilletia caries*) resistance loci in bread wheat (*Triticum aestivum*) accessions of the USDA National Small Grains Collection

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Author contributions:

- Magdalena Ehn collected and analyzed the data and wrote the original draft
- Sebastian Michel supervised the study and edited the original draft
- Laura Morales supervised the study and edited the original draft
- Tyler Gordon conceptualized the study and provided additional data
- Hermann Gregor Dallinger reviewed and edited the original draft
- Hermann Buerstmayr conceptualized and supervised the study and edited the original draft

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ORIGINAL ARTICLE

Genome-wide association mapping identifies common bunt (*Tilletia caries*) resistance loci in bread wheat (*Triticum aestivum*) accessions of the USDA National Small Grains Collection

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Abstract

Key message **Association mapping and phenotypic analysis of a diversity panel of 238 bread wheat accessions highlights differences in resistance against common vs. dwarf bunt and identifies genotypes valuable for bi-parental crosses.**

Abstract Common bunt caused by *Tilletia caries* and *T. laevis* was successfully controlled by seed dressings with systemic fungicides for decades, but has become a renewed threat to wheat yield and quality in organic agriculture where such treatments are forbidden. As the most efficient way to address this problem is the use of resistant cultivars, this study aims to broaden the spectrum of resistance sources available for breeders by identifying resistance loci against common bunt in bread wheat accessions of the USDA National Small Grains Collection. We conducted three years of artificially inoculated field trials to assess common bunt infection levels in a diversity panel comprising 238 wheat accessions for which data on resistance against the closely related pathogen *Tilletia controversa* causing dwarf bunt was already available. Resistance levels against common bunt were higher compared to dwarf bunt with 99 accessions showing ≤ 1% incidence. Genome-wide association mapping identified six markers significantly associated with common bunt incidence in regions already known to confer resistance on chromosomes 1A and 1B and novel loci on 2B and 7A. Our results show that resistance against common and dwarf bunt is not necessarily controlled by the same loci but we identified twenty accessions with high resistance against both diseases. These represent valuable new resources for research and breeding programs since several bunt races have already been reported to overcome known resistance genes.

Keywords *Tilletia caries* · *Triticum aestivum* · Genome-wide association mapping · Resistance breeding · Diversity panel

Introduction

More than 100 years ago, at the beginning of the twentieth century, common bunt was a common a disease in wheat growing areas all around the world as its name suggests. In regions like the Pacific North West in the US, wheat fields were so heavily infected that the average number of spores in a spore-trap at Pullman, WA, was 36.111 per square inch in 1916, which equals almost 600 spores per gram of soil. The region therefore became known as the smut capital of the world (Bruehl 1990) since common bunt is also called stinking smut - a name hinting at the production of trimethylamines resulting in a fishy smell already at very low contamination levels (Laroche et al. 2000). In consequence, a lot of effort was put into research on the causal agents of the disease, the two closely related fungi *Tilletia caries* (D.C.) Tul. & C. Tul. (also called *Tilletia tritici* (Bjerk.) G. Winter) and *T. laevis* J.G. Kühn (also called *T. foetida* (Wallr.) Liro) and on measures to prevent them from infecting wheat plants (Bruehl 1990). The development of seed treatments with hexachlorobenzenes (HCB) during the 1950s finally provided farmers with an efficient and reliable tool to keep

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bunt infections at stake (Line 1993). During the following decades, research activities ceased quickly and common bunt was largely neglected until the onset of the twenty-first century and the increasing popularity of organic farming. This is reflected in the number of publications on all aspects of common bunt which were as low as 18 from 1960 to 1990 and then suddenly rose to 249 from 1991 until the end of 2020 (www.scopus.com). The concept of organic farming had already been known for a long time, but it was not until the last two decades of the twentieth century that a considerable number of farmers started adopting these practices (Kuepper 2010). As the research at hand is a collaboration between groups in the U.S. and Austria, these two countries shall serve as examples for the current significance of organic agriculture on two different continents. In the United States, 2.33 million hectares were farmed organically in 2019, leading to rank four in the list of countries with the largest organic farming areas. However, Austria as a very small country took rank 14 on this list, with 669.921 hectares (FiBL survey 2021). This makes Austria the country with the second highest percentage (26.1%) of organically managed farming area relative to the total arable land (FiBL survey 2021), while this value was only 0.6% in the U.S. in 2019 (Meier et al. 2021). The importance of wheat breeding for organic agriculture is emphasized by the fact that cereals are the key arable crop for organic production in both North America (The World of Organic Agriculture 2021) and Europe (Willer et al. 2021).

Treatments against common bunt infection are available for organically managed farms, but they are in most cases not as easy in their application as seed dressings for conventional farming and only provide limited control (Borgen and Davanlou 2001). Voit et al. (2012) reported that in years with high disease pressure, organic treatments only showed 65% efficiency on farms in Germany and Austria. In consequence, resistant wheat varieties can be considered the most economically efficient and environmentally friendly way of disease prevention (Borgen and Davanlou 2001; Voit et al. 2012; Matanguihan et al. 2011). A range of genes (*Bt*-genes) conferring resistance to common and/or dwarf bunt (*Tilletia controversa* J.G. Kühn) via gene-for-gene interaction have been identified in wheat (Goates 2012; Goates and Bockelman 2012; Steffan et al. 2017; Muellner et al. 2020). Apart from these qualitative resistances, also quantitative trait loci (QTL) with effect against one or both fungal diseases have recently been mapped (Bhatta et al. 2018; Mourad et al. 2018; Muellner et al. 2021; Singh et al. 2015; Fofana et al. 2008; Wang et al. 2009; Dumalasová et al. 2012). Considering results by Goates (2012) and Hoffman and Metzger (1976) who found that several of the bunt races examined in their experiments were able to overcome genotypes with known *Bt*-genes and also recent reports of resistance breakdowns against certain bunt isolates (e.g. Gladysz et al. 2021; Dumalasová 2021; Orgeur et al. 2021), the urge of identifying new resistance sources and possibly also combining several loci in a single cultivar becomes evident. One way of searching for novel resistance alleles that has become possible with the availability of high-density molecular markers for wheat is to conduct a genome-wide association study (GWAS). Successful applications of this technique have already identified SNP-markers significantly associated with bunt resistance on chromosomes 2B, 7A (Schmidt et al. 2021), 6DS (Gordon et al. 2020), 2A, 3D and 4A (Bhatta et al. 2018). A study by Mourad et al. (2018) identified more than 120 SNPs significantly associated with common bunt resistance of which SNPs on chromosomes 1A, 1B, 4A, 5B and 6A showed the highest R^2 values (between 5% and 9%).

All these studies were focused on common bunt, except for Gordon et al. (2020) who examined a diversity panel with 292 bread wheat accessions of the USDA National Small Grains Collection (NSGC) for resistance against dwarf bunt. Caused by *Tilletia controversa* J.G. Kühn, dwarf bunt is closely related to the *Tilletia* species causing common bunt. Several publications have stated that resistance against both these diseases is controlled by the same genes (Metzger and Hoffman 1978; Goates 1996, 2012), but recent findings support the hypothesis that resistance to common bunt does not automatically confer resistance to dwarf bunt and vice versa (Muellner et al. 2021). To shed more light on this question, we aim to identify marker-trait associations for common bunt resistance in the same diversity panel that was used by Gordon et al. (2020) and compare the results. Furthermore, we want to determine whether the NSGC comprises accessions which have the potential to broaden the genetic resources for common bunt resistance that can be exploited for resistance breeding in bread wheat.

Methods

In order to test the postulated hypotheses, we evaluated a panel of 292 bread wheat accessions from the USDA NSGC for common bunt resistance. The panel is described in detail in Gordon et al. (2020). Common bunt infection data from field trials in Austria was combined with data on dwarf bunt infection levels from field trials in Utah, U.S. and genome-wide marker data generated with a 90K SNP-chip (Wang et al. 2014). Information on both phenotypic data on dwarf bunt infection and genotyping is also available in Gordon et al. (2020).

Field trials

The NSGC panel was phenotypically evaluated in three subsequent years at the experimental station of IFA Tulln (48°19'05"N, 16°04'10"E, elevation 177 m above sea level).

Sowing took place in autumn and all seed samples were artificially inoculated prior to sowing. Teliospores were harvested from infected wheat ears in previous field trials from a variety of moderately susceptible genotypes showing typical common bunt symptoms and stored at room temperature under dry conditions. The original inoculum for bunt testing at IFA Tulln consisted of a mixture of spores collected at three different locations in eastern and western Austria which represents the common bunt race spectrum in this region. Following a protocol adapted from Goates (1996) and Muellner et al. (2020) grain samples were artificially inoculated with a suspension of teliospores in a solution of methylcellulose in water (2 g of methylcellulose in 1000 ml of water). For 10 g of seeds, 0.09 g of spores were used (= 0.3 ml of spore suspension) which were added to the grain samples with a multi-dispense pipette and distributed onto the seeds by shaking.

For the trial in 2019, seeds for all genotypes were received from Tyler Gordon and sown in double-row plots of 65 cm in length in a non-replicated field trial with 17 cm spacing between rows, 33 cm spacing between plots and 50 cm spacing to the next row of plots. To facilitate sowing of further field trials, seeds were multiplied in a separate trial. Field experiments for 2020 and 2021 were sown as randomized complete block designs in two replications with single-row plots of 160 cm accompanied by a support row of equal length. This support row consisted of different short, sturdy cultivars ('Balaton', 'Balitus') and was intended to stabilize lodging-prone accessions. Spacings between rows and plots were the same as in 2019. In each year, 5 g of seeds were used per plot. Growth regulators were applied in 2020 and 2021 to prevent extensive lodging because scoring of lodged accessions becomes more complicated and error-prone.

Heading date was scored when 50% of all tillers had reached BBCH 55 (half of inflorescence emerged from flag leaf) as days after May 1. Plant height was measured as the average height per plot in cm excluding awns.

Common bunt incidence was determined in 75 randomly chosen spikes per row by cutting each spike with scissors and checking for bunted kernels. Spikes were considered infected if at least one bunt sorus was detected and incidence was calculated as the percentage of diseased spikes out of 75 spikes. Incidence was normalized (common bunt normalized incidence, CB–NI) to a range between zero and the average of susceptible cultivar 'Capo' which we assessed in two plots as 100%.

Molecular marker data

The final panel used for genotypic analysis in the publication at hand contained 238 accessions, after removing duplicated entries as in Gordon et al. (2020) and genotypes without phenotypic information from the Austrian field trials. All SNPs with $\leq 5\%$ minor allele frequency (MAF) in this reduced panel were excluded and missing values were imputed as zero. The final set of markers contained 18953 SNPs.

Statistical analysis

All statistical analyses were carried out using R (R Core Team 2021). Best Linear Unbiased Estimates (BLUEs) were calculated for each trait observed in the replicated field trials of 2020 and 2021 using a linear mixed model of the form

$$P_{ik} = \mu + G_i + R_k + e_{ik} \tag{1}$$

with P_{ik} denoting the observed phenotypic value for the respective trait, μ being the grand mean, G_i representing the effect of the i^{th} genotype, R_k being the effect of the k^{th} replication and e_{ik} denoting the error term. For analysis across all three environments, this model was extended to

$$P_{ijk} = \mu + G_i + E_j + E_j(R_k) + GE_{ij} + e_{ijk} \tag{2}$$

to calculate BLUEs which also take the effect of the j^{th} year E_j, the nested effect of replication k in year j ($E_j(R_k)$) and the genotype-environment-interaction GE_{ij} into account. In both models (Eqs. 1, 2) the grand mean and the genotype effect were treated as fixed effects while all other effects were modelled as random. Based on across-year BLUEs, mean values and standard errors for all phenotypic traits observed in field trials in Tulln, Austria, as well as for dwarf bunt normalized incidence were calculated for each subpopulation identified in the data by Gordon et al. (2020).

Variance components were determined using the same linear mixed model as described in Eq. 2 but only the grand mean (μ) was treated as a fixed effect and all other effects were modelled as random. All models were fit with the remlf90 function from package breedR (Munoz and Sanchez 2020).

Broad-sense heritability was calculated as

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{\sigma_{G\times E}^2}{n_E} + \frac{\sigma_e^2}{n_R \cdot n_E}} \tag{3}$$

with σ_G^2 being the genotypic variance, $\sigma_{G\times E}^2$ denoting the genotype-environment-interaction, σ_e^2 as the residual variance, n_R as the number of replications in each year and n_E denoting the number of test environments (Schmidt et al. 2019; Hallauer and Miranda 1986).

A principal component analysis of the genotypic data of all 238 lines was conducted using the prcomp function from the stats-package in R (R Core Team 2021) to investigate population structure.

Genome-wide association analysis

Genome-wide linkage disequilibrium (LD) for the markers and population structure in the panel are described in Gordon et al. (2020). For detection of marker-trait associations with dwarf bunt incidence, a mixed linear model controlling for familial relationships with a kinship covariance matrix and for population stratification with two principal components (Yu et al. 2006) was the best performing model (Gordon et al. 2020). However, such a model did not perform equally well when trying to find marker-trait associations for common bunt incidence according to QQ-plots (Online Resource 10b). We therefore applied compression of the kinship covariance matrix and determined marker-trait associations using a compressed mixed linear model [CMLM, (Zhang et al. 2010)].

Compression was achieved through partitioning around medoids clustering (Kaufman and Rousseeuw 1990) of the SNP marker data using the pamk function in R package fpc (Hennig 2020). Cluster solutions for two to 238 clusters were obtained and the optimum compression level was determined for each data set separately by fitting mixed linear models with CB–NI as the response variable, the grand mean as a fixed effect and the cluster-assignment of each genotype. Allele calls for all 18,953 markers were averaged across all genotypes assigned to a single cluster and this averaged marker profile was then assigned to each genotype in the respective cluster so that they became identical in terms of their allele calls. A similar approach has been suggested for analysis of pooled DNA of family bulks in the context of applied plant breeding programs by Michel et al. (2021) and is also described by Baller et al. (2020) for genomic predictions on pooled DNA in animal breeding. The additive relationship matrix K was calculated based on the averaged, clustered marker data for all 238 accessions with the A.mat function from the rrBLUP package (Endelman 2011).

Models were fitted with the mmer function of the R package sommer (Covarrubias-Pazaran 2016) and the Bayesian information criterion (BIC) was used to choose the most suitable model. For each data set (2019 to 2021 and BLUEs across years), a marker matrix as genotypic input for the final GWAS-model was prepared according to the optimal clustering solution (i.e. compression level). Genome-wide marker-trait associations were estimated using the sommer package. Mixed models with CB–NI as the response, SNP as fixed effect and genotype as a random effect, with variance-covariance specified by the K matrix were fitted and variance components were estimated with the *P3D* method described in Zhang et al. (2010). P-values, SNP effect estimates and R^2 values for each SNP in each data set were extracted from the GWA-models and multiple test correction was applied on the p-values using the $q$value package (Storey et al. 2020). Significant marker-trait associations were identified using a false discovery rate (FDR) of $\alpha = 0.05$.

To identify marker-trait associations for plant height and heading date, the same type of model was used as described for common bunt but the K matrix was calculated based on the original, non-clustered marker data.

Evaluation of panel composition

The composition of the experimental population was initially based on data on dwarf bunt infection levels of individual accessions available in the GRIN database (https://www.ars-grin.gov/) and optimized to comprise approximately 50% dwarf bunt resistant and susceptible accessions, respectively (Gordon et al. 2020). It has been shown that races of dwarf bunt and common bunt exhibit different virulence patterns against various bunt resistance sources (Goates and Bockelman 2012; Muellner et al. 2021) and therefore, a panel optimized for dwarf bunt reactions cannot be expected to also provide optimal conditions for common bunt association mapping. To investigate how the ratio of susceptible vs. resistant accessions in an experimental population influences GWA results, we conducted a leave-one-out cross-validation based on the classification of accessions into subpopulations described in Gordon et al. (2020). Of the six subpopulations, one at a time was excluded and the GWA-procedure was repeated as described above with accessions belonging to the other five subpopulations. Since some subpopulations were composed of almost exclusively highly resistant accessions, the ratio of susceptible vs. resistant genotypes in the reduced panel changed when individual subpopulations were excluded. For this cross-validation, a non-compressed kinship matrix and the original genotypic data were used.

Results

Field trials

In the whole panel 66.8% of the lines were resistant to common bunt infection with ≤ 10% CB–NI BLUE (Table 1). Two out of three trials were replicated and Pearson correlation coefficients between replications were $r = 0.90$ for 2020 and $r = 0.60$ for 2021, both significant at $p < 0.0001$. Mean CB–NI was significantly ($\alpha = 0.05$) higher in the second replication in 2021. This could be traced back to scoring errors in the field trial and strongly deviating data points were excluded from the analysis. After this correction which concerned 18 out of 303 genotypes, correlation between replications in 2021 improved from $r = 0.60$ to $r = 0.88$. CB–NI was highly correlated between individual years ($r = 0.90$ to 0.94) with all estimates being significant at the $p < 0.0001$ level and showed strongly right-skewed distributions.

Table 1 Classification of bread wheat accessions by their country of origin into resistance classes based on data across three subsequent years

Accession origin	HR[a]	*R*[b]	*S*[c]
Azerbaijan	0	0	3
Germany	0	0	1
Iran	6	4	13
Montenegro	3	0	1
Russia	0	0	2
Serbia	9	2	7
Sweden	0	1	0
Turkey	21	8	29
USA	86	19	23
Total	125	34	79

[a]Highly resistant, ≤ 1% CB infection

[b]Resistant, > 1% and ≤ 10% CB infection

[c]Susceptible, > 10% CB infection

Significant negative correlations were observed across years for CB–NI with plant height ($r = -0.16$, $p = 0.012$) and heading date ($r = -0.20$, $p = 0.002$), respectively (Fig. 1). A positive correlation of $r = 0.37$ ($p \leq 0.0001$) was observed between across-year BLUEs for CB–NI assessed in Tulln, Austria and across-year BLUEs for DB-NI assessed in Logan, UT and in the GRIN database. The corresponding scatterplot (top left of the scatterplots in Fig. 1) also indicates that there are a lot of lines resistant to common bunt but highly susceptible to dwarf bunt and only very few which show a reversed pattern. Twenty lines showed ≤ 1% incidence across years for both common (2019–2021) and dwarf bunt (2017–2019, Gordon et al. (2020)) (Table 2).

Infection levels in the bunt differential lines were inconsistent between years for *Bt8*, *Bt9*, *Bt15*, *BtP* and 'PI 173438' possessing an unknown type of resistance (Table 4). In general, more of the known resistance sources are effective against common bunt compared to dwarf bunt as shown in columns "BLUE" and "DB-BLUE" in Table 4. Only for *Bt8*, *Bt14*, *Bt15*, *BtP* and the unknown resistance source of 'PI 173438', CB–NI was higher than DB-NI across years.

Heritability of all observed traits across data sets was high (≥ 0.84) and highest for common bunt incidence ($H^2 = 0.96$). For both plant height and CB–NI, the genotype effect explained the largest part of the observed phenotypic variance whereas variance in heading date was mainly explained by the environmental effect (Table 3).

Table 2 Accessions with high resistance levels (≤ 1% incidence) against both common and dwarf bunt (Gordon et al. 2020)

Accession	HD[a]	PH[b]	Status	Origin	Source/pedigree
CItr 17727	31.13	105.88	Cultivar	U.S., Idaho	From PI 178383
PI 178383	35.45	98.93	Landrace	Turkey, Hakkari	*Bt8*, *Bt9*, *Bt10*
PI 345102	36.87	120.15	Landrace	Serbia	
PI 345106	32.80	115.28	Landrace	Serbia	
PI 345428	38.13	138.19	Landrace	Montenegro	
PI 374540	33.94	103.83	Landrace	Serbia	
PI 470395	34.62	100.88	Landrace	Turkey, Hakkari	
PI 518914	39.94	108.68	Breeding line	U.S., Idaho	From PI 178383
PI 560601	32.68	88.56	Landrace	Turkey, Hakkari	
PI 560792	37.87	97.83	Landrace	Turkey, Hakkari	
PI 560842	34.06	100.64	Landrace	Turkey, Hakkari	
PI 560843	36.82	95.76	Landrace	Turkey, Hakkari	
PI 620655	32.87	91.13	Breeding line	U.S., Oregon	
PI 622967	33.24	98.44	Landrace	Iran, Esfahan	
PI 636145	38.80	97.83	Breeding line	U.S., Idaho	From PI 560603
PI 636147	38.17	96.73	Breeding line	U.S., Idaho	From PI 560603
PI 636156	38.10	92.83	Breeding line	U.S., Idaho	From PI 560795
PI 636169	36.66	96.00	Breeding line	U.S., Idaho	From PI 560843
PI 636170	36.10	88.68	Breeding line	U.S., Idaho	From PI 560843
PI638644	31.13	102.71	Breeding line	U.S., Washington	

Values for heading date and plant height are best linear unbiased estimates (BLUEs) across trials in Tulln, Austria, from 2019–2021

[a]Heading date in days after May 1

[b]Plant height in cm

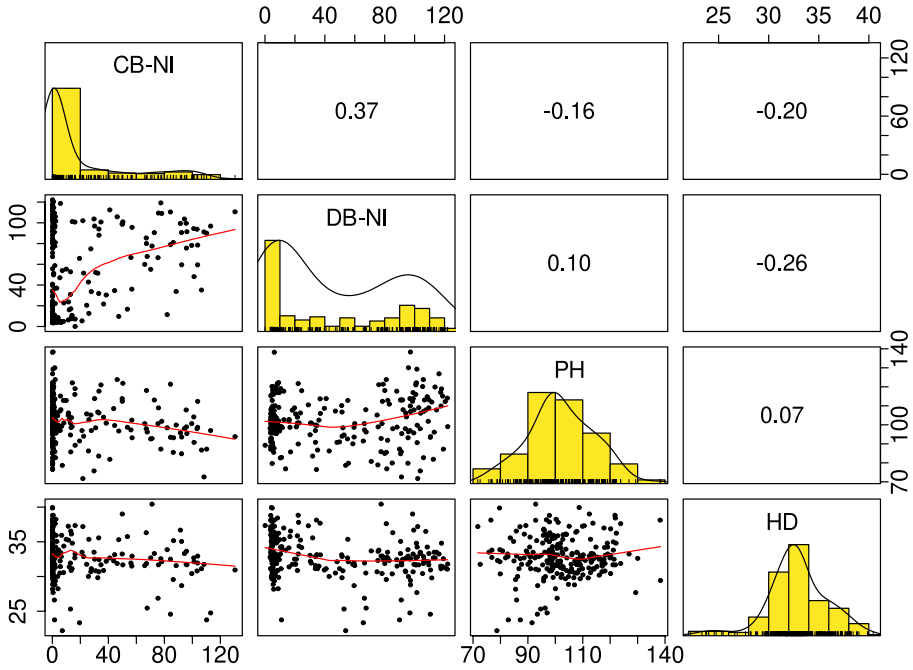

Fig. 1 Pearson correlation coefficients, histograms and scatterplots between across-year best linear unbiased estimates (BLUEs) for normalized incidences of dwarf bunt (DB-NI) and common bunt (CB–NI) as well as plant height (PH) and heading date (HD) in the common bunt trials across all years (2019–2021) (Gordon et al. 2020)

Table 3 Average, minimum and maximum values for individual years and BLUEs across years , variance components (rows 5–9) and broad-sense heritability estimates (H^2) for phenotypic traits observed in field trials from 2019 to 2021

	HD[a]	PH[b]	CB–NI[c]
2019	34.2 (25–42)	109.0 (55–150)	18.0 (0–131)
2020	25.39 (16–40)	100.1 (65–145)	15.57 (0–123.7)
2021	39.4 (25–48)	97.63 (55–150)	22.7 (0–158.7)
BLUE	33.08 (22.2–43.5)	102.3 (55–143.8)	18.88 (0–130.6)
$V_{Genotype}$	8.9	127.1	888.0
$V_{Environment}$	50.5	48.9	12.0
$V_{Replication}$	0	2.7	4.5
$V_{G\times E}$	0.8	14.0	44.7
V_{error}	1.2	113.8	118.8
H^2	0.95	0.84	0.96

[a]Heading date in days after May 1

[b]Plant height in cm

[c]Common bunt normalized incidence

Subpopulations and disease reaction

Accessions from Iran, Serbia and Turkey showed the highest proportions of susceptible lines whereas accessions originating from the U.S. were for the most part highly resistant (Table 1). Individual subpopulations reacted differently to common bunt compared to dwarf bunt (Fig. 2b). While genotypes assigned to subpopulations three and four showed the second and third highest dwarf bunt infection levels, they had low average CB–NI (Online Resource 1).

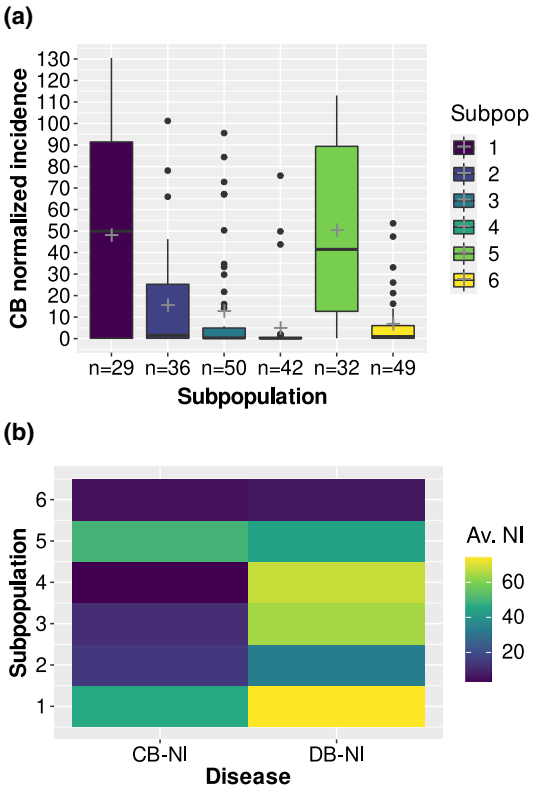

Fig. 2 **a** Best linear unbiased estimates (BLUEs) across three years for common bunt normalized incidence (CB–NI) in percentages for genotypes assigned to different subpopulations. Number of genotypes per subpopulation is shown on the *x*-axis, crosses mark average CB–NI. **b** Heatmap comparing subpopulation averages of BLUEs across years for normalized incidence (NI) of dwarf bunt (DB-NI) and CB–NI (Gordon et al. 2020)

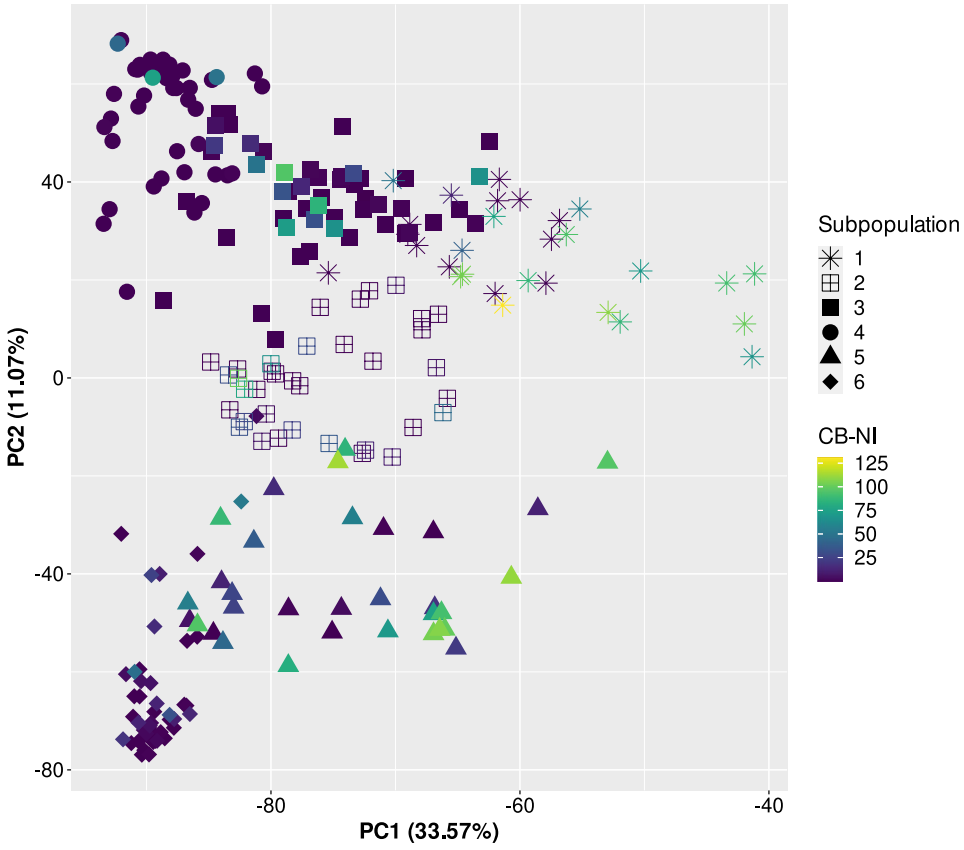

Fig. 3 Scatterplot of the first two principal components of the 238 accessions used for association mapping. Individual subpopulations in the panel are discriminated by shapes of the data points. Colours of individual data points indicate across-year best linear unbiased estimates (BLUEs) of normalized common bunt incidence (CB–NI) levels of the respective genotypes (Gordon et al. 2020)

Table 4 Phenotypic scores for dwarf bunt (DB) and common bunt (CB) normalized incidence for the bunt differential set and the susceptible cultivar 'Capo' used for normalization

Accession	Name	*Bt*-gene	DB-BLUE[a]	CB 2019	CB 2020	CB 2021	CB BLUE[b]
.	Capo	Susceptible	.	100	100	100	100
PI 209794	Heines VII	Susceptible	111.2	82.7	78.0	100.8	86.5
PI 554101	Selection 2092	*Bt1*	104.0	0	0	0	0.1
PI 554097	Selection 1102	*Bt2*	119.2	84.7	56.8	93.1	77.5
CItr 6730	Ridit	*Bt3*	51.2	0	0	2.1	0.9
PI 11610	CI 1558	*Bt4*	120.4	0	1.7	0	0.8
CItr 11458	Hohenheimer	*Bt5*	32.1	0	2.5	0	4.0
CItr 10061	Rio	*Bt6*	67.4	0	0	0	0.1
PI 554100	Selection 50077	*Bt7*	112.7	44.6	48.7	.	49.9
PI 554120	M72-1250	*Bt8*	7.5	0	1.7	33.9	13.6
PI 554099	R63-6968	*Bt9*	55.3	13.4	3.4	13.8	9.9
PI 554118	R63-6982	*Bt10*	37.0	0	2.5	5.3	3.1
PI 554119	M82-2123	*Bt11*	4.5	0	0.8	0	1.0
PI 119333	1696	*Bt12*	3.4	0	0	0	0.6
PI 181463	Thule III	*Bt13*	11.0	0	2.7	1.1	1.6
CItr 13711	Doubbi	*Bt14*	3.7	0	6.6	3.2	3.9
CItr 12064	Carleton	*Bt15*	14.8	37.4	6.2	21.2	19.6
PI 173437	7838	*BtP*	0.1	18.6	4.4	29.6	16.2
PI 173438	7845	Unknown	0.1	0	16.1	19.0	13.5
PI 178383	6256	*Bt8,9,10*	4.5	0	0	0	0.1
PI 476212	SM Selection 4	Unknown	4.0	0	0	2.1	0.9

[a]Normalized dwarf bunt incidence across four data sets derived from Gordon et al. (2020)

[b]Normalized common bunt incidence across three data sets (2019–2021)

Subpopulations two and six showed similar reactions to both diseases. High variation in CB–NI was observed for subpopulations one and five (Figs. 2a and 3). Average infection levels for common bunt were lower than for dwarf bunt in all subpopulations except subpopulation five. Please note that only dwarf bunt data on those genotypes for which CB–NI could be assessed in all three years was used for the analysis, resulting in 238 accessions compared to 246 used by Gordon et al. (2020). While accessions in subpopulations two, four and six, respectively, clustered together and showed low variation in CB–NI in a PCA heatmap (Fig. 3), genotypes belonging to subpopulations one, three and five, respectively, were more scattered across the principal component plot. Variation in plant height also differed between subpopulations with highest variation in subpopulation three and a comparably narrow range of observed heights in subpopulation six. Such patterns were not found for heading date. Variation in heading date was similar and standard errors were low across all subpopulations (Online Resource 1).

Marker-trait associations

Based on model fit in terms of BIC for models with compressed kinship matrices, we chose the best fitting model and thereby the ideal number of clusters, i.e. groups, for each data set (2019–2021 and BLUEs across years). While for 2020 data, the ideal number of clusters was approximately the same as the number of genotypes (236), higher compression was optimal for 2021 (155 clusters). The ideal number of clusters for 2019 was 176 and across years, the optimum compression was reached with 230 clusters (Online Resource 8).

With a model that corrected for relatedness using a compressed kinship matrix with the optimum compression level for each year, six SNP markers were found to be significantly (FDR-adjusted p-value ≤ 0.05) associated with CB–NI in at least one out of four data sets (Fig. 4) and thereof, four SNPs (in the following called *CB-1A*, *CB-2B*, *CB-7A1* and *CB-7A2*) showed significant associations in two of the data sets (Table 5). The resistance conferring allele was the prevalent one for all four SNPs in the panel under investigation and allele frequencies ranged from 91.2% to 94.1%. Differences in average CB–NI levels between accessions carrying the resistant vs. the susceptible allele ranged from 29.4% for *CB-7A1* to 52.1% for *CB-2B*. In the data set for 2021, no markers showed significant associations with CB–NI, but p-values indicated that SNPs *CB-1A* and *CB-2B* which were significantly associated with resistance in other years also might play a role in bunt resistance in 2021 (Online Resource 9).

Allele calls at the four SNP positions associated with CB–NI in more than one data set for all lines in the bunt differential set for which both genotypic and phenotypic data was available reflect the high allele frequencies of the resistance conferring alleles. Those three differential lines showing the highest CB–NI and DB-NI levels (*Bt0*, *Bt2* and *Bt7*) are the only genotypes which lack two of the resistance conferring alleles, all other lines in the differential set have the resistant allele in at least three out of four SNP positions (Online Resource 2).

Association mapping for heading date identified two markers on chromosome 7B in an interval of 9.753 to 9.754 Mbp and one marker on chromosome 7D at 72.95 Mbp to be significantly (FDR-adjusted p-value ≤ 0.05) associated with time to heading in at least two out of four data sets (Online Resource 4). No significant marker-trait associations were detected for plant height.

Table 5 SNP markers significantly (FDR-adjusted p-value ≤ 0.05) associated with normalized common bunt incidence in data from individual field trials in Tulln, Austria, from 2019 to 2021 or best linear unbiased estimates (BLUEs) across all three trials

SNP	Chromosome	bp[a]	Data set	AF[b]	R_{CB-NI}[c]	S_{CB-NI}[d]	LOD[e]	r^2
RAC875_c31133_464	1A	473.965.765	BLUEs	91.6	16.2	51.5	4.82	0.08
RAC875_c31133_77	1A	473.966.540	2020, BLUEs	91.2	15.9	53.2	5.18, 5.44	0.08, 0.09
BS00032266_51	1B	11.181.473	BLUEs	92.9	17.0	58.8	5.05	0.08
Ku_c71357_859	2B	581.704.044	2019, BLUEs	94.1	16.4	68.5	5.80, 5.01	0.09, 0.08
Ku_c5529_824	7A	335.991.471	2020, BLUEs	93.3	17.1	46.5	5.61, 5.58	0.09
RAC875_c23665_68	7A	629.801.516	2020, BLUEs	91.2	15.7	57.5	5.46, 5.53	0.08, 0.09

[a]Position in bp

[b]Allele frequency of the resistant allele in %

[c]Average CB–NI score (based on BLUEs) for accessions carrying the resistant allele

[d]Average CB–NI score (based on BLUEs) for accessions carrying the susceptible allele

[e]FDR-adjusted $-log_{10}(p)$-value

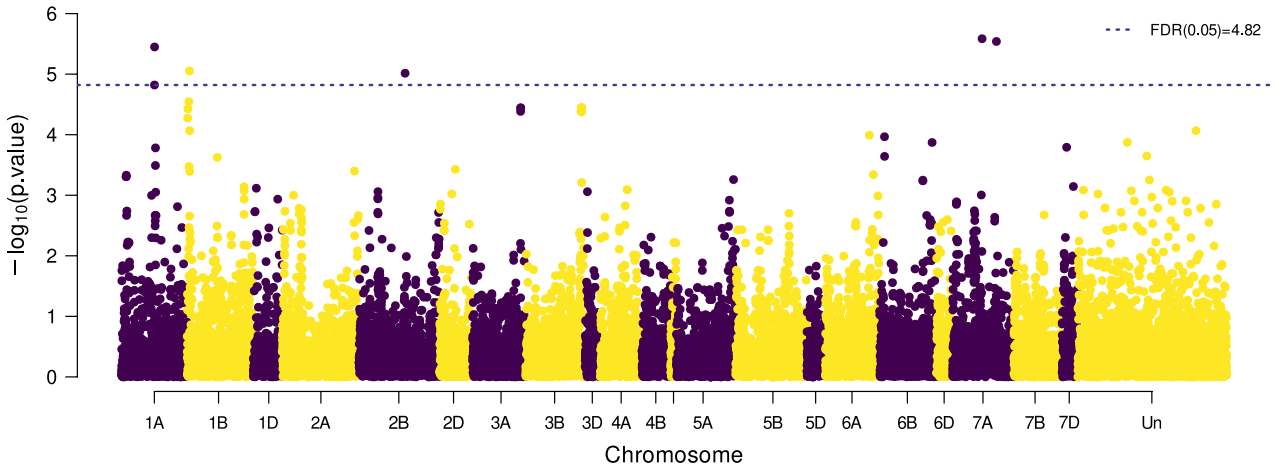

Fig. 4 Manhattan plot showing marker-trait associations for best linear unbiased estimates (BLUEs) of normalized common bunt incidence across all three years (2019–2021). The dashed line marks a significance threshold of $\alpha = 0.05$

Discussion

The inoculation method to provoke common bunt infections used at IFA Tulln was proven to be effective over several years of field trials and led to successful infestation of experimental genotypes with common bunt in all years (2019–2021). The susceptible cultivar 'Capo' and the susceptible control line in the bunt differential set, 'Heines VII' showed high infection levels in all field trials (Table 4). The comparably low correlation between replications in 2021 resulting from scoring errors that had to be corrected remains unexplained. Methods and procedures used in 2021 were no different from previous years which showed good correlations between replications and no plausible causes for the observed discrepancy could be identified.

Of the genotypes tested in this study, 42% (99 out of 238 lines) were highly resistant ($\leq$ 1% CB–NI) to common bunt in each data set. Compared to 11.38% of consistently highly dwarf bunt resistant lines (Gordon et al. 2020), this ratio is very high. Accessions were originally chosen to be 50% dwarf bunt resistant and susceptible, respectively, and to represent many different geographic origins. An ideal situation would be to have an approximately equal number of resistant and susceptible accessions from each geographic region, but this was already not the case for dwarf bunt. The six subpopulations identified in the diversity panel showed variation in their mean DB-NI levels as described in Gordon et al. (2020) and the same problem occurred for CB–NI levels (Fig. 3, Online Resource 1). Contrary to DB-NI, though, the majority of all subpopulations showed very low CB–NI below the overall average of 19.4% (values based on BLUEs across years) while only two subpopulations had higher than average CB–NI. As variation for CB–NI was low in the panel and alleles that confer susceptibility were rare, fitting a standard kinship matrix lead to overfitting, leaving no variation to be explained by putative QTL. We tackled this problem by using compressed kinship matrices as described in Zhang et al. (2010), reducing matrix complexity and facilitating association of observed variation with genetic loci. Compressing kinship matrices has been shown to improve model fit and increase statistical power compared to general linear models (GLM) and non-compressed mixed linear models (MLM) if the optimum number of groups for clustering is chosen as compression level (Zhang et al. 2010).

In view of these challenges and in line with the suggestions by Gordon et al. (2020), we would therefore recommend to take extra care when assembling a diversity panel intended for GWA analysis in order to ensure approximately equal percentages of resistant and susceptible accessions in the overall panel as well as for individual regions of origin. The benefits of a balanced data set with approximately equal variation for a certain trait in each subpopulation also become visible when considering the other traits assessed in the common bunt trials in Tulln. While variation in plant height also differed between subpopulations, variation in heading date was more evenly distributed. With a mixed model correcting for familial relationships with a standard kinship matrix, QQ-plots indicated appropriate modelling of the data and significant marker-trait associations were detected (Online Resources 4 and 10c). For plant height, on the other hand, similar problems as for CB–NI were encountered and would have to be addressed in a separate analysis.

Subpopulation six was the only one exhibiting consistently low incidence levels for both DB-NI and CB–NI. It is mainly composed of landraces from Hakkari province in Turkey and U.S. breeding lines that incorporate such landraces in their pedigrees as described in Gordon et al.

(2020). The province is located in a mountainous region characterized by a continental climate with snowy winters and dry summers matching the Köppen-Geiger climate classifications of *Dsa* to *Dsc* (Beck et al. 2018; Turkish State Meteorological Service 2022). Such climatic conditions are especially favourable for dwarf bunt infections and have lead to the evolution of highly dwarf bunt resistant landraces in this region (Bonman et al. 2006). Common bunt needs less specific conditions to infect its host and the occurrence of resistant genetic resources is not limited to narrow geographic regions as shown in the study by Bonman et al. (2006). We therefore hypothesize that genotypes from Hakkari province might be of special interest to breeders and scientists searching for high levels of resistance against both types of bunt diseases.

CB–NI showed high heritability ($H^2 = 0.96$) which is comparable to previous studies using data from artificially inoculated field trials (Muellner et al. 2020, 2021; Chen et al. 2016; Wang et al. 2019). Correlations between individual years were also high, but no SNP was found to be significantly associated with CB–NI in more than two of the four data sets. There are only few studies dealing with GWA for common bunt so far (Mourad et al. 2018; Bhatta et al. 2019). To our knowledge, the only one providing results for multiple years is the one by Gordon et al. (2020), working with the same panel as the study at hand but investigating dwarf bunt resistance on the accessions. They observed a similar pattern of differing results in marker-trait associations across years which could possibly be caused by factors like marker-by-environment interactions or the application of the stringent FDR-threshold of $\alpha = 0.05$.

Four markers were significantly associated with CB–NI in two data sets out of which two markers on chromosomes 1A and 7A, respectively, overlap with or are in proximity of regions previously reported to be associated with bunt resistance. Marker *CB-1A* is located at 473.97 Mbp on chromosome 1A. In addition, marker *RAC875_c31133_464* which is only 775 bp away from *CB-1A* was also found to be significantly associated with common bunt resistance in BLUEs across years. Muellner et al. (2020) have mapped a locus conferring dwarf bunt resistance to this chromosomal region between 380.97 and 516.67 Mbp while Chen et al. (2016) mapped a dwarf bunt resistance locus to a region between 74 and 76 cM on chromosome 1A. The peak marker for this 1A locus was *Xcfa2129* in their study. Marker *IWA6553* is neighbouring *Xcfa2129* and is located at 503.31 Mbp according to the *Triticeae Toolbox* (available via https://wheat.triticeaetoolbox.org) (Blake et al. 2016). Muellner et al. (2020) also included common bunt resistance in their study and detected a QTL in close proximity (starting at 490.09 Mbp) of *CB-1A* which conferred high levels of resistance to common bunt in their mapping populations.

Two markers on different positions of chromosome 7A were found to be associated with CB–NI in this study. Wang et al. (2019) mapped dwarf bunt resistance to a region on chromosome 7A approximately 100 Mbp away from marker *CB-7A2* at 629.80 Mbp. Chromosome 7A was also identified to be associated with common bunt resistance in earlier studies. Fofana et al. (2008) mapped a QTL with a small but consistent effect against common bunt infection to a region on the long arm of chromosome 7A. The location of the second marker found on 7A in this study, *CB-7A1* located at 336.00 Mbp, is ambigous. While reported to be on 7A in the annotation data of the wheat 90K SNP chip, this marker is recorded on chromosome 7B at 339.21 Mbp in the wheat 90K Array Consensus and RefSeq v1.0 (Blake et al. 2016; IWGSC et al. 2018) and also on 7B but at 342.46 Mbp in the wheat RefSeq v2.1 (Zhu et al. 2021). This discrepancy might be a possible explanation why no association with common or dwarf bunt has been reported for this location on chromosome 7A in any study published to date.

To our knowledge, marker *CB-2B* identified to confer common bunt resistance in this study has not yet been reported in any other publication. Bhatta et al. (2018) report marker-trait associations for common bunt for two markers at 795.3 Mbp and 799.3 Mbp, respectively. Chromosome 2B has been reported to harbour bunt resistance gene *Bt1*, which has not yet been mapped or further characterized (Sears et al. 1960; McIntosh et al. 1998). *Bt1* has been shown to provide resistance against several isolates of *T. caries* and *T. laevis* (Goates 2012) as well as against prevalent isolate mixtures in Austria used in field tests at IFA Tulln (data not shown). *PI 554101*, the accession for *Bt1* in the differential set, possesses the resistant allele for all four SNPs associated with CB–NI in more than one year in our study (Online Resource 2), so further work would be required to determine if marker *CB-2B* could be linked to resistance gene *Bt1*.

Marker *BS00032266_51* on chromosome 1B (located at 11.18 Mbp) was only found to be associated with CB–NI in the BLUEs across all years and corresponds with regions identified to confer common bunt resistance by Muellner et al. (2020) (4.35 to 38.91 Mbp) and Singh et al. (2015) (peak marker at 13.0 Mbp). Fofana et al. (2008) also detected a QTL on the short arm of chromosome 1B at ~ 19.3 cM in a mapping population derived from the cross RL4452 × 'AC Domain'. While the 1B-marker only crossed the significance threshold of $FDR = 0.05$ in a single data set and showed comparably low effect sizes in our experiment, it was the most effective locus explaining the largest part of the phenotypic variance in the three studies mentioned above. In general, wheat chromosome 1B plays an important role in bunt resistance as several other authors have also reported markers or QTL associated with bunt incidence at different positions on 1B (Dumalasová et al. 2012; Wang et al. 2009; Zou et al. 2017; Bhatta et al. 2019; Mourad et al. 2018;

Galaev et al. 2018). Furthermore, reports on the initial set of ten bunt differentials (Hoffman and Metzger 1976) state that three different resistance genes (*Bt4*, *Bt5* and *Bt6*) are located on chromosome 1B (Schmidt et al. 1969; McIntosh et al. 1998).

Allele frequencies for all markers passing the significance threshold in this study were very high - both in the overall population but also in individual subpopulations (Online Resource 3). It has been discussed by multiple authors (Dickson et al. 2010; Gibson 2012; Zhu et al. 2011) that the detection of rare variants acting as causal agents for the trait of interest in association mapping is difficult and comes with challenges. In our study, the rare variant is the one causing infection while the desired genotype leading to resistance is abundant. This would be unexpected in most other experimental or natural populations where bunt resistance would be caused by rare alleles. Nevertheless, the pre-selection for dwarf bunt resistance applied while assembling the diversity panel used in this study caused a strong deviation in allele frequencies of loci conferring common bunt resistance compared to what would be expected without pre-selection. By conducting a kind of leave-one-out cross-validation with exclusion of one subpopulation at a time, we investigated the influence of high percentages of highly resistant lines on the GWA results. The robustness of our results is supported, as loci found to be significantly associated with CB–NI levels in the full panel of lines also frequently passed the significance threshold of an FDR-adjusted p-value of 0.05 if one of the subpopulations was excluded. Especially exclusions of subpopulations three and four, consisting almost entirely of highly resistant genotypes (Fig. 2) and showing allele frequencies of close to or equal to 100% for the resistance conferring allele (Online Resource 3), were of interest as these diminished the number of highly resistant lines by 26 and 30, respectively - i.e. by more than a quarter of the total number. Since the reported markers were also found to be significantly associated with CB–NI in this cross-validation process (Online Resource 11), we conclude that our methodology was appropriate in terms of coping with the rare nature of susceptible variants and results can be regarded as robust.

Comprehensive data on both common and dwarf bunt incidence levels is now available for the diversity panel investigated in this study which consists of accessions from the USDA National Small Grains Collection. This gives both the scientific community and breeders access to genotypes with high levels of resistance to both bunt diseases. In total, 20 accessions have been identified which had a mean DB-NI of $\leq 1\%$ according to Gordon et al. (2020) and at the same time showed $\leq 1\%$ CB–NI in each of the four data sets used in our study (Table 2). These accessions originate from various geographic origins and thereby may provide valuable new genetic variation for research and breeding programs aimed at creating bunt resistant material. To validate the identified common bunt resistance loci in other wheat populations or panels and to facilitate their usage in (pre-)breeding programs, KASP markers for the respective QTL regions should be developed. This will be subject of future bunt research projects at IFA Tulln.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00122-022-04171-3.

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Author Contribution statement HB and TG conceptualized the study. Data collection and analysis was done by ME, who also wrote the original draft. TG provided additional data for the study. HGD reviewed and edited the original draft. HB, SM and LM supervised the study and edited the original draft. All authors read and approved the manuscript.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information files. Data on common bunt phenotypes has been provided to Harold E. Bockelman for addition to the GRIN database.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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4 Publication 2

Wheat (*Triticum aestivum*) chromosome 6D harbours the broad spectrum common bunt resistance gene *Bt11*

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Author contributions:

- Magdalena Lunzer collected data in 2020, 2021 and 2022, carried out data analysis and wrote the original draft
- Maria Buerstmayr supervised linkage map construction and edited the original draft
- Heinrich Grausgruber reviewed and edited the original draft and acquired funding
- Almuth Elise Müllner provided data from 2015 and 2016
- Iris Fallbacher carried out data collection in 2019
- Hermann Buerstmayr conceptualized and supervised the study, generated the populations and edited the original draft

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ORIGINAL ARTICLE

Wheat (*Triticum aestivum*) chromosome 6D harbours the broad spectrum common bunt resistance gene *Bt11*

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Abstract

Key message **A major QTL on chromosome 6DL corresponding to bunt resistance gene *Bt11* was identified in four mapping populations generated through crosses with *Bt11*-carriers PI 166910 and M822123.**

Abstract Common bunt in wheat has witnessed a renaissance with the rise of organic agriculture that began in the 1980s. The abandonment of systemic fungicides in organic farming, together with a lack of resistant cultivars, has led to wide-spread problems due to common bunt infections. Knowledge about genetic sources for resistance is still scarce and only few of the known bunt resistance factors are currently used in breeding. We therefore aimed to map the resistance factor harboured by the Turkish landrace PI 166910, which is the resistance donor for the *Bt11* bunt differential line. Four mapping populations (MPs) with 96–132 recombinant inbred lines (RILs) were phenotyped for common bunt resistance over 2, 3 or 4 years with one or two local bunt populations and genotyped with the 25K SNP array. A major bunt resistance locus on the distal end of chromosome 6D designated *QBt.ifa-6DL* was identified in all MPs and experiments. Additional QTL contributing to resistance were detected on chromosomes 4B, 1A, 1B, 2A and 7B. *QBt.ifa-6DL* mapped to a region overlapping with the *Bt9*-locus identified in previous studies, but results indicate that *QBt.ifa-6DL* is different from *Bt9* and convincing evidence from haplotype comparisons suggests that it represents the *Bt11* resistance allele. Markers for the distal region of chromosome 6D between 492.6 and 495.2 Mbp can be used to select for *QBt.ifa-6DL*. This resistance factor confers high and stable resistance against common bunt and should be integrated into organic and low-input wheat breeding programs.

Introduction

Organic farming aims to reduce problems in modern agriculture like soil erosion, soil depletion or decreasing diversity in crop plants (Kuepper 2010) but at the same time faces almost forgotten challenges which endanger crop yields. One prominent example of such a challenge is common bunt (CB) in wheat, caused by the fungal pathogens *Tilletia caries* (D.C.) Tul. & C. Tul. (*Tilletia tritici* (Bjerk.) G. Winter) and *T. laevis* J.G. Kühn (*T. foetida* (Wallr.) Liro). Until the first half of the nineteenth century, common bunt was among the most devastating wheat diseases destroying whole fields and causing so-called "black harvests," e.g. in the Pacific North West of the U.S.A (Bruehl 1990; Matanguihan et al. 2011). With the introduction of highly efficient systemic fungicide treatments during the 1950s, this seed-borne disease was not given a lot of attention in wheat breeding programs any more (Line 1993). For organic agriculture, these seed treatments are not an option and farmers are faced with challenges due to a lack of breeding activities for resistance to common bunt over many decades (Saari and Mamluk 1996). This negligence of common bunt results in the availability of only few cultivars providing complete or at least partial resistance against the disease, especially in Europe and the U.S.A (Goates and Bockelman 2012; Liatukas and Ruzgas

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2008). The (re-)consideration of common bunt resistance as a high priority goal for breeding is in fact beneficial for both conventional and organic farming. The deployment of resistant cultivars is the most economically efficient and at the same time environmentally friendly way of managing bunt diseases and provides advantages for conventional and low-input farming systems (Matanguihan et al. 2011; Saari and Mamluk 1996).

Already very low infection levels of 0.05% of grain weight can lead to quality reduction (Martens et al. 1984) and especially to a build-up of disease incidence over the following years if contaminated, untreated grain is used for sowing. Therefore, control measures need to have an efficiency of over 99% to provide protection (Borgen and Davanlou 2000). Apart from quality loss, infestation with common bunt also leads to yield losses in the same quantity as disease incidence since the wheat grains get replaced by fungal teliospores, resulting in so-called bunt balls or *sori* (Cherewick 1953; Hoffman 1982). Yield losses due to common bunt infections in wheat producing states of the U.S.A and Ontario, Canada, were estimated to amount to 2,215,441 bushels (30,294.3 tons) in 2021 and 519,051 bushels (14,126.2 tons) in 2022 (source: Wheat Disease Loss Calculator by the Crop Protection Network, available via https://loss.cropprotectionnetwork.org/crops/wheat-diseases [accessed 2023-07-19]). The disease's common name "stinking smut" is derived from a strong smell of rotten fish caused by trimethylamines produced in the fungal spores (Chen et al. 2016; Matanguihan et al. 2011).

Resistance against common bunt has long been regarded as being of mainly qualitative genetic nature and based on the gene-for-gene concept of matching virulence and avirulence genes (Flor 1956; Goates 1996; Goates and Bockelman 2012; Hoffman and Metzger 1976). In recent years, though, also quantitative trait loci conferring a more complex and quantitative type of resistance have been detected (Bhatta et al. 2018; Mourad et al. 2018; Muellner et al. 2021). A bunt differential set consisting of wheat accessions putatively monogenic for bunt resistance genes *Bt1–Bt15* and *BtP* developed by Hoffman and Metzger (1976) and extended by Goates (2012) has been widely used for gene postulation and virulence monitoring of bunt populations by research groups around the world (Gordon et al. 2020; Liatukas and Ruzgas 2008; Muellner et al. 2020). Distinct patterns of virulence/avirulence against the individual *Bt* resistance genes present in the differential lines can be observed for bunt races from different origins. Blazkova and Bartos (2002) tested bunt races from several European countries and Syria and found that the only differential lines affected by none of these races were those carrying *Bt8*, *Bt11* and *Bt12*. These findings are in line with previous studies that could also not detect bunt races virulent to one of these three resistance factors (Goates 1996; Hoffman and Metzger 1976; Metzger and Hoffman 1978). In recent years, virulence of common bunt against both *Bt9* and *Bt10* has been detected in several European countries (Bengtsson et al. 2023; Dumalasová 2021; Ritzer et al. 2022). While this is still a rather rare phenomenon, virulence against *Bt2* and *Bt7* has been reported in a wide range of environments (Cadot et al. 2021; Ehn et al. 2022; Goates and Bockelman 2012). Recent studies conducted in Sweden and the U.S.A additionally identified common bunt races virulent to *Bt1*, *Bt3*, *Bt4*, *Bt8*, *Bt13* and *BtP* (Bengtsson et al. 2023; Joshi et al. 2023).

Out of the 17 *Bt*-genes postulated to date, only *Bt9*, *Bt10* and *Bt12* have been mapped and have linked markers available facilitating their use in practical breeding programs. The first bunt resistance gene to be mapped was *Bt10* which is located on the short arm of chromosome 6D (Laroche et al. 2000; Menzies et al. 2006). The donor for both *Bt9* and *Bt10* is PI 178383, a landrace collected in Turkey (Harlan 1950) and crossed to 'Elgin' to develop the respective lines for the differential set (Goates 1996). *Bt9* was shown to be distinct from *Bt10* by Steffan et al. (2017) and mapped to the long arm of chromosome 6D. Its position was refined by Wang et al. (2019) to a region between 456 and 471 Mbp. Muellner et al. (2020) mapped *Bt12* originating from PI 911333, a Turkish landrace (Goates 2004), to the short arm of chromosome 7D.

In order to avoid frequent breakdowns of resistances in cultivars, it is necessary to make new resistance sources available to breeders and broaden the genetic basis for bunt resistance. As *Bt11* has so far been overcome by only two races of the closely related dwarf bunt pathogen (*Tilletia controversa*), it is considered the most durable of the known bunt resistance genes (Goates 2012). A donor for *Bt11* is PI 166910 (Goates and Bockelman 2012; Goates 2012), a wheat accession collected in Tokat, Turkey, in 1948 (Harlan 1950). Despite its valuable characteristics in terms of bunt resistance (Goates and Bockelman 2012), *Bt11* has not been deployed in breeding yet (Goates 2012). We therefore developed segregating populations with wheat accessions PI 166910 and PI 554119 (M822123) as the resistance donors to map the resistance factor *Bt11* and thus contribute to the development of highly and durably resistant cultivars.

Materials and methods

Plant material

Mapping populations

We investigated four different bi-parental populations putatively segregating for bunt resistance gene *Bt11*. Two mapping populations (MPs) were developed through a reciprocal cross with PI 166910 as the resistant and 'Rainer'

as the susceptible parent. Mapping populations MP-PR1 and MP-PR2 resulting from this reciprocal cross each consist of 120 $F_{5:7}$ recombinant inbred lines (RILs). The third mapping population, MP-PL, was composed of 160 $F_{5:7}$ RILs derived from the cross PI 166910 × 'Lukullus'. The resistance donor for these first three populations, PI 166910, is an awned Turkish landrace with a winter growth habit collected in 1948 (Harlan 1950) and contains a unique resistance factor designated *Bt11* by Robert Metzger in 1986 (Goates and Bockelman 2012; Goates 2012). In addition, PI 166910 was postulated to harbour the *Bt7* and *Bt9* resistance alleles (Abdalla 1984). 'Rainer' and 'Lukullus' are Austrian winter wheat cultivars released by Saatzucht Donau GesmbH and CoKG in 2006 and 2008, respectively. They are adapted to Central European growing conditions and highly susceptible to common bunt.

The fourth mapping population MP-MM with 106 $F_{5:7}$ RILs was developed from a cross between the bunt differential line M822123 (wheat accession PI 554119, *Bt11*) and 'Mulan'. M822123 was developed by Robert Metzger in Oregon, U.S.A from a cross between 'Elgin' and PI 166910. 'Mulan' is a bunt susceptible winter wheat cultivar released by Nordsaat Saatzucht GmbH in 2007 with adaptation to a broad range of environments ranging from the Scandinavian region to Eastern Europe.

Additionally, 16 wheat accessions (Online Resource 3) were sourced for genotyping and haplotype comparison only (see below).

Field experiments and disease scoring

RILs of MP-PR1, MP-PR2 and MP-PL were evaluated for CB resistance in 2019 and 2020, while population MP-MM was evaluated in 2015, 2016, 2020 and 2021. Field trials were artificially inoculated and sown as randomized complete block designs with two replications. Populations MP-PR1 and MP-PR2 were additionally evaluated in 2022 in a field trial sown as an augmented balanced incomplete block design with 23 and 33 out of 120 lines in each population, respectively, in two replications and the remaining 97 or 87 lines, respectively, in unreplicated plots. For this trial, a slightly different spore mixture was used compared to all other seasons of bunt evaluation. This bunt population was derived from infected spikes of the cultivar 'Tilliko' [released in 2016 by Cultivari gGmbH Darzau, AGES (2022)], a cultivar registered as bunt resistant. This bunt population is exceptionally virulent against resistance gene *Bt10* (Ritzer et al. 2022). Parental lines of the MPs, the set of bunt differential lines and the susceptible cultivar 'Capo' as a control were included in each of the field trials in order to monitor the virulence spectrum of the used inoculum. The experimental fields were located in Tulln, Austria (48° 19′ 05″ N, 16°04′ 10″ E, elevation 177 m above sea level).

Field trials were sown in autumn, and all grain samples were inoculated prior to sowing with a teliospore mix representing the prevailing common bunt population in Austria in all years except 2022, were the bunt population described above was used. Inoculation was carried out following a protocol adapted from Muellner et al. (2020) and Goates (1996). Infected wheat ears were harvested from field plots with medium infection levels (20–50% incidence) and stored in a dry place at room temperature. A wide range of genotypes was used as a source for spore collection to avoid unintended selection. Spores were extracted from the ears, cleaned from plant residuals and mixed with a 0.2%-solution of methylcellulose in water. The resulting spore suspension was applied to grain samples in a concentration of 0.09 g of spores (≡ 0.3 ml of spore suspension) per 10 g of seeds. Field plots were sown with 10 g of seeds for each plot as double rows of 1.6 m in length and spaced 25 cm apart, resulting in plot sizes of approximately 1 m^2.

Heading date (HD) was scored when 50% of all spikes in a single plot had reached BBCH 55 and plant height (PH) was measured at maturity in cm excluding awns. CB incidence (CBI) was recorded as the percentage of infected spikes out of 150 randomly chosen spikes per plot. Whether a spike was infected or not was determined by cutting it open and checking for bunt balls inside. Spikes were recorded as infected when at least one bunted spikelet was spotted.

Phenotypic analysis

Best linear unbiased estimates (BLUEs) were calculated for each genotype and trait in each individual experiment using a model of the form

$$P_{ik} = \mu + G_i + R_k + e_{ik} \quad (1)$$

with P_{ik} as the observed phenotypic value for the respective trait, μ as the grand mean, G_i as the effect of the i^{th} genotype, R_k as the effect of the k^{th} replication and e_{ik} as the error term (BLUEs for each experiment are available in Online Resource 5). For analysis across environments, the model was extended to

$$P_{ijk} = \mu + G_i + E_j + E_j(R_k) + GE_{ij} + e_{ijk} \quad (2)$$

taking also the effect of the j^{th} year E_j, the nested effect of replication k in year j ($E_j(R_k)$) and the genotype-environment-interaction GE_{ij} into account. The grand mean and the genotype effect were treated as fixed effects, while all other effects were modelled as random in both models (1 and 2). For calculation of variance components, only the grand mean was modelled as a fixed effect. Models were fit with the remlf90 function in the R (R Core Team 2021) package breedR (Muñoz and Sanchez 2020).

Broad-sense heritability was calculated as suggested by Strube (1967) as

$$H^2 = \frac{\sigma^2_G}{\sigma^2_G + \frac{\sigma^2_{G\times E}}{n_E} + \frac{\sigma^2_e}{n_R \cdot n_E}} \quad (3)$$

with σ^2_G as the genotypic variance, $\sigma^2_{G\times E}$ as the genotype-environment-interaction, σ^2_e as the residual variance, n_R as the number of replications in each year and n_E as the number of test environments. All statistical analyses were carried out in R (R Core Team 2021).

Genotypic data

Genome-wide SNP (single-nucleotide polymorphism) marker data was obtained for all parental lines and RILs in the MPs (raw data available in Online Resource 9). Additionally, 16 wheat accessions were genotyped with the purpose of confirming the pedigree of resistant lines and comparing haplotypes for QTL regions between carriers of *Bt11*, *Bt9* and susceptible lines, respectively (raw data available in Online Resource 10). Fresh leaf samples were used to extract genomic DNA following a protocol adapted from Saghai-Maroof et al. (1984). Ten samples were collected from each genotype, dried and pooled for DNA extraction. Genotyping was performed by TraitGenetics GmbH (Gatersleben, Germany, https://traitgenetics.com) with the Illumina Infinium 25 K XT array (Gogna et al. 2022) yielding 24145 SNP markers. Quality control was performed on the marker data for RILs prior to the construction of linkage maps and QTL anaylsis. For each MP, markers with more than 20% missing calls and markers showing significant ($p \leq 0.001$) segregation distortion were discarded. Genotypes with more than 20% missing marker data were excluded from the analysis and genotypes with more than 95% identical marker calls were combined. Co-located markers were generally excluded, but one randomly chosen SNP of each set of co-located markers was kept to ensure maximum mapping resolution.

Linkage map construction

The R package ASMap (Taylor and Butler 2017) was used to determine linkage groups (LGs). For map construction, the sum of recombination events between markers was minimized by keeping the default setting of the objective function in the call to mstmap. LGs were identified with a stringent *p*-value threshold (from $p < 1 \times e^{-7}$ to $p < 1 \times e^{-11}$ depending on the MP) for marker clustering. LGs were assigned to wheat chromosomes by BLASTing markers against the IWGSC RefSeq v2.1 (Zhu et al. 2021) and obtaining their chromosome and basepair positions. Reordering of markers within robust LGs was performed using a less stringent *p*-value threshold. Map distances were calculated with the Kosambi mapping function based on recombination frequencies between markers. After a preliminary QTL scan, LGs identified as harbouring bunt resistance loci in this first scan were refined. LGs belonging to the same chromosome were combined to determine the location of respective QTL based on the whole chromosome. The order of these LGs was determined based on physical marker positions obtained from the IWGSC RefSeq v2.1 (Zhu et al. 2021) for markers in the individual LGs. Gaps between LGs forming a chromosome were defined by re-estimating the linkage map after merging individual LGs and thereby calculating the total chromosome length in cM. Marker coverage on chromosome 6D was comparably low and large gaps were present between individual LGs in all genetic maps on this chromosome. While ordering markers based on their physical positions in the IWGSC RefSeq v2.1 generally worked well, the marker orders within the LGs on the distal end of chromosome 6D harbouring a QTL were not in full agreement with marker orders based on physical positions in the reference sequence. To evaluate reliability of physical positions, we aligned markers in this specific 6DL region against other published chromosome assembled wheat genomes from the 10+ Wheat Genomes Project (https://10wheatgenomes.org) (Walkowiak et al. 2020). We blasted markers using BLASTn at https://galaxy-web.ipk-gatersleben.de and selected those hits on the reference sequences that had the highest query coverage and lowest expectation value per marker and genome. Genome sequences of CDC Stanley v1.2, Jagger v1.1, Julius v1.0, Mace v1.0, Norin61 v1.1 and SY Mattis v1.0 were used. Graphical representations of LGs were compiled using R package LinkageMapView (Ouellette et al. 2018).

QTL analysis

QTL analyses for common bunt resistance QTL were performed for each MP separately employing the population-specific linkage map. All analyses were conducted with the R package R/qtl (Broman et al. 2003). Analyses for individual years were conducted using BLUEs across replications while BLUEs across experiments were used for analyses across years. Main effect, heavy and light interaction penalties for LOD (logarithm of the odds) scores at different significance levels were determined by running 1000 permutations (Manichaikul et al. 2009) using Haley-Knott regression (Haley and Knott 1992). The multiple imputation method proposed by Sen and Churchill (2001) was applied to impute missing genotype information. To determine the optimal QTL model, the automated search algorithm implemented in the stepwiseqtl-function of the R/qtl-package was employed. The maximum number of

QTL was set to seven. This procedure first applies forward selection, searching for additional additive or interacting QTL in each step until the maximum number of QTL is reached. Subsequently, backward elimination down to the null model is performed. Among all models, the one with the maximum penalized LOD score according to the penalties derived by permutation analysis is chosen as the optimal model. The penalized LOD criterion implemented in the stepwise-function requires strong evidence for additional QTL and as the detection of linked QTL is essential in to our study, we followed the suggestion in Broman and Sen (2011) and applied the most liberal penalties in the model search. Based on the results from the stepwise selection procedure, multiple QTL models (MQM) were fitted for individual experiments as well as for BLUEs across experiments. LOD scores, additive effect estimates and the amount of phenotypic variance explained by each QTL or interaction were derived from the drop-one-ANOVA table of the MQM analyses. As Broman and Sen (2011) recommend, Bayes' credible interval was used to derive interval estimates for individual QTL. The nominal Bayes' fraction was set to 95%.

Haplotype comparison

Physical positions and chromosome assignments for marker data of the 16 additional wheat accessions were obtained by BLASTing markers against the IWGSC RefSeq v2.1 as described in the section on linkage map construction. Accessions were sorted based on their *Bt*-gene postulations in order to compare allele calls between carriers of different resistance sources in specific chromosomal regions showing significant association with CBI in the QTL analyses. Gene postulations were either obtained from the GRIN database (available at https://npgsweb.ars-grin.gov/gringlobal/search), scientific publications or personal communications with breeders. The full list of accessions and references to *Bt*-gene postulations is available in Online Resource 3.

Results

Field trials

Common bunt race spectrum

To monitor behaviour of the bunt population across years, the spore mixture used for artificial inoculation of the MPs was tested in parallel on the bunt differential set in each year. Virulence patterns against the individual bunt resistance genes were comparable across years with some quantitative variation observed on the differential lines for *Bt2*, *Bt7*, *Bt13* and *BtP* (Table 1). Reactions of the differential lines for *Bt6*, *Bt9* and *Bt10* indicated slight changes in the virulence spectrum of the applied inoculum between 2020 and 2021. In 2022, a different and more aggressive spore mixture was applied to the seed samples, showing increased aggressiveness against *Bt3*, *Bt5* and *Bt10*. It has to be noted that field infection levels were generally higher in 2022 compared to all other years, presumably due to specific environmental conditions in this season (Online Resource 8). The differential line for *Bt11*, M822123, showed complete resistance in all plots. 'Capo', the cultivar used as the susceptible control,

Table 1 Virulence patterns of common bunt inocula on genotypes of the bunt differential set and the susceptible control 'Capo' across six years. Mean values of common bunt incidence (CBI) from two replications in each year (2015, 2016, 2019–2022)

Genotype	CBI 15	CBI 16	CBI 19	CBI 20	CBI 21	CBI 22[a]
Capo (susceptible)	62.1	81.2	71.7	78.7	70	91.5
Sel2092 (*Bt1*)	0	1.9	0	0	0	0
Sel1102 (*Bt2*)	60.2	44.5	49	70.3	27.7	98
Ridit (*Bt3*)	1.6	2	5	0.7	4	15.5
CI1558B (*Bt4*)	3.7	2.2	5	2	2.7	4
Hohenheimer (*Bt5*)	0	0	0	1	0	10
Rio (*Bt6*)	1.8	0.2	2	0	5	4
Sel500-77 (*Bt7*)	71.5	51.5	52	50	26.7	98
M822161 (*Bt8*)	1	0.2	2.3	1.3	3.7	4
M90387 (*Bt9*)	0	0	4.3	0	10	5.5
M822102 (*Bt10*)	0	0.2	2	0	6.3	50
M822123 (*Bt11*)	0	0	0	0	0	0
PI199333 (*Bt12*)	0	0.5	0	8[b]	0.3	0
Thule-III (*Bt13*)	6.8	2.5	28	7.3	12.3	30
PI173437 (*BtP*)	1.1	1	10	3.7	6	15.5

[a] A different, more aggressive inoculum was used for inoculation in 2022

[b] Contamination in the seed sample, score not reliable

was highly infected in all years with CBI levels ranging between 62.1 and 91.5%.

Phenotypic traits

CBI was highly heritable across years with estimates of $H^2 = 0.95$ or $H^2 = 0.97$ for individual MPs (Table 2). Estimates of H^2 for PH and HD showed more variability compared to CBI and ranged between 0.77 and 0.97. Average CBI levels were similar across populations and experiments except for trials conducted in 2022 which showed levels of CBI approximately 50% higher compared to previous years. High variation in phenotypic values was observed for all traits and MPs with CBI ranging between 0 and 99%, PH ranging from 65 to 145 cm and HD varying between May 21 and June 13. The resistant parental lines of the MPs (PI 166910 and M822123) were taller and had a slightly later HD than the susceptible parents ('Rainer', 'Lukullus' or 'Mulan'). MPs with PI 166910 as the resistance donor had similar average PH and HD, while RILs in MP-MM with M822123 as the donor line were on average shorter and had later HD. Both resistant parents

Table 2 Means of parents, means and ranges for individual years and/or BLUEs across years, broad-sense heritability (H^2, across years) or repeatability (r, for individual experiments) and variance components for all analysed traits of all mapping populations (PR1 = PI 166910 × 'Rainer', PR2 = 'Rainer' × PI 166910, PL = PI 166910 × 'Lukullus', MM = M822123 × 'Mulan').

Experiment		Parents		Population					
		PI	Rai	Mean (range)	H^2(r)	V_G	V_{Env}	V_{GE}	V_e
PR1 (n = 120)									
CBI[b]	2019	0	67.6	9.0 (0–74.7)	0.99[a]	262			14.9
	2020	0	73.5	10.6 (0–90.0)	0.97[a]	366			47.3
	2022	0	99	24.6 (0–99)	0.84[a]	519			198
	Mean	0	79.1	12.9 (0–79.1)	0.97	383	29.6	32.5	49.3
PH[c]	Mean	115	93	112 (91–127)	0.77	29	83.2	16.9	34.6
HD[d]	Mean	29.3	26.5	28.2 (25.5–31.7)	0.92	1.5	29.6	0.2	0.7
PR2 (n = 120)									
CBI	2019	0	67.6	9.2 (0–74.7)	0.98[a]	305			24
	2020	0	73.5	11.5 (0–98)	0.97[a]	443			56
	2022	0	99	21.9 (0–99)	0.92[a]	594			107
	Mean	0	79.1	12.2 (0–79)	0.97	424	17	24.5	49.7
PH[c]	Mean	116	93	109 (93–126)	0.85	30.8	72	7.9	34.6
HD[d]	Mean	29.3	26.5	28.2 (25.8–32)	0.89	1.1	30.3	0.3	0.6
PL (n = 160)									
CBI	2019	0	43.3	7.1 (0-72)	0.96[a]	204			15.2
	2020	0	65.8	9.4 (0−88.7)	0.98[a]	359			23.8
	Mean	0	55.5	8.1 (0−74.2)	0.95	261.6	1.8	20.4	19.4
PH	Mean	117	88	110 (88-128)	0.79	29.7	149.9	6.8	34.5
HD	Mean	30.1	29.1	29.8 (22.5-35)	0.91	3.8	39.8	0.5	0.8
MM (n = 106)									
CB	2015	0	40.5	7.9 (0−85.5)	0.99[a]	297			2.7
	2016	0	28.1	6.6 (0−73.5)	0.99[a]	239			15
	2020	0	25.3	6.6 (0−91.3)	0.97[a]	213			31.2
	2021	0	13.7	7.1 (0−69.3)	0.94[a]	165			22.5
	Mean	0	26.8	7.2 (0−70.5)	0.96	197.5	0.3	27.4	18.2
PH	Mean	95	82	96 (71-127)	0.97	78.3	58.6	8.6	16.4
HD	Mean	32.7	32	31.7 (27.7−37.3)	0.93	2.3	33.6	0.5	1.6

The number of RILs in each population is given in brackets. V_g is the genotypic variance component, V_{env} denotes the variance component explained by the year effect, V_{GE} is the variance component for genotype-environment interaction and V_e denotes the residual variance component

In 2022 (CB22), a spore mixture with different virulence was used for artificial inoculation

[a] repeatability

[b] common bunt incidence in %

[c] plant height in cm

[d] heading date in days after April 30

showed no bunt infections at all, while especially 'Rainer' was highly infected (67.6−99%), followed by 'Lukullus' (43.3–65.8%) and 'Mulan', the latter being moderately infected (13.7–40.5%, Fig. 1). ANOVA revealed that the genotypic effect explained most of the variance observed for CBI, while variance components for environmental effects, genotype-environment interaction and error variance were small. Variation in PH and HD, on the other hand, was for the largest part explained by the environmental effect. The only exception from this pattern was PH in MP-MM, for which the genotypic variance was larger than the environmental variance. CBI was negatively correlated with PH in all MPs with correlation coefficients ranging from $r = -0.13$ in MP-MM to $r = -0.25$ in MP-PR1 across years (Table 3). No correlation was observed for CBI with HD except in MP-PR1 where a low but significant negative correlation of $r = -0.12$ was found. HD and PH were positively correlated (coefficients ranging from $r = 0.55$ in MP-PR2 to $r = 0.71$ in MP-PL) in all MPs except for MP-MM in which the two traits were negatively correlated ($r = -0.18$). The parental lines for RILs in MP-MM had approximately the same HD and were also more similar in PH compared to parents in all other MPs.

QTL analysis

Linkage maps

During the data preparation for linkage map construction, we noticed that the set of markers being polymorphic between the parental genotypes differed by more than 1000 markers when comparing MP-PR1 and MP-PR2. We therefore conclude that the two crosses between 'Rainer' and PI 166910 were actually not reciprocal, but that the individuals of PI 166910 used as mother/father in the crosses rather have to be treated as two sublines. All subsequent analyses were therefore performed separately in the two MPs instead of treating all RILs as one homogeneous MP. The linkage map for population MP-PR1 was built on data from 105 RILs with 2430 markers and comprised 32 LGs,

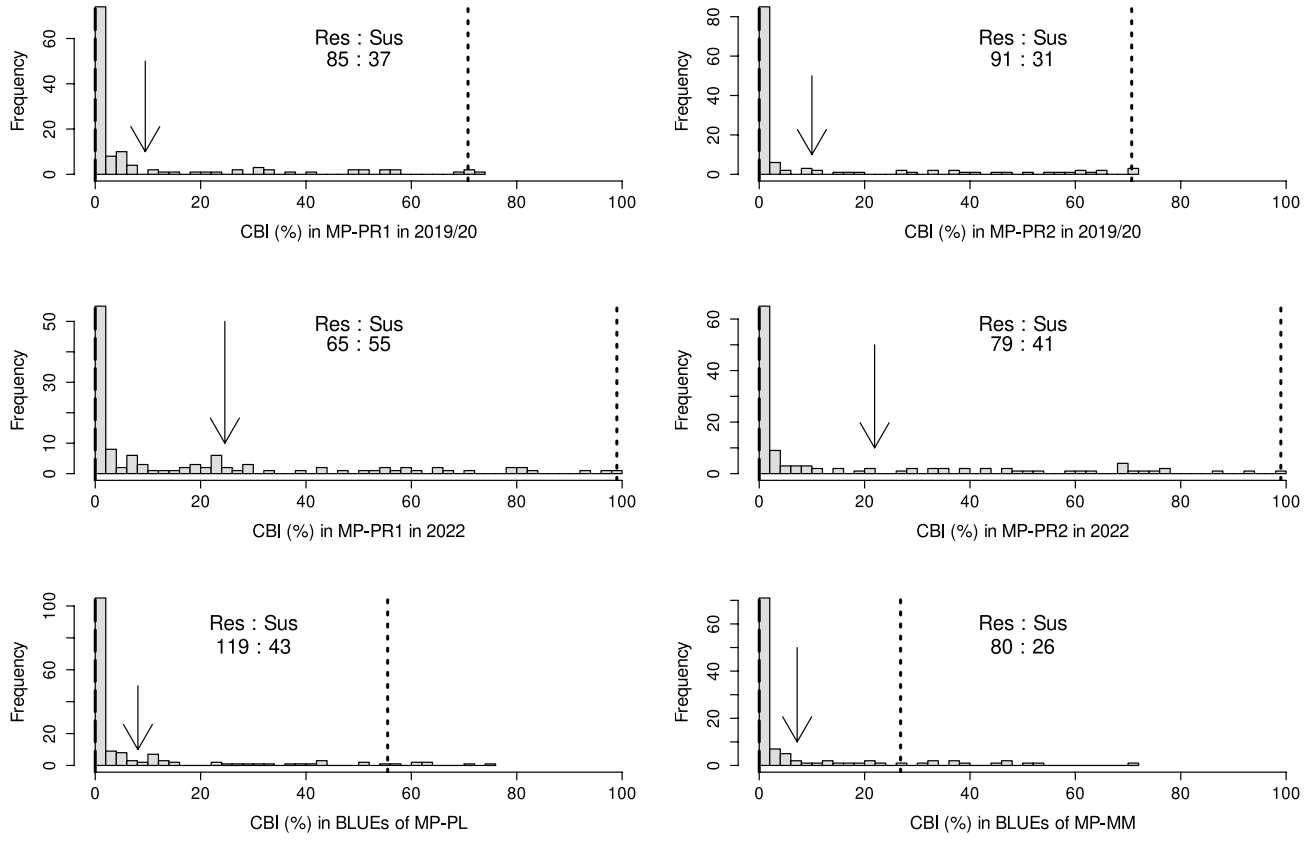

Fig. 1 Histograms for all mapping populations (MP) with ratios indicating the proportion of resistant (Res, < 5% infection) to susceptible (Sus, ≥ 5% infection) RILs per population. MP-PR1 and MP-PR2 were inoculated with a different bunt population in 2022 which was more aggressive, and therefore, histograms are shown for BLUEs across 2019 and 2020 (row 1) and for 2022 (row 2) separately for these two MPs. For MP-PL and MP-MM, histograms for best linear unbiased estimators (BLUEs) across years are shown (row 3). Arrows indicate the population mean in the respective data set, dashed lines indicate CB levels of the resistant and dotted lines of the susceptible parent

resulting in a total map length of 3792.4 cM. In MP-PR2, 100 genotypes with 2795 markers were used to construct a linkage map comprising 44 LGs, summing up to a total length of 4574 cM. For population MP-PL, 132 RILs were available after quality control. The map for MP-PL consisted of 2114 markers in 38 LGs with a total map length of 4230.1 cM. In population MP-MM, 96 RILs and 1821 markers passed quality control. The linkage map consisted of 31 LGs and was 3160.1 cM long. All wheat chromosomes were represented by the LGs in each of the four individual maps, which are available in Online Resource 1.

QTL identification and analysis

Wheat chromosome 6D was identified as harbouring a locus controlling resistance against common bunt in all four MPs (Table 4, Fig. 3, Online Resource 6). The QTL mapped to the long arm of chromosome 6D and is subsequently referred to as *QBt.ifa-6DL*. It was detected consistently in all experiments and MPs, had a major effect on CBI and explained between 18.5% (MP-PL, 2020) and 49.6% (MP-PR1, 2022) of the phenotypic variation with an average of 33.9%. The QTL spanned a maximum genetic distance of 13.9 cM (482.8–495.2 Mbp) across MPs, flanked by markers *wsnp_Ex_c14691_22763609* and *AX-94841369*. Comparison of the *QBt.ifa-6DL* region with six additional reference sequences of the 10+ Wheat Genomes Project suggested that the QTL interval might be smaller than the region based on IWGSC RefSeq v2.1 (Zhu et al. 2021). Maximum physical distance between peak markers in individual MPs was approximately 2.580 Mbp in RefSeq v2.1, while distances between these markers ranged between 0.865 Mbp (CDC Stanley) and 2.369 Mbp (Julius) in the additional reference sequences (Online Resource 4).

On chromosome 4B, two regions were associated with common bunt resistance. The first locus, hereafter referred to as *QBt.ifa-4BS*, is located on the short arm of chromosome 4B and was found in all data sets for MP-PR1 and MP-PL as well as in MP-MM in 2015. The second locus will be referred to as *QBt.ifa-4BL* and mapped to the long arm of chromosome 4B. It was identified in MP-MM in 2016. In MP-PR2, a locus with a peak marker on the short arm of chromosome 4B was associated with CBI, but the estimate derived using Bayes' credible interval for this locus spanned almost the whole LG, so no unambiguous assignment to either *QBt.ifa-4BS* or *QBt.ifa-4BL* was possible. However, if the interval estimate was based on LOD drop-off, the region was corresponding to *QBt.ifa-4BS*. The effect of *QBt.ifa-4BS* on CBI was on average smaller compared to *QBt.ifa-6DL*. *QBt.ifa-4BS* explained between 16.5% (MP-PR1, 2022) and 28.3% (MP-MM, 2015) of the total phenotypic variance, averaging 22.0% across experiments. In MP-PL, *QBt.ifa-4BS* explained a larger part of the total phenotypic variance than *QBt.ifa-6DL* in two out of three data sets, while *QBt.ifa-6DL* explained higher amounts of the total variation in all other MPs and experiments. *QBt.ifa-4BS* mapped to a region between 11.5 and 28.6 Mbp across MPs, spanning a maximum distance of 38 cM on the short arm of chromosome 4B flanked by markers *wsnp_BF483640B_Ta_2_2* and *AX-94707905*. Resistance improving alleles for these loci on chromosomes 4B and 6D descended from M822123 (MP-MM) or PI 166910 (all other MPs), respectively.

Apart from *QBt.ifa-6DL* and *QBt.ifa-4BS* that were associated with CBI in three or all four MPs, additional loci were detected in single MPs. QTL on chromosomes 1A (*QBt.ifa-1A*), 1B (*QBt.ifa-1B*) and 7B (*QBt.ifa-7B*, on LG 7.2) were identified in MP-PR2. *QBt.ifa-1B* was detected in all years and BLUEs across years and had the second largest effect on CBI levels in MP-PR2, while the loci on chromosomes 1A and 7B were significant in two out of four experiments. Both *QBt.ifa-1A* and *QBt.ifa-7B* had minor effects on CBI with an average of 6.1% (*QBt.ifa-1A*) and 14.2% (*QBt.ifa-7B*) of the total variance explained by the QTL. *QBt.ifa-1B* had a larger effect and explained 31.1% (2019) to 42.3% (2020) of the total phenotypic variance in MP-PR2. The interval estimate for *QBt.ifa-1B* varied between data sets, with the genetic distance spanned by the QTL ranging between 7.6-18 cM in 2019 and 31-34 cM in 2020 and corresponding to a region between 12.2 and 46.9 Mbp on the short arm of chromosome 1B. This region was flanked by markers *BS00004903_51* and *Tdurum_contig9762_314*. An additional QTL on chromosome 2A (*QBt.ifa-2A*) was identified in MP-MM. It was detected in two out of five experiments and was located on the proximal end of the short arm of chromosome 2A , flanked by markers *Tdurum_contig29983_490* and *AX-94381641*. *QBt.ifa-2A* had the second largest effect in MP-MM in 2020 and explained 26.5% of the total variation across data sets. *QBt.ifa-4BL* was only detected in MP-MM in 2016 and explained 10.2% of the phenotypic variance. The QTL spanned a distance of 14 cM on chromosome 4BL, corresponding to a region between 662.9 Mbp and 671.4 Mbp. Epistatic interactions were found for *QBt.ifa-6DL* with all other bunt QTL except for *QBt.ifa-1A* and *QBt.ifa-4BL* as well as between *QBt.ifa-1B* and *QBt.ifa-7B* (Fig. 2).

Flanking markers for all identified QTL in all mapping populations are provided in Online Resource 2.

Haplotype comparisons

A comparison of SNP haplotypes of the chromosomal region at and surrounding *QBt.ifa-6DL* was performed. For this purpose, the SNP allele calls of 21 wheat genotypes comprising the five parents of the MPs and 16 additional wheat accessions were compared side by side (Online Resource

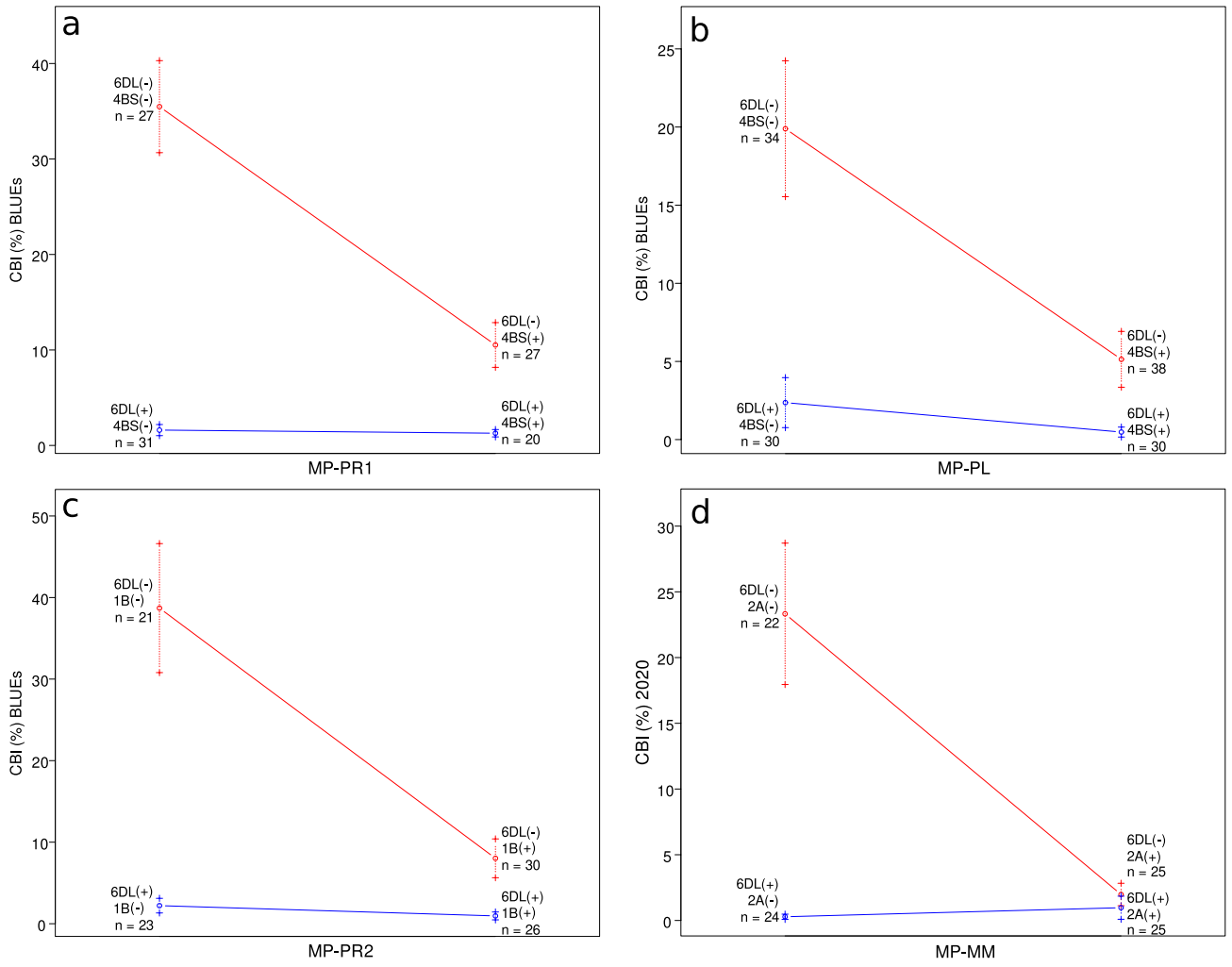

Fig. 2 Epistatic interactions between the two QTL with the largest effects in the individual mapping populations (MPs). **a** and **b** in the first row show epistatic interactions between *QBt.ifa-6DL* and *QBt.ifa-4BS* in MP-PR1 (**a**) and MP-PL (**b**). Interactions between *QBt.ifa-6DL* and *QBt.ifa-1BS* in MP-PR2 (**c**) and *QBt.ifa-2A* in MP-MM (**d**) are shown in the second row. All effectplots show data for BLUEs across experiments, except **d** which shows data from 2020 as *QBt.ifa-2A* was not detected in BLUEs across years in MP-MM. Standard errors are indicated by error bars and numbers next to the bars designate the number of lines harbouring the respective QTL(-combination)

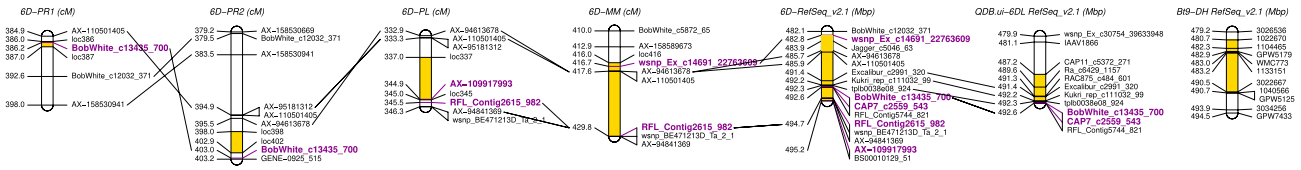

Fig. 3 Comparison of linkage maps for the distal end of chromosome 6D from our mapping populations (6D-PR1, 6D-PR2, 6D-PL and 6D-MM) with physical positions (Mbp) of the markers on the IWGSC RefSeq v2.1 (Zhu et al. 2021) (6D-RefSeq_v2.1), the physical positions (RefSeq v2.1) of markers in the linkage map for the 6D QTL identified by Wang et al. (2019) (QDB.ui-6DL RefSeq_v2.1) and markers to the 6D QTL published by Steffan et al. (2017) (Bt9-DH RefSeq_v2.1). Markers highlighted in magenta indicate peak markers in individual MPs: *BobWhite_c13435_700* in MP-PR1 and MP-PR2; *AX-109917993* in MP-PL; *wsnp_Ex_c14691_22763609* and *RFL_Contig2615_982* in MP-MM; *CAP7_c2559_543* for *QDB.ui-6DL*. Regions marked in yellow indicate the 6D QTL-region across experiments in the individual MP or study

Table 3 Pearson's correlation coefficients between heading date in days after April 30 (HD), plant height in centimeters (PH) and common bunt incidence levels in percent (CBI) of individual mapping populations

MP-PR1: 'Rainer' × PI 166910 (n = 122)			MP-PR2: PI 166910 × 'Rainer' (n = 122)		
	PH	CBI		PH	CBI
HD	0.62	− 0.12	HD	0.55	n.s.
CBI	− 0.25		CBI	− 0.19	
MP-PL: PI 166910 × 'Lukullus' (n = 162)			MP-MM: M822123 × 'Mulan' (n = 106)		
	PH	CBI		PH	CBI
HD	0.71	n.s.	HD	− 0.18	n.s.
CBI	− 0.19		CBI	− 0.13	

The number of lines in each population is given in brackets. Correlation coefficients were calculated based on best linear unbiased estimators across years. Non-significant correlations are indicated by n.s., all other correlation coefficients were significant at the $p < 0.001$ level

3). Four genotypes postulated to possess the *Bt11* allele, two genotypes with *Bt8*, three genotypes with *Bt9*, the *Bt10* differential line as well as five genotypes with different combinations of *Bt8*, *Bt9* and *Bt10* were included. In addition, we also compared haplotypes of six susceptible cultivars. Differences in haplotypes in the 6DL region were observed between *Bt9* genotypes and accessions harbouring *Bt11*, while no clear pattern was found for *Bt8*- and *Bt10*-lines. The SNP haplotype of PI 166910 in the *QBt.ifa-6DL* region was found in all the genotypes postulated to harbour *Bt11*, including the *Bt11* differential line.

Discussion

With a constant increase in organic or low-input farming systems and reports of resistance break-downs of common bunt resistant wheat cultivars, more and more stakeholders recognize that bunt pathogens should be considered in their agenda. In order to achieve sustainable bunt management that is successful in the long term, we regard the diversification of resistance sources available for applied breeding as essential. In this study, we therefore aimed at unlocking resistance loci originating from the Turkish landrace PI 166910 which was used to develop the differential line for bunt resistance gene *Bt11*. PI 166910 is postulated to carry three different *Bt*-genes: *Bt7*, *Bt9* and *Bt11* (Abdalla 1984). Goates (2012) called *Bt11* "the most difficult bunt resistance gene to overcome". PI 166910 therefore constitutes an ideal source for additions to the range of bunt resistance sources for wheat breeding.

High infection levels in the susceptible check cultivar 'Capo' in all experiments showed that artificial infection was successful and disease pressure in all trials was high (Table 1). Unusually high infection levels in the susceptible control as well as in some of the bunt differential lines in 2022 can be explained by two main factors: (1) weather conditions in the critical time period for field infections were ideal in autumn 2021 with dry and cool soil into which seed samples were sown (Borgen 2000; Johnsson 1992) (Online Resource 8); (2) a slightly different spore mixture was used for artificial inoculation of seed samples for 2022 field trials. The primary source for this spore mixture were infected spikes of the cultivar 'Tilliko' which was bred to be bunt tolerant (AGES 2022; RWA 2023). As bunt populations with the ability to overcome previously effective host plant resistance factors are of special interest for research projects, this inoculum source was multiplied and used as an infection source for field trials. Although it is not known what kind of genetic source confers the tolerance of 'Tilliko', a comparison with the standard bunt population used before 2021 shows that the inoculum collected from 'Tilliko' is especially virulent against the differential lines for *Bt2* and *Bt10* (Table 1).

Repeatabilities for individual experiments were high, as well as heritabilities across years for individual MPs (Table 2). The largest part of the total observed variation in CBI levels was due to the genetic component, while year effects were the main source of variation for PH and HD. Similar results were obtained by other mapping studies investigating bunt diseases in wheat, e.g. Muellner et al. (2020, 2021); Steffan et al. (2017) or Wang et al. (2019). Distributions of CBI levels were strongly right-skewed in all MPs with around three quarters of all RILs per MP showing less than 5% CBI (Fig. 1). Such distributions are indicative of two resistance factors—in our MPs these were *QBt.ifa-6DL* and *QBt.ifa-4BS*, *QBt.ifa-1B* or *QBt.ifa-2A*, respectively, acting together (Fig. 2, Online Resource 7).

QTL for common bunt resistance

QBt.ifa-6DL

Steffan et al. (2017) mapped bunt resistance gene *Bt9* to the distal end of wheat chromosome 6D using DArT markers

Table 4 Effect estimates and chromosomal locations in cM and Mbp as well as peak markers for common bunt QTL identified in individual mapping populations (MPs) using a forward-backward iteration for multiple QTL mapping

Experiment	Chrom.	Interval estimate		Peak position					Peak marker
		cM	Mbp[c]	cM	Mbp	Add[a]	PV (%)[b]	LOD	
MP-PR1[d] ($n = 105$)									
2019	4B	8.7–23	13.6–26.2	13.0	13.6	5.6	23.1	8.2	Excalibur_c64418_447
2020	— " —	8.7–23	13.6–26.2	1 6.0	13.6	6.8	24.1	8.5	— " —
2022	— " —	6–20	11.5–26.2	8.0	13.6	7.8	16.5	7.1	— " —
BLUEs	— " —	8–22	13.0–26.2	8.7	13.6	6.4	21.3	8.5	— " —
2019	6D	386–387	492.6	386.2	492.6	8.4	39.1	12.5	BobWhite_c13435_700
2020	— " —	386–387	492.6	386.2	492.6	9.6	38.6	12.4	— " —
2022	— " —	386–387	492.6	386.2	492.6	16.0	49.6	16.8	— " —
BLUEs	— " —	386–387	492.6	386.2	492.6	11.0	45.7	15.4	— " —
Epistatic Interactions				Add	PV%	LOD			
2019	4B:6D			5.5	10.8	4.2	46.5	14.3	Simultaneous fit
2020	— " —			6.7	10.6	4.1	46.5	14.2	— " —
2022	— " —			7.5	7.7	3.6	54.5	17.9	— " —
BLUEs	— " —			6.4	10.2	4.4	52.6	17.0	— " —
MP-PR2[d] ($n = 100$)	Chr	cM	Mbp	cM	Mbp	Add	PV%	LOD	Marker
2022	1A	65–162	549.1–94.8	126.1	500.3	5.7	5.4	4.0	wsnp_Ex_rep_c81556_76277906
BLUEs	— " —	116.8–165.4	515.2–355.2	158.0	403.9	5.2	6.7	4.2	AX-94404955
2019	1B	7.6–18	12.2–41.5	11.8	28.2	7.0	31.1	12.0	AX-158570920
2020	— " —	31–34	44.6–46.9	31	46.1	7.2	42.3	19.4	Tdurum_contig55639_241
2022	— " —	11.8–16	28.2–43.2	11.8	28.2	10.6	32.0	16.9	AX-158570920
BLUEs	— " —	14–18	28.2–43.2	11.8	28.2	8.6	33.9	15.9	— " —
2022	4B	3.8–184	9.2–664.0	28.0	19.0	4.7	3.6	2.7	tplb0035d20_506
BLUEs	— " —	18–205.8	15.0–671.0	28.0	19.0	4.4	5.0	3.2	— " —
2019	6D	398–400	485.7–492.6	403.0	492.6	9.0	39.3	14.3	BobWhite_c13435_700
2020	— " —	398–402	485.7–492.6	403.0	492.6	8.6	36.9	17.7	— " —
2022	— " —	400–402	485.7–492.6	403.0	492.6	16.0	49.1	22.4	— " —
BLUEs	— " —	398–400	485.7–492.6	403.0	492.6	11.4	44.3	19.1	— " —
2019	7B	108–132.9	26.4–7.1	116.5	12.8	4.4	6.8	3.3	AX-158593396
2020	— " —	118–122	12.8–10.1	121.7	10.1	4.3	21.6	12.0	AX-94810990
Epistatic Interactions				Add	PV%	LOD			
2019	1B:6D			8.3	16.8	7.3	57.9	18.8	Simultaneous fit
2020	— " —			6.6	9.6	6.1	70.6	26.6	— " —
2020	1B:7B			7.5	13.0	8.0			
2020	7B:6D			5.6	6.3	4.2			
2022	1B:6D			10.6	14.2	9.1	72.8	28.3	Simultaneous fit
BLUEs	— " —			9.8	17.7	9.7	68.7	25.2	— " —
MP-PL[d] ($n = 132$)	Chr	cM	Mbp	cM	Mbp	Add	PV%	LOD	marker
2019	4B	10–45	15.8–28.6	14.9	15.8	4.6	21.5	8.1	AX-94629926
2020	— " —	7–15	13.1–18.9	14.9	15.8	6.3	20.2	7.3	— " —
BLUEs	— " —	7–40	13.1–28.6	14.9	15.8	5.5	21.2	7.8	— " —
2019	6D	338–345	486.1–494.7	344.9	495.2	6.0	23.0	8.6	AX-109917993
2020	— " —	337–344.9	486.1–495.2	344.9	495.2	7.2	18.5	6.8	— " —
BLUEs	— " —	338–345	486.1–494.7	344.9	495.2	6.6	20.8	7.7	— " —
Epistatic Interactions				Add	PV%	LOD			
2019	4B:6D			5.0	9.2	3.7	34.4	12.1	Simultaneous fit
2020	— " —			6.0	7.5	2.9	30.3	10.3	— " —
BLUEs	— " —			5.5	8.4	3.4	32.6	11.3	— " —

Table 4 (continued)

Experiment	Chrom.	Interval estimate		Peak position					Peak marker
		cM	Mbp[c]	cM	Mbp	Add[a]	PV (%)[b]	LOD	
MP-MM[d] ($n = 96$)	Chr	cM	Mbp	cM	Mbp	Add	PV%	LOD	Marker
2016	2A	0–4	0.3–35.1	0.4	31.8	5.0	28.3	10.5	AX-94684111
2020	— " —	0–5	0.3–35.1	0.4	31.8	4.8	24.7	7.1	— " —
2015	4B	24–34	19.3–27.3	24.6	17.8	7.9	28.3	8.4	tplb0060n12_565
2016	— " —	116–130	662.9–671.4	124.0	671.4	5.9	10.2	4.4	AX-158542232
2015	6D	416–428	482.8–494.7	416.7	482.8	6.0	29.7	8.8	wsnp_Ex_c14691_22763609
2016	— " —	423–428	486–494.7	429.9	494.7	8.8	39.3	13.5	RFL_Contig2615_982
2020	— " —	424–429.9	486.1–494.7	429.9	494.7	6.9	29.7	8.3	— " —
2021	— " —	421–429.9	486–494.7	429.9	494.7	6.4	19.1	4.4	— " —
BLUEs	— " —	420–429.9	486–494.7	429.9	494.7	7.3	19.7	4.6	— " —
Epistatic Interactions				Add	PV%	LOD			
2015	4B:6D			7.5	13.4	4.4	43.1	11.7	Simultaneous fit
2016	2A:6D			7.7	18.1	7.3	56.7	17.5	— " —
2020	2A:6D			6.4	14.5	4.5	39.3	10.4	— " —
2021							19.1	4.4	— " —
BLUEs							19.7	4.6	— " —

The number of lines in each MP is given in brackets. Rows showing 'simultaneous fit' in the last column indicate the amount of phenotypic variance (column 8) and the LOD score (column 9) for the respective experiment (column 1) if all QTL significant in the data set were fit together

In 2022, a spore mixture with different virulence was used for artificial inoculation

[a]Positive additive effects indicate a decreasing effect of the resistance-conferring allele

[b]Percentage of phenotypic variance explained by the respective QTL

[c]markers closest to the cM locations of the estimated borders for each QTL interval were used to determine interval regions in Mbp

[d]PR1 = PI 166910 × 'Rainer'; PR2 = 'Rainer' × PI 166910; PL = PI 166910 × 'Lukullus'; MM = M822123 × 'Mulan'

(SNPs and presence-absence variants) in a doubled haploid (DH) population resulting from the cross PI 554099 (*Bt9*-differential) × 'Cortez'. This *Bt9*-locus explained between 37.7 and 53.7% of the phenotypic variation for CBI in their trials. Wang et al. (2019) identified a QTL conferring dwarf bunt resistance in a DH population derived from a cross between IDO835 (a resistant breeding line) and 'Moreland' (susceptible cultivar (Souza et al. 2004)). The QTL was located on chromosome 6DL and explained 17–53% of the phenotypic variation in dwarf bunt incidence in their study. Resistance in these DH lines originated from UT944157, a sib-selection to the highly resistant cultivar 'Golden Spike' (Chen et al. 2018). We obtained physical positions according to IWGSC RefSeq v2.1 (Zhu et al. 2021) for markers to the 6DL loci published by Steffan et al. (2017) and Wang et al. (2019) via GrainGenes BLAST (Yao et al. 2022) (available at https://wheat.pw.usda.gov/). For markers to *QDB.ui-6DL* identified by Wang et al. (2019), FASTA sequences were obtained from JBrowse at the Wheat@URGI portal (Alaux et al. 2018) (available at https://urgi.versailles.inra.fr/jbrowseiwgsc/gmod_jbrowse/?data=myData/IWGSC_RefSeq_v1.0) before blasting them on GrainGenes. These most recent physical positions for the two previously published 6DL loci were compared to the QTL region of *QBt.ifa-6DL* and visualized in Figure 3. Both Wang et al. (2019) and Steffan et al. (2017) suggest that the QTL they found on 6DL corresponds to *Bt9*. *QBt.ifa-6DL* detected in our experiments is located very close to or partially overlapping with these two previously identified 6D loci (Fig. 3, Online Resource 3). The exact location cannot be determined in our MPs as polymorphic markers at the distal end of chromosome 6D are scarce. In MP-PR1 and MP-PR2, the most distal marker according to physical positions in RefSeq v2.1 was *BobWhite_c13435_700*. Figure 3 shows other markers mapping to positions beyond *BobWhite_c13435_700* in the linkage maps for MP-PR1 and MP-PR2, but these are possibly incorrectly ordered in the linkage group when compared to physical positions. As no polymporhic SNPs are available beyond the 492.6 Mbp-position, it cannot be determined whether the LOD-peak would appear at a more distal locus in MP-PR1 and MP-PR2 if more polymorphisms were available, or if *BobWhite_c13435_700* would still remain the peak marker in that case. Such a shift of the LOD peak to a more distal position in MP-PR1 and MP-PR2 seems plausible because of the peak marker locations in MP-PL (495.2 Mbp) and MP-MM (494.7 Mbp). Nevertheless, *BobWhite_c13435_700*

is neither polymorphic in MP-PL and MP-MM, nor are polymorphic markers at very similar physical positions available in the linkage maps. In consequence, we hypothesize that LOD peaks would appear in more similar positions in the four MPs with a higher density of polymorphic SNPs at the distal end of 6DL. This marker scarcity at the distal chromosome end of 6D is a common problem, as Wang et al. (2019) similarly found no polymorphic markers in their MP beyond 492.6 Mbp on chromosome 6D (Jianli Chen and Pabitra Joshi, personal communication). Interestingly, Wang et al. (2019) described that their resistance donor is a sib-selection to the bunt resistant cultivar 'Golden Spike' (Hole et al. 2002a) and has the resistance conferring allele for their 6DL-2 marker (*Cap7_c2559_543*). They state that 'Golden Spike' has the *Bt9* resistance according to B.J. Goates, but no empirical evidence for this gene postulation is available to our knowledge. Nevertheless, it seems plausible based on the 'Golden Spike' pedigree and is supported by a matching haplotype of 'Golden Spike' with the haplotypes of the *Bt9*-differential, PI 554099 and Golden Spike's resistance donor, PI 178383, in the *QDB.ui-6DL* region (469.83 Mbp - 471.02 Mbp based on IWGSC RefSeq v1.0) mapped by Wang et al. (2019) (Online Resource 3). PI 166910, the resistance donor for both our MPs in which *Cap7_c2559_543* and *BobWhite_c13435_700* are polymorphic and segregating, has the contrasting alleles to 'Golden Spike' (Online Resource 3) for these markers. This allele contrast can be interpreted as an indication that, as hypothesized above, the true peak location for *QBt.ifa-6DL* could actually be located in a more distal position in MP-PR1 and MP-PR2 and *BobWhite_c13435_700* was only identified as peak marker because it was the most distal SNP available in these MPs. In conclusion, PI 166910 most likely does not harbour *Bt9* since it has the susceptible alleles for the *Bt9*-markers identified by Wang et al. (2019). The causal gene for its resistance could be located at the more distal position on 6DL indicated by MP-PL and MP-MM. When comparing haplotypes in the 6DL region, it is striking that all genotypes postulated to harbour *Bt11* show a distinct allele pattern for markers between 494.58 Mbp and 494.69 Mbp which is different from all genotypes indicated to have the *Bt9* allele (Online Resource 3). Based on SNP markers on the 25K array, no universal functional markers for *QBt.ifa-6DL* were found. Therefore, for breeding purposes in new populations, parental testing and choosing appropriate selection markers is necessary. We recommend searching for markers in the potential *Bt11* region on the distal end of chromosome 6DL flanked by markers *BS00070856_51* and *AX-94841369*. According to the haplotypes of the resistance donors of our four MPs, PI 166910 and M822123 (PI 554119), we propose *Bt11* as the most likely causal gene underlying *QBt.ifa-6DL*. The fact that *QBt.ifa-6DL* is partially overlapping with the loci identified by Wang et al. (2019) and Steffan et al. (2017) does not necessarily contradict this hypothesis but could be explained by the coarse mapping resolution on 6DL resulting from marker scarcity. Our data suggest that *Bt9* and *Bt11* comprise either two genes in close neighborhood or two different alleles of the same locus on the distal end of chromosome 6DL. Except for accession PI 211657, all *Bt11*-lines listed in Online Resource 3 descend from PI 166910. Its genetic background can be expected to be present in all our RILs because M822123 is a cross between PI 166910 and 'Elgin'. PI 166910 is also designated to harbour *Bt7*, but we can exclude this resistance type as the underlying factor for *QBt.ifa-6DL* since *Bt7* is not active against our inoculum (Table 1). Another source for bunt resistance that has been identified on wheat chromosome 6D is *Bt10*. Menzies et al. (2006) mapped *Bt10* to the short arm of 6D, which was confirmed in a study by Singh et al. (2015) investigating offspring from the cross 'Carberry' × 'AC Cadillac'. This 6DS locus found in both studies is distinct from *QBt.ifa-6DL* and maps to a different location on the chromosome.

QBt.ifa-4BS

The QTL region for *QBt.ifa-4BS*, identified in all MPs, is partially overlapping with *QBt.ifa-4B* published by Muellner et al. (2020) (20.6–706.5 Mbp vs. 11.5–28.6 Mbp in our study). Similar to our results, Muellner et al. (2020) also had difficulties narrowing down the QTL region on the 4B chromosome as shown by the large physical interval. In their MPs, *QBt.ifa-4B* was detected in two out of five data sets (2015 and 2016) and explained 10.8 and 11.2% of the phenotypic variance. Interestingly, in MP-MM *QBt.ifa-4BS* was also only detected in data from 2015 and 2016 in our study. Across all MPs, it explained between 10.2 and 24.1% of the total variance in CBI. While *QBt.ifa-4B* was classified as a minor QTL by Muellner et al. (2020) which was not verified in their validation populations, it had a larger effect on CBI in all MPs except for MP-PR2 in our study. In MP-PL, the effect of *QBt.ifa-4BS* was even larger in some years than the effect of *QBt.ifa-6DL*, which was the main resistance source in all other MPs. We also observed epistatic interactions between *QBt.ifa-4BS* and *QBt.ifa-6DL* which was not the case in Muellner et al. (2020). Singh et al. (2015) also mapped a QTL conferring common bunt resistance to a region on the short arm of chromosome 4B which is overlapping with *QBt.ifa-4BS*, but they detected a significant effect of this locus in a single year only. The 4B locus explained 7.6% of the phenotypic variation in that year, its effect being thereby considerably smaller compared to *QBt.ifa-4BS*.

QBt.ifa-1B

In MP-PR2, which consists of RILs from a cross between 'Rainer' and a subline of PI 166910, we found a locus conferring resistance to common bunt on the short arm of chromosome 1B. This QTL had a strong effect on CBI in MP-PR2, explaining between 31.3 and 42.3% of the total phenotypic variation. In 2019, its effect was even stronger than the one of *QBt.ifa-6DL*. In 2022, RILs from MP-PR1 and MP-PR2 were tested with a different, more aggressive inoculum, which led to a higher number of susceptible RILs (Fig. 1). The increase in RILs with CBI levels above the 5%-threshold was lower in MP-PR2 compared to MP-PR1. Possibly, *QBt.ifa-1B*, due to its strong effect on CBI, together with *QBt.ifa-6DL* led to a more stable resistance in MP-PR2 RILs compared to lines in MP-PR1. The effect of *QBt.ifa-4BS* as an additional resistance source besides *QBt.ifa-6DL* in MP-PR1 RILs was weaker than the effect of *QBt.ifa-1B* which might be an explanation for the difference observed between the two MPs in 2022 (Online Resource 7). A locus conferring resistance to CBI in a similar region as *QBt.ifa-1B* was also found by Singh et al. (2015) in two out of three data sets included in their study. It explained 5 and 18% of the total phenotypic variation, respectively, and showed epistatic interactions with other bunt resistance loci detected on chromosomes 4B and 6D. Common bunt resistance QTL in overlapping or neighbouring locations to *QBt.ifa-1B* were also identified by Fofana et al. (2008), Dumalasová et al. (2012) and Muellner et al. (2021). In all three studies, QTL on other chromosomes were also detected but the 1B-locus had the largest effect on CBI levels. As *QBt.ifa-1B* was only detected in MP-PR2 but not in any other MP with PI 166910 as the resistant parent, we conclude that it is an additional resistance factor present only in this specific subline used as parent in this cross, but not for MP-PR1 or MP-PL.

Additional QTL

QBt.ifa-2A was the second largest effect QTL in MP-MM and located at the very proximal end of the chromosome arm. Bokore et al. (2019) found markers on chromosome 2A associated with CBI, but their QTL was located at 746 Mbp and is therefore not corresponding to the region we identified. To our knowledge, no other study investigating common bunt resistance has detected QTL on chromosome 2A, so we conclude that *QBt.ifa-2A* represents a new bunt resistance source, possibly specific to M822123.

A minor effect QTL close to the centromere of chromosome 7B was detected by Dumalasová et al. (2012) using SSR markers. Blasting the publicly available markers against the latest wheat reference sequence yielded physical positions between 417 and 544 Mbp on chromosome 7B, though, leading to the conclusion that the QTL detected by Dumalasová et al. (2012) is not corresponding to *QBt.ifa-7B*. Mourad et al. (2018) detected a significant marker-trait-association between common bunt resistance and a SNP at 18.1 Mbp on chromosome 7B in their genome-wide association study. This marker had an allele effect of −0.17 and its position is overlapping with the QTL interval for *QBt.ifa-7B*. Both Dumalasová et al. (2012) and Mourad et al. (2018) report that none of the known bunt resistance genes has been mapped to the short arm of chromosome 7B and this is still true in 2023 according to our literature research.

Loci conferring bunt resistance in regions corresponding to *QBt.ifa-1A* have been identified in previous studies. Ehn et al. (2022) found significant marker-trait associations of two SNPs at 473.97 Mbp on chromosome 1A with common bunt incidence in a GWA study conducted on a diversity panel. Muellner et al. (2021) investigated both common and dwarf bunt resistance in their study and identified a QTL at 380.97–516.67 Mbp (based on IWGSC RefSeq v1.0 (Appels et al. 2018)) on chromosome 1A which was effective against both diseases. A locus conferring dwarf bunt resistance in this region was also mapped by Chen et al. (2016), indicating that *QBt.ifa-1A*, despite its comparably small effect in MP-PR2, may contribute to resistance against both bunt diseases.

Conclusion

The Turkish landrace PI 166910 has been described as a source of efficient bunt resistance which is only overcome by very few of the currently known isolates (Goates and Bockelman 2012). Goates (2012) lists only two isolates of dwarf bunt that show virulence against the resistance factor of PI 166910, *Bt11*, in his experiments. We confirm the high and stable resistance of this wheat accession in our study. Large proportions of lines in all MPs showed complete resistance in trials across six years in total and against two different local common bunt inocula. A QTL on the long arm of chromosome 6D designated *QBt.ifa-6DL* was identified in all MPs and all data sets. It had a consistently significant and decreasing effect on CBI and showed epistatic interactions with additional QTL on other chromosomes. The obtained data combined suggest that *QBt.ifa-6DL* corresponds to the bunt resistance factor *Bt11* postulated to be present in the resistant crossing partners of our MPs. Based on the evidence we collected, *QBt.ifa-6DL* is likely different from *Bt9* mapped by Steffan et al. (2017) and Wang et al. (2019), although a final proof is not yet possible due to sparse marker polymorphisms on the distal end of 6DL. The provided lines and SNP markers between 492.6 and 495.2 Mbp on chromosome 6D pave the way to deploy the promising allele *QBt.ifa-6DL—Bt11* in bunt resistance breeding through marker assisted selection.

PI 166910 inherits additional resistance loci, notably *QBt.ifa-4BS* and *QBt.ifa-1BS*, which contribute to the high and robust bunt resistance response of this accession and its descendants.

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Author contribution statement HB conceptualized and supervised the study, generated the populations and edited the original draft. Data collection in 2020, 2021 and 2022, and data analysis was done by ML, who also wrote the original draft. AEM provided data from years 2015 and 2016. IF carried out data collection in 2019. MB supervised linkage map construction and edited the original draft. HG reviewed and edited the original draft and acquired funding. All authors read and approved the manuscript.

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Data availability All data generated during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest Hermann Buerstmayr is member of the editorial board of Theoretical and Applied Genetics. The authors declare no conflicts of interest.

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5 Publication 3

Common bunt in organic wheat: unvravelling infection characteristics relevant for resistance breeding

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- Hermann Buerstmayr supervised marker-assisted selection and edited the original draft
- Heinrich Grausgruber organised funding, curated the data, conceptualized and supervised the study and edited the original draft

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Abstract

After decades off the radar of breeders and producers, common bunt has re-emerged as a major threat to wheat yield and quality, especially in organic farming. Resistance against its causal agents *Tilletia tritici* and *T. laevis* is present in the wheat gene pool and can be deployed in resistance breeding. Molecular markers for resistance loci help to accelerate the time-consuming breeding process and are therefore crucial for the rapid development of resistant cultivars. These pose the most economically efficient and sustainable way to combat the disease since seed treatments approved for organic farming are rare and do not provide full protection. Many aspects of bunt infection characteristics are still unknown or have not been addressed since the mid-20th century when seed treatment was not yet routine. We tested a winter wheat diversity panel with 128 lines for common bunt resistance in Austria and Czechia, and evaluated the applicability of marker-assisted selection (MAS) via Kompetitive Allele-Specific PCR (KASP) markers in genotypes with high variation in their genetic background. Field trials were conducted across two years and artificially inoculated with local bunt populations. The virulence patterns of these inocula differed between locations and only 15% of the tested genotypes showed stable resistance across test sites. Number and weight of bunt sori relative to the total number and weight of wheat grains in sampled ears revealed that partial infections of ears were frequently appearing. Forty-two breeding lines harbouring combinations of four different resistance QTL were developed through MAS. Out of these, a quarter was resistant with a maximum of 5% common bunt incidence. We thereby showed that MAS is a useful tool to speed up selection of resistant breeding lines even in populations with highly diverse genetic backgrounds in which the availability of informative markers may become scarce. MAS is efficient in pyramiding resistance loci and thereby improving the level of resistance as shown by lines with multiple resistance loci having significantly lower disease incidence. Only six out of 46 tested commercial cultivars and breeding lines showed no infection with common bunt, underlining the present scarcity of bunt resistant cultivars for organic wheat production.

1. Introduction

Common bunt of wheat caused by *Tilletia tritici* (Bjerk.) G. Winter (also called *Tilletia caries* (D.C.) Tul. & C. Tul.) and *T. laevis* J.G. Kühn (also called *T. foetida* (Wallr.) Liro) is experiencing a come-back on the fields after decades off the radar of researchers, breeders and farmers. The causal agents of this fungal disease belong to the division of the Basidiomycota and show differences in teliospore morphology. Despite this phenotypic variation, genetic studies suggest that *T. tritici* and *T. laevis* might be the same species (Carris, 2010; Sedaghatjoo et al., 2022). They also have identical life cycles with teliospores germinating at temperatures between 5°C and 20°C, relatively independent of light conditions (Lowther, 1950). This characteristic enables common bunt fungi to infect wheat seedlings also in the absence of continuous snow cover which is required for successful dwarf bunt (*T. controversa*) infections (Gassner and Niemann, 1954). Common bunt can therefore occur in both autumn- and spring-sown wheat given that temperatures after sowing are conducive for infection of the young seedlings (Goates and Bockelman, 2012). Optimum infection temperatures occur between 5°C and 10°C according to Hoffmann and Schmutterer (1983) while Johnsson (1992) narrowed down the ideal temperature interval to 6-7°C. Especially the first ten days after sowing were a critical period for bunt infections in his field experiments conducted in Sweden. If environmental conditions were suitable during these first few days, bunt infections were high while temperatures, precipitation or snow cover after the initial ten days had no influence on infection levels (Johnsson, 1992). Hansen (1959) conducted experiments in controlled conditions in the greenhouse and found lower sensitivity of common bunt spores to environmental temperatures. In her study, fungal hyphae were able to penetrate seedlings already four days after inoculation both at 3°C and 15°C. This highlights that the crucial period for bunt infections is restricted to a short time after seedling emergence. Even though the main inoculum source is usually contaminated grain, common bunt teliospores are also able to remain viable in the soil for years and thereby cause soil-borne infections of clean, healthy grain (Johnsson, 1990; Borgen, 2000; Goates and Bockelman, 2012). For this type of infections, the proximity between bunt spores and wheat grain is essential. Only if teliospores are within one-centimetre distance from sown grains, infection can occur (Johnsson, 1990). In an experiment conducted under standard farming practices, Borgen (2000) observed higher infection levels resulting from soil-borne teliospores two years after inoculum was introduced into the soil compared to the first year. He concluded that this rise in infection levels was likely caused by teliospores being buried too deep in the soil by ploughing in the first year but being ploughed up again in the second year, resulting in closer proximity to the sown grains. In his multi-year experiments, Borgen (2000) observed that resting common bunt spores are able to survive under the plough layer and remain viable enough to have practical implications under organic conditions for at least five years. Increased use of untreated seeds and minimum tillage practices are therefore boosting soil- and seed-borne diseases like common bunt if prevention measures such as appropriate hygiene in seed production, good crop rotation and cultivation of resistant varieties are neglected.

Resistance against bunt diseases is naturally occurring in the wheat gene pool but resistance genes are often found in landraces or non-adapted exotic genotypes. Based on phenotypic evaluation of reactions to different bunt races, a set of wheat differential lines harbouring distinct types of resistances has been assembled by Hoffman and Metzger (1976) and extended by Goates (2012). For a long time, resistance to common bunt was seen as being only qualitative and based on gene-for-gene interaction (Hoffman and Metzger, 1976; Goates, 1996; Goates and Bockelman, 2012) but during recent years, also quantitative resistances have been identified (Wang et al., 2009; Dumalasová et al., 2012; Muellner et al., 2021). To make both resistance genes and quantitative trait loci (QTL) available for applied breeding, molecular markers for the selection of the respective chromosomal regions are essential. Kompetitive allele-specific PCR-markers (KASP-markers)

provide a fast and easy method for screening large numbers of lines for the presence of resistance loci. Such KASP-markers have been developed and published for a range of bunt resistance sources (Wang et al., 2019; Muellner et al., 2020; 2021). As chromosomal positions and markers for selection of more and more resistance loci become available, bunt resistance is being re-considered as a breeding goal in several wheat breeding programs, especially in those focused on organic farming.

Common bunt causes not only losses in grain yield through the replacement of grains by so-called 'bunt balls' (i.e. sori filled with fungal teliospores), but also deteriorates end-use quality by the typical rotten fish-like odour caused by trimethylamine, a volatile compound present in the teliospores (Hanna et al., 1932). Already low infection levels – Canadian studies mention 0.1% by volume and/or 0.05% by weight (Laroche et al., 2000; Menzies et al., 2006) – allow olfactory assessments as a mean for common bunt detection (Börjesson and Johnsson, 1998). Another aspect of the typical bunt balls that has been discussed in a few works published in the mid-20th century are partial infections of wheat kernels (Sampson, 1927; Gieseke, 1929; Gassner, 1938; Hansen, 1959). Information about this phenomenon is, according to our literature study, not found in any more recent publications on bunt diseases. Gassner (1938) questioned the until then widely accepted hypothesis that infections occurred through the ovules. Instead, he concluded from extensive microscopic analysis of partially bunted kernels that ovules remained intact in partially infected grains but that they were seriously inhibited in their development and only ultimately replaced in cases of fully bunted kernels. Partially infected kernels were also investigated by Hansen (1959) who described that the pericarp was for the largest part replaced by bunt spores while endosperm and embryo were free from fungal cells. While Gassner (1938) considered fully bunted kernels the final stage of a transition from partial to full infections, Hansen (1959) assumes that the difference between fully and partially infected kernels is that only in the latter, successful pollination had occurred, leading to the development of embryo, endosperm and seed coat. Such partially infected kernels, mixed with completely healthy ones in a single ear, are hard to detect in a wheat field. Fully bunted ears can be spotted with a little experience and training because of their modified appearance. They are usually shorter and spikelets are spread apart so that ears appear both flattened and stilted. If only partial infections occur, these symptoms are a lot harder to recognize or ears might even look completely healthy from the outside. Field trials with partially infected grains proved that the patches of bunt teliospores present inside otherwise healthy-looking kernels with unspoiled embryos were able to infect the seedlings emerging from these seeds. On the other hand, the removal of partially bunted grains from the seed lot via mechanical separation or washing was not possible (Gassner, 1938). These investigations were already conducted decades ago, but their conclusions can still be taken as valid today.

In order to add to the rather scarce knowledge about partial bunt infections, we wanted to study (i) whether partial infections occur in a diversity panel composed of multi-parent breeding lines and European cultivars, (ii) how measures for phenotypic evaluation of partial bunt infections were correlated to standard qualitative scoring of common bunt incidence, (iii) how common bunt infections in our panel differed between test locations in two European countries using different inocula, and (iv) whether marker-assisted selection can be applied as a tool for screening multi-parent breeding lines for bunt resistance QTL.

2. Materials and Methods

2.1 Plant material

A panel of 128 genotypes was assessed for different aspects of common bunt infection. A full list of all genotypes is available in Supplementary Table S1. The panel comprised 67 multi-parent winter wheat breeding lines developed at the Institute of Plant Breeding, BOKU, Tulln. The bunt resistance sources for these breeding lines were, on the one hand, three donors with mapped resistance loci, i.e. the differential line for bunt resistance gene *Bt12*, PI 199333 (Muellner et al., 2020) and the two cultivars 'Blizzard' and 'Bonneville' (Muellner et al., 2021). On the other hand, registered cultivars with unmapped bunt resistances were used. The donor lines were crossed to cultivars registered in various European countries provided by partners from the ECOBREED project. Depending on the number of crosses, each breeding line comprised between two and ten different genotypes in its pedigree. In addition, a set of 46 registered cultivars and commercial breeding lines originating from different countries was included in the test panel to evaluate the presence of bunt resistance in breeding programs across Europe. For monitoring virulence of the applied bunt inocula across years, we also included the bunt differential set consisting of 14 wheat accessions each indicative for one of the known bunt resistance types (*Bt1* to *Bt13*, plus *BtP*) (Goates, 2012). Genotypes for *Bt14* and *Bt15* were excluded as these are tetraploid durum wheats. Instead, we included the susceptible controls 'Heines VII' (*Bt0*) and 'Capo'.

2.2 Field trials

Artificially inoculated field trials were conducted in two locations in Austria and the Czech Republic. The experimental site in Austria was located in Tulln (48°19'05''N, 16°04'10''E) at an elevation of 177 m a.s.l. Mean annual temperature and precipitation in 2021 and 2022 were 10.2°C and 11.2°C, and 450 and 504 mm, respectively. Seed samples were artificially inoculated before sowing using a suspension of common bunt teliospores in a solution of 2% methylcellulose in water following a protocol adapted from Goates (1996) and Muellner et al. (2020). Teliospores were extracted from infected wheat ears harvested in field trials of the previous seasons, cleaned from all plant residues and stored in a dry place at room temperature. When harvesting the infected ears, a wide range of medium infected genotypes (20-50% infection) was used as spore sources to avoid unintended selection and to make sure that the inoculum represented the local bunt population. The spore suspension for artificial inoculation was applied in a concentration of 0.09 g of spores (= 0.3 mL of spore suspension) per 10 g of seeds and distributed onto the seeds by shaking. Double-rows of 1.6 m length and spaced 25 cm apart were sown in the first two weeks of November. In 2021 and 2022, 98 and 84 genotypes were tested in Tulln, respectively. Herbicide treatment and fertilizer applications were carried out following standard agricultural practices. The experiments were laid out as augmented designs with two replicates for check cultivars and unreplicated test entries in both years.

Experimental fields in the Czech Republic were located at the Crop Research Institute in Prague-Ruzyne (50°05'05''N, 14°17'58''E) at 280 m a.s.l. Mean annual temperatures in 2021 and 2022 were 9.1°C and 10.1°C, respectively. Annual precipitation was 835 and 867 mm in the two test years, respectively. Seed samples were inoculated by shaking 250 seeds of each genotype together with 0.1 g of common bunt teliospores in an Erlenmeyer flask by hand for one to two minutes. Teliospores originated from a mixture of two Czech common bunt samples that were collected in 2014 and re-inoculated since then on the susceptible variety 'Heines VII'. Field plots were sown by hand in mid-October as double-rows of 1 m length and spaced 20 cm apart. In 2021, 55 genotypes were tested and in 2022, 60 genotypes were tested. Weed removal in the field experiments was done by hand; no

fertilizer or pesticides were applied. The trials were laid out as unreplicated randomized designs in both years.

2.3 Disease scorings

Common bunt infections (CB) were scored as disease incidence in 150 randomly selected ears per plot (Austria) or all ears per plot (Czech Republic) and the results were converted to percentages. The different number of scored ears between the two locations was due to the smaller plot size in Czechia, resulting in less than 150 ears for some plots. Ears were cut open and recorded as infected if a single bunt ball was spotted. If an ear was not obviously completely infected, a diagonal cut was first applied in the upper third of the ear and then a second diagonal cut was performed in the lower third of the ear to ensure that partial infections would be recognized. Scoring was done at the time of ripening between growth stages BBCH 80 and 89 in June and July.

In the Austrian field trials, 50 randomly chosen, non-cut ears were harvested from each plot after incidence socring and subjected to further analyses. First, the number of bunt sori (BS) relative to the total number of ovules in the ear (i.e. healthy kernels plus bunt sori) was determined by manually removing all grains and bunt balls from wheat spikes. Bunt sori were then weighed and their weight relative to the total yield of the ear (i.e. healthy kernels plus bunt sori) was assessed (WBS). BS and WBS were determined on 82 of the 98 genotypes tested in 2021 and on 66 of the total 84 genotypes tested in 2022.

2.4 Marker-assisted selection in multi-parent breeding lines

Marker-assisted selection (MAS) for known bunt resistance QTL (quantitative trait loci) was applied in the development of 42 out of the total 57 multi-parent breeding lines using Kompetitive Allele-Specific PCR (KASP) markers. Selection was carried out for four loci on chromosomes 1A, 1B, 7A and 7D which were mapped in the bunt resistant cultivars ‘Blizzard’ and ‘Bonneville’ by Muellner et al. (2021). ’Blizzard’ was present in the pedigree of all 42 lines and 4 lines additionally contained ’Bonneville’ as a parent. The 42 breeding lines originated from ten crosses. These ten crosses were conducted between nine pre-selected breeding lines, themselves originating from either three-way or four-way crosses, which harboured bunt resistance loci in heterozygous allelic states. The pre-selection of these nine lines based on their heterozygosity at the resistance loci was carried out using 14 KASP markers published by Muellner et al. (2021). Progeny from the ten crosses between the heterozygous breeding lines had complex pedigrees consisting of up to ten different genotypes. As this led to a loss of polymorphism for some of the KASP markers applied in the pre-selection, a slightly different set of markers with similar physical positions had to be used for MAS in the multi-parent breeding lines (Supplementary Table S2). We generally aimed to use markers at flanking positions of the QTL regions to achieve good selection accuracy. The full list of markers used for MAS in each genotype is available in Supplementary Table S3.

Prior to the process of screening all lines with KASP markers, DNA was extracted from fresh leaf samples of eight to 14 plants per cross following a protocol adapted from Saghai-Maroof et al. (1984). DNA concentrations were normalized to 50 ng μL^{-1} and PCR reactions were carried out following the protocol for KASP PCR provided by LGC Biosearch Technologies (Berlin, Germany). Allelic discrimination results were obtained by reading fluorescence signals with a CFX384 TM Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA).

2.5 Data Analysis

All statistical analyses were carried out in R (R Foundation for Statistical Computing, Vienna, Austria). Correlations between trials were calculated using Pearson's correlation coefficient. Analysis of variance (ANOVA) was carried out for individual locations separately using a model of the form

$$P_{ij} = \mu + G_i + E_j + GE_{ij} + e_{ij}$$

where P_{ij} is the phenotypic value observed for the respective trait, μ is the grand mean, G_{ij} is the genotype effect of the i^{th} line, E_j is the effect of the j^{th} environment (i.e. year), GE_{ij} is the genotype-environment interaction of the i^{th} genotype with environment j and e_{ij} is the residual effect. For analysis across both locations, the model was extended to

$$P_{ij} = \mu + G_i + E_j + L_k + GE_{ij} + GL_{ik} + EL_{jk} + e_{ij}$$

where L_k is the effect of location k, GL_{ik} is the interaction effect between genotype i and the k^{th} location and EL_{jk} is the interaction between the j^{th} environment and location k. All effects were modeled as random except for the grand mean, which was treated as a fixed effect. Models were fit using R package *breedR* (Muñoz and Sanchez, 2020) with the *remlf90* function. Broad-sense heritability ('operative heritability') was calculated following Strube (1967) as

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{\sigma_{GxE}^2}{n_E} + \frac{\sigma_e^2}{n_E}}$$

with σ^2 as the genotypic variance, σ_{GxE}^2 as the genotype-environment interaction, σ_e^2 as the residual variance and n_E as the number of test locations.

3. Results

3.1 Phenotypic evaluation of common bunt infections

Differential set and cultivars

To monitor the virulence spectrum of the applied inoculum, the bunt differential set consisting of 14 differential lines plus two susceptible controls was tested in both locations (Table 1). Inocula used for artificial infection showed different virulence patterns against the *Bt*-genes represented in the differential set between Austria and the Czech Republic. The Austrian inoculum was not virulent (0-1% CB) against *Bt1*, *Bt8*, *Bt11* and *Bt12* and showed low aggressiveness against *Bt4*, *Bt5*, *Bt6* and *Bt9* (1-10% CB) across two years (2021-2022). Infection levels were generally elevated in 2022 compared to 2021 in Austria. Qualitative differences were observed for *Bt5* and *BtP* with differential lines for these two genes being resistant in 2021 but infected in 2022. In the Czech Republic, the inoculum was avirulent to *Bt8*, *Bt9*, *Bt10*, *Bt12*, *Bt13* and *BtP* and showed low aggressiveness against *Bt11* in 2021. The bunt differential set was not tested in the Czech Republic in 2022.

Out of the 46 commercial cultivars in the panel, five (i.e. ‘Aristaro’, ‘Blizzard’, ‘Bonneville’, ‘Deloris’, ‘UI SRG’) showed resistance to common bunt across years and/or locations with up to 5% infection and one cultivar (i.e. ‘Unitar’) had up to 10% incidence. All other cultivars were moderately to highly infected (Figure 1, Table S1).

Evaluation traits of common bunt infections

High variation was observed in the levels of CB in both test locations (Table 2). CB ranged between 0 and 98% in the Austrian field trials and between 0 and 91.5% in the Czech experiments. Based on scorings in 2021, multi-parent breeding lines and cultivars that showed elevated infection levels and therefore did not qualify as interesting material for resistance breeding were excluded from the panel to enable testing of additional breeding lines and cultivars in 2022 (Figure 1). This led to a lower mean CB in both locations in 2022 because many highly susceptible cultivars were eliminated from the trials in this year. This down-shifting of average infection levels occurred also in Austria although CB was generally elevated by approximately 50% in 2022 due to environmental conditions highly favourable for bunt infections as obvious from higher CB values in the bunt differential set (Table 1). High variation was observed between the difference of CB to BS scores of individual genotypes, ranging from –24.9% to 27.7% in 2021 and between -5.1% and 48.3% in 2022 (Table 2). The negative relationships between CB and BS in 2021 were primarily due to four cultivars (i.e. ‘Alessio’, ‘Sheriff’, ‘Tillexus’ and ‘Tillstop’) which had high levels of CB but the number of sori relative to the total number of grains was even higher. These four cultivars were excluded from the 2022 trials. On average, CB scores were 4.1% (2021) and 7.4% (2022) higher than BS scores and 14.6% (2021) and 12.8% (2022) higher than WBS scores. While BS was on average 10.3% higher than WBS in 2021, this ratio dropped to 5.4% in 2022.

3.2 Heritabilities and trait correlations

ANOVA results showed that the largest part of the total phenotypic variation in CB as well as in BS was explained by the genotype if data were analysed for each location separately (Table 3). For WBS, the residual component explained the largest part of the total phenotypic variance, followed by the genotype by environment interaction and the genotypic variance. If analysis was performed across trial sites, the largest part of the variation was also accounted for by the residual variance,

followed by the interaction of year (environment) and location. Broad-sense heritability estimates were highest for CB (H^2 = 0.68 in Czech trials and H^2 = 0.63 in Austrian trials) and lower for BS (H^2 = 0.59) and WBS (H^2 = 0.44). Both ANOVA and estimation of broad-sense heritability were calculated on reduced data sets taking only genotypes into account that were tested in both years and/or locations, respectively. The same subsets of 42 (Austria), 40 (Czech Republic) and 22 (across years and locations) genotypes were used to estimate Pearson's correlation coefficients between the different traits. Correlation coefficients for CB between 2021 and 2022 were similar between locations and significant at α = 0.001 (r = 0.59 in Austria and r = 0.63 in the Czech Republic; Figure 2). Correlation coefficients for BS and WBS were lower and significant at α = 0.01 (BS: r = 0.48; WBS: r = 0.39). In 2022, correlation coefficients between CB and BS/WBS were higher than in 2021. In addition, CB was more correlated to BS than to WBS in 2022, while correlation coefficients between CB and the two bunt sori parameters were almost equal in 2021. No significant correlation was observed for CB scorings between the two test sites in any year.

3.3 Marker-assisted selection in multi-parent breeding lines

For each cross, between eight and 14 progenies were screened with two to six KASP markers for one to four different bunt resistance QTL (Supplementary Table S2). If possible, two flanking markers for each QTL were used, but due to missing polymorphisms, some QTL could only be tested for with a single marker or could not be selected at all. Between two and four F_2 lines from each cross were positively selected to harbour different QTL or combinations of QTL. A set of eight to 15 negatively selected lines were included in each test year as a control panel (Figure 3). In 2021, nine breeding lines out of 42 tested in Austria were found to be resistant with less than 5% CB and two of these were also completely resistant in the Czech Republic. In 2022, eleven lines out of 33 tested in Austria showed resistance and nine of these lines were also resistant in the Czech Republic. Six of these lines were tested in both seasons in Austria and one season in the Czech Republic and showed stable resistance across years and environments. These genotypes all harboured combinations of two or three different bunt resistance loci according to MAS results (Supplementary Table S1). In both test locations, genotypes selected to harbour bunt resistance loci were on average more resistant compared to negative controls and cultivars (Figure 3).

4. Discussion

A diversity panel consisting of the bunt differential set, a range of cultivars and breeding lines from European breeding companies and experimental multi-parent breeding lines developed at the Institute of Plant Breeding, BOKU, Tulln, was analysed for common bunt resistance in two environments. The panel comprised a total of 128 genotypes out of which several subsets were used to assess different characteristics of common bunt infections. Especially for the multi-parent breeding lines, we aimed at determining differences between standard scoring and two alternative methods providing more detailed information about the degree of infection in individual ears.

4.1 Partially bunted ears

Scoring of common bunt incidence is usually done by cutting wheat ears and checking for the presence of bunt balls. Incidence is then scored in a qualitative manner, recording an ear as infected if at least a single bunted spikelet is spotted. Triggered by the observation of partially bunted ears with only a few bunt balls among otherwise healthy grains in field trials in Tulln, Austria, we adapted our scoring method by cutting ears at least two times, once in the upper third and once in the bottom third of the ear. Thereby, we achieved a more accurate scoring of incidence which covered partially bunted ears. In addition, we applied two more methods of bunt assessment, i.e. the number (BS) and weight (WBS) of bunt sori relative to the total number/weight of grains plus bunt sori in a sample. Combining these measures, we were able to determine in which genotypes partially bunted ears occurred more frequently than in others as the BS scores of such partially infected lines would be considerably lower than CB obtained from standard scoring. Our results confirm the observations already made by Sampson (1927) and Gieseke (1929) that partial bunt infections occur primarily in genotypes with a certain level of disease resistance. Both studies were conducted with a cultivar called ‘Heils Dickkopfweizen’ (syn. ‘Dornburger Heils Dickkopf’) which was known to harbour resistance to bunt. In our experiments, heavily infected genotypes had similar levels of BS compared to CB with BS scores sometimes even exceeding CB levels. These rare cases of BS > CB were due to the fact that scoring of CB and BS was not done as repeated measurements on the same ears. Instead, new ears on which BS and WBS were scored were randomly selected from non-cut ears in plots that had previously been assessed for CB. Genotypes showing at least some levels of resistance with CB scores below or around 30%, on the other hand, frequently had BS scores which were only up to 40% of the CB levels. Gassner (1938) attributed these observations to a kind of race between the fungus and the ovule taking place in early stages of grain development.

The lower weight of bunt sori compared to healthy grains was well reflected in WBS scores, which were in most cases 50-60% lower than BS scores. According to our results, assessing BS is sufficient to determine the extent of partial infections while additional scoring of WBS is not necessary as the two traits were highly correlated in both years of data collection. Based on the bunt characteristics assessed in our study, we were able to determine the extent of partially bunted ears but did not obtain data on partially bunted wheat grains. To draw further conclusions on partial infections of single grains, sowing healthy seeds harvested from partially bunted ears in field trials would be an appropriate strategy.

4.2 Variation between common bunt populations

Common bunt infections were observed in two test locations for this study: one in the north-east of Austria and one in the north-west of Czechia. The distance between these two locations is approximately 300 km, but both can be regarded as Central European environments. Two different,

locally collected bunt populations which showed distinctly different virulence patterns against lines in the diversity panel were used for artificial inoculations in these two environments. High virulence against differential lines for *Bt1*, *Bt4* and *Bt6* was observed in the Czech Republic, while these genes were still effective against the Austrian bunt population (Table 1). The high infection levels on the *Bt5*-differential with the Czech inoculum are most likely the result of admixture in the seed sample as tests of the inoculum on seed samples for the *Bt5*-differential in Denmark did not yield any infection (Borgen and Christensen, 2023). Several cultivars tested in both locations were moderately to highly susceptible in Austria but resistant against the Czech inoculum. Although the source of resistance is not known for most of these cultivars, many of them might possess *Bt10* or also *BtP* as the Austrian inoculum was virulent against these genes but they showed resistance in the Czech trials.

Seventeen genotypes of the whole panel showed bunt resistance across locations. Among the multi-parent breeding lines selected with KASP-markers, most of those harbouring the QTL on chromosome 1A were infected in Austria but resistant in the Czech Republic (Figure 1). Lines harbouring combinations of QTL on chromosomes 1A and 1B or only the QTL on chromosome 1B, on the other hand, were more strongly affected by the Czech bunt population. Infection levels varied not only between locations but also between years: while CB incidence was approximately 50% elevated in Austria in 2022 compared to 2021, infection levels in the Czech Republic were lower in 2022. Some genotypes that had moderate levels of CB in the Czech trials in 2021 were resistant in the same location in 2022. These results indicate that breeding for common bunt resistance needs to be done with strong emphasis on regional adaptation. Although the two trial sites are not very far away from each other and have a similar climate, local bunt populations show clear differences in their virulence against various resistance sources.

4.3 Applicability and efficiency of marker-assisted selection

The molecular markers used to select bunt resistance QTL in multi-parent breeding lines in our diversity panel were not diagnostic for individual loci but rather flanking the region to which the QTL had been mapped. As far as possible, at least two markers, one on each end of the chromosomal region, were applied to select for a specific locus. Due to the complex pedigrees of the breeding lines with up to ten different genotypes per line, some markers which yielded good selection results in previous studies (Muellner et al., 2021; Lunzer et al., 2023b) were not informative in individual lines. The more parental genotypes are added to the pedigree, the higher the chance becomes that one of these genotypes has the same allele call for a certain marker as the original resistance donor. If this is the case, the polymorphism of this marker is lost and it cannot be used for MAS. In Supplementary Table S1, QTL for which MAS could be conducted only with a single marker are indicated. This was the case in 13 out of 27 multi-parent breeding lines. Selection accuracy is negatively affected if screening is performed with just one flanking marker per locus. Therefore, outliers and high variance in lines selected through MAS as shown in Figure 3 were expected. Even if two markers per QTL are used for selection, recombination events could occur in the region between these markers, leading to a loss of resistance in individual positively selected lines. Despite these challenges, MAS was effective in our panel with negatively selected and unselected lines showing higher infection levels compared to positively selected ones (Figure 1 and Figure 3). Combinations of two or more QTL in a single line lead to on average higher resistance levels than inheritance of only one QTL, which is in line with the findings of Wang et al. (2019) and Muellner et al. (2020; 2021).

4.4 Conclusions for organic breeding

As scores for BS were on average lower than for CB in our diversity panel, this indicates that partial infections of wheat ears were rather the rule than an exception. It should therefore be re-considered whether the qualitative scoring of bunt incidence done in most experiments at the moment is really the most appropriate method or if rather a scoring method, also taking partial infections into account, would provide better knowledge about resistance levels in different genotypes. A first step for improvement could be cutting wheat ears several times as it was done in this study to make sure that partial infections of single ears do not go undetected. This is more time-consuming than the standard scoring, but still less tedious than assessing BS or WBS.

Among the 46 cultivars and breeding lines from breeding companies that were tested for this study, only six cultivars and one breeding line showed resistance to common bunt across years and locations. Four out of them are U.S. cultivars selected for bunt resistance, i.e. 'Blizzard' (Sunderman et al., 1991), 'Bonneville' (Souza et al., 1995), 'Deloris' (Hole et al., 2004) and 'UI SRG' (Chen et al., 2012). Only two cultivars come from European breeding programs, one (i.e. 'Aristaro') indeed from an organic program. The other, i.e. 'Unitar', a breeding line developed at NARDI, Romania, carries a 1AL·1RS wheat-rye chromosome translocation, introduced via a cross between wheat and triticale. The line shows stable resistance against bunt across a wide range of locations that is attributed to a gene on the rye chromosome (Ciuca et al., 2023). Resistance to bunt was also described for wild wheat species, wheat wild relatives (Mamluk, 1998; Babayants et al., 2006), and tritordeum (Rubiales et al., 1996). However, no *Bt* genes from these wild relatives were yet characterized or exploited widely in commercial breeding. This is in contrast to e.g. leaf rust resistance where ∼50% of the more than 80 described *Lr* genes were derived from alien species and some of them successfully exploited in commercial breeding programs (Kumar et al., 2022). When searching for resistance sources against bunt diseases, alien species and wheat wild relatives should not be neglected in pre-breeding programs and characterization of resistance genes. Resistance to common bunt is currently mainly a problem of organic wheat production due to the lack of effective organic seed treatments, however, the European Union is aiming to halve the use of pesticides and increase the share of organic farms by 2030 within its Green Deal. Some fungicides used today in conventional agriculture might therefore be banned in the future. Hence, the incorporation of resistance genes against bunt diseases shall be a general breeding target for sustainable wheat production.

The six multi-parent breeding lines identified as being resistant across years and locations in this study represent genotypes that could be directly used in commercial breeding programs. Breeding lines with such complex pedigrees have advantages because of their high variation in the elite genetic background, but these come with the drawback of high chances for losing polymorphic markers for MAS. The selection accuracy with a quarter of all selected lines being actually resistant (0-5% infection) in our diversity panel corresponds to other experiments for MAS in multi-parent breeding lines conducted at BOKU (unpublished data). As genotypes harbouring multiple resistance loci have been shown to possess superior resistance to bunt infections in previous works (Wang et al., 2019; Muellner et al., 2020; 2021) and also in this study, pyramiding of bunt resistance genes should be a major focus in organic breeding. To achieve such a stacking, informative molecular markers for the loci of interest are essential. Common bunt is a serious problem in organic wheat production. Based on the results of this study, breeding of resistant varieties should be conducted regionally and sped up through the application of MAS to secure further organic and sustainable wheat production.

5. References

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6. Tables

TABLE 1 Common bunt incidence (%) for genotypes of the bunt differential set across two years and locations: Austria (Tulln, AT) in 2021 and 2022; Czech Republic (Prague, CZ) in 2021. Two different susceptible controls were used in the two locations, i.e. 'Capo' in Tulln and 'Heines VII' in Prague.

Bt gene	Accession	AT 2021	AT 2022	CZ 2021
susceptible	Capo	70.0	91.5	-
Bt0	Heines VII	-	-	50.9
Bt1	PI 554101	0.0	0.0	39.9
Bt2	PI 554097	56.0	96.0	15.5
Bt3	CI 6703	10.3	18.0	11.9
Bt4	PI 16610	1.3	0.0	60.6
Bt5	CI 11458	0.7	10.0	63.0
Bt6	CI 10061	1.7	1.0	21.8
Bt7	PI 554100	35.3	98.0	48.2
Bt8	PI 554120	1.0	0.0	0.0
Bt9	PI 554099	9.0	6.0	0.0
Bt10	PI 554118	32.7	44.0	0.0
Bt11	PI 554119	0.0	0.0	8.2
Bt12	PI 119333	0.0	0.0	0.0
Bt13	PI 181463	19.7	22.0	0.0
BtP	PI 173437	0.0	20.0	0.0

TABLE 2 Minima, maxima and mean values of common bunt scorings in individual locations and years: common bunt incidence (CB) in 150 ears per plot (Austria) or all ears per plot (Czech Republic); number of bunt sori relative to the total number of grains in 50 ears per plot (BS); and weight of bunt sori relative to the total grain weight of 50 ears per plot (WBS). All values given as percentages, as well as differences between scorings of each trait relative to the other two.

	Austria			Czech Republic		
	Min	Max	Mean	Min	Max	Mean
2021						
CB	0	81.3	25.5	0	91.5	18.9
BS	0	88.6	24.2			
WBS	0	74.7	13.3			
CB to BS	-24.9	27.7	4.1			
CB to WBS	0	47.4	14.6			
BS to WBS	0	29.0	10.3			
2022						
CB	0	98	22.1	0	50.5	5.4
BS	0	91.1	13.5			
WBS	0	81.2	8.1			
CB to BS	-5.1	48.3	7.4			
CB to WBS	0	53.4	12.8			
BS to WBS	0	24.6	12.8			

TABLE 3 Variance components and broad-sense heritability estimates for common bunt assessment in individual locations and across locations. For within-location analyses, 42 and 40 genotypes tested in both years were included in the Austrian and Czech data, respectively. Analysis across locations comprised 22 genotypes that were tested in both years and locations.

Location	Trait [a]	σ_G [b]	σ_E	σ_L	σ_{GxE}	σ_{GxL}	σ_{ExL}	σ_{error}	H^2
Austria	CB	81.2	5.6		39.6			55.7	0.63
	BS	29.9	0.02		17.3			24.5	0.59
	WBS	5.7	0.01		5.9			8.5	0.44
Czechia	CB	186.9	73.6		63.6			108.7	0.68
Across	CB	8.9	0.02	10.8	0.5	18.4	60.3	80.9	0.18

[a] Common bunt assessments: CB, common bunt incidence in 150 ears per plot (Austria) or all ears per plot (Czech Republic); BS, number of bunt sori relative to the total number of grains in 50 earsper plot; WBS, weight of bunt sori relative to the total grain weight of 50 ears per plot

[b] variance components for: σ_G, genotype; σ_E, year (environment); σ_L, location; σ_{GxE}, genotype by environment interaction; σ_{GxL}, genotype by location interaction; σ_{ExL}, environment by location interaction; σ_{error}, residual; H^2, broad-sense heritability.

7. Figure legends

FIGURE 1 Heatmap of common bunt incidence (CB, %) across two years (2021, row 1 and 2022, row 2) and two locations (AT: Tulln, Austria; CZ: Prague, Czech Republic) for a diversity panel of 113 wheat genotypes. The left-hand side of the heatmap shows lines harbouring different QTL according to marker-assisted selection using KASP markers; the chromosomal locations of the QTL are indicated on the x-axis. These QTL are known bunt resistance loci mapped by Muellner et al. (2020; 2021) and originate from cultivars 'Blizzard' and 'Bonneville'. Lines which were negatively selected and included as negative controls are indicated as "negative", lines which were not subjected to MAS are indicated by "no MAS", and a panel of cultivars and breeding lines is shown on the right-hand side with genotype names indicated on the x-axis.

FIGURE 2 Scatterplots (below diagonal), histograms (diagonal) and Pearson's correlation coefficients (above diagonal) for different common bunt infection traits evaluated in 2021 and 2022: CB, common bunt incidence in 150 ears per plot; BS, number of bunt sori relative to the total number of kernels in 50 ears per plot; WBS, weight of bunt sori relative to the total grain weight in 50 ears per plot. All values in percentages.

FIGURE 3 Boxplots showing common bunt incidence (CB, %) in different sub-groups of a diversity panel in field trials in (**a**) Austria and (**b**) Czech Republic in 2021 and 2022: genotypes identified with marker-assisted selection (MAS) to harbour different known bunt resistance loci originating from 'Blizzard' or 'Bonneville' (Muellner et al., 2020, 2021) are indicated by the chromosomal locations of resistance QTL or the designation "threeQTL" on the x-axis; genotypes harbouring no QTL according to MAS are marked as "none"; results for the bunt differential set including differentials for *Bt0-Bt13* and *BtP* are shown in group "Bt"; registered cultivars and breeding lines are shown as group "check". The number of genotypes tested per group, location and year is indicated below the respective box; outliers are shown as dots.

8. Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

9. Author contributions

Laboratory analysis and preparation of the initial draft for the manuscript was done by ML. VD and KP carried out field trials and collection of phenotypic data. HB supervised marker-assisted selection and edited the original draft. HG organised funding, curated the data, conceptualized and supervised the study and edited the original draft. All authors read and approved the manuscript.

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12. Supplementary Material

Supplementary information is provided in a separate file.

13. Data Availability Statement

All data generated or analysed during this study are either included in this article or are available via Zenodo repository (DOI will be included here upon acceptance of the manuscript).

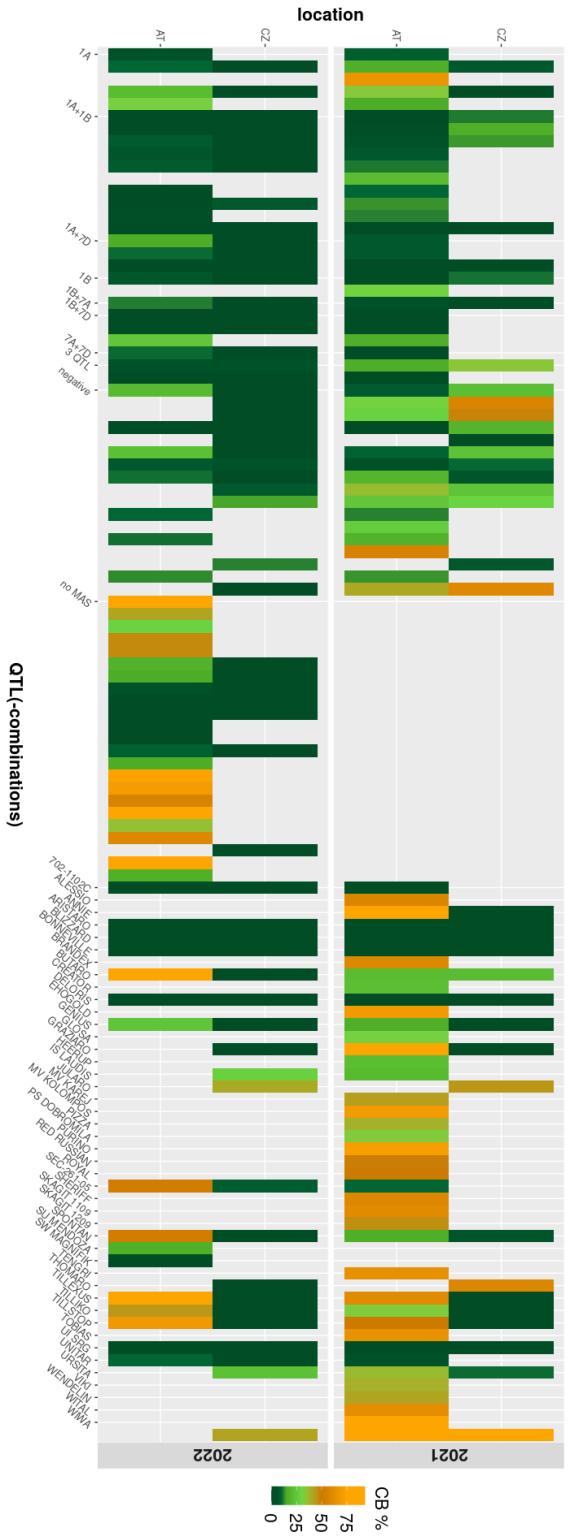
location
AT
CZ
AT
CZ
QTL(-combinations)
1A
1A+1B
1A+7D
1B
1B+7A
1B+7D
7A+7D
3 QTL
negative
no MAS
2022
2021
CB %
0
25
50
75

Figure 1

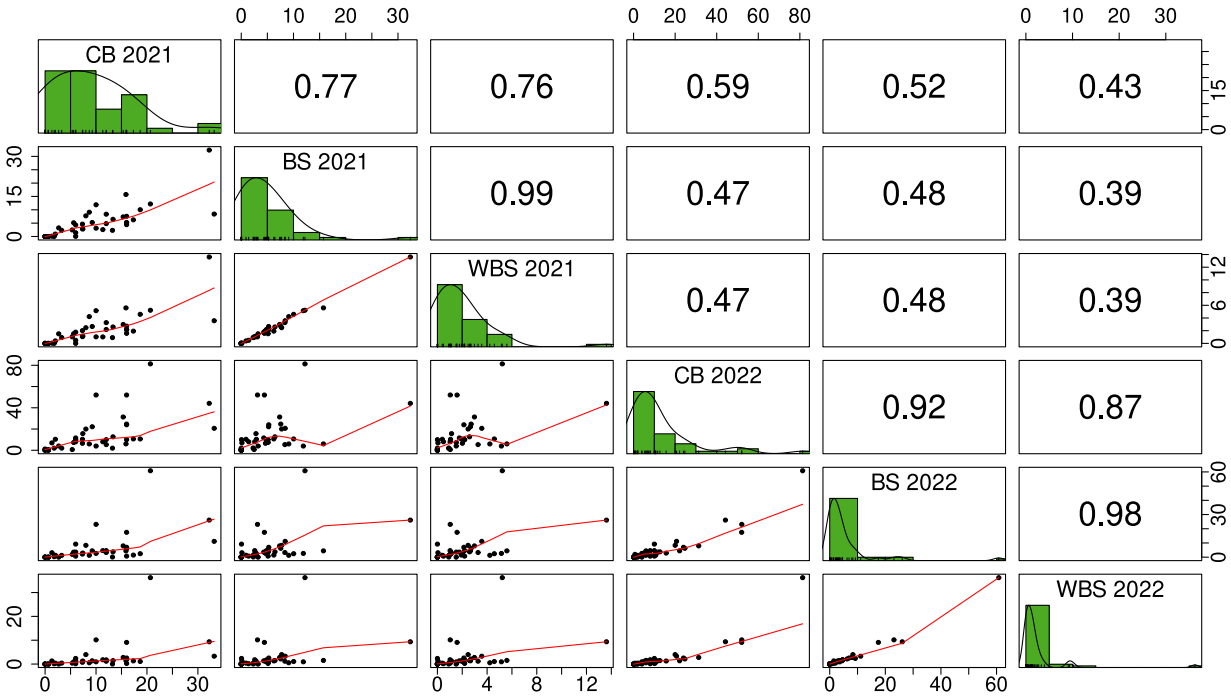
CB 2021
BS 2021
WBS 2021
CB 2022
BS 2022
WBS 2022
0.77
0.76
0.59
0.52
0.43
0.99
0.47
0.48
0.39
0.47
0.48
0.39
0.92
0.87
0.98

Figure 2

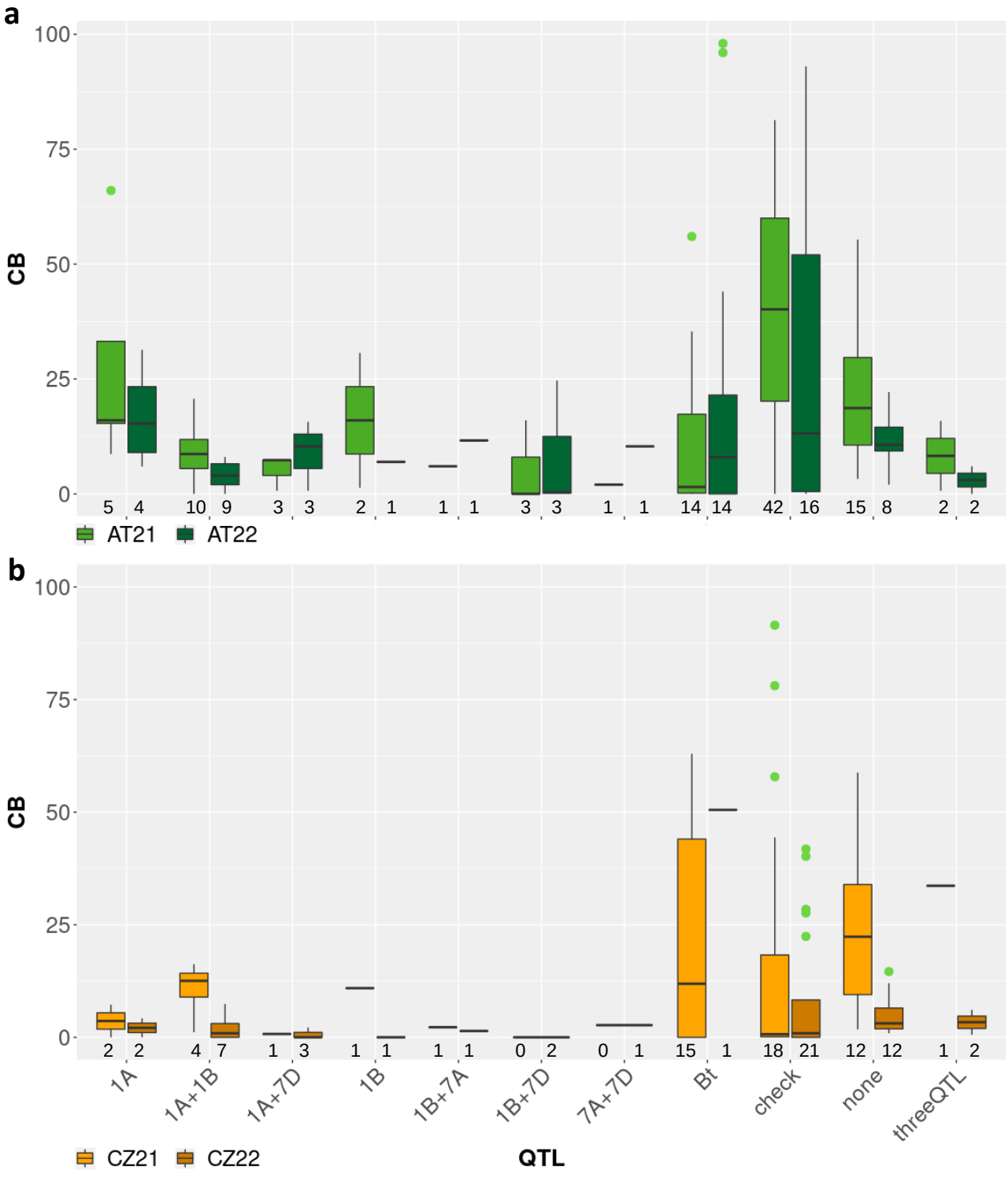
a
100
75
50
25
0
CB
5 4 10 9 3 3 2 1 1 1 3 3 1 1 14 14 42 16 15 8 2 2
AT21
AT22
b
100
75
50
25
0
CB
2 2 4 7 1 3 1 1 1 1 0 2 0 1 15 1 18 21 12 12 1 2
1A
1A+1B
1A+7D
1B
1B+7A
1B+7D
7A+7D
Bt
check
none
threeQTL
CZ21
CZ22
QTL

Figure 3

6 Additional contribution

How long does it take to develop high performing and common bunt resistant winter wheat lines using organics-compliant methods?

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Available online at: `https://zenodo.org/record/7875597`

Author contributions:

- Magdalena Lunzer carried out field trials, marker-assisted selection and data analysis and wrote the original draft
- Sebastian Michel generated genomic-estimated breeding values
- Maria Buerstmayr supervised marker-assisted selection
- Heinrich Grausgruber acquired funding and edited the original draft
- Hermann Buerstmayr conceptualized and supervised the study, generated the population and edited the original draft

73. Tagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs
21.-23. November 2022, Raumberg-Gumpenstein, Österreich

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Abstract

Once among the most devastating wheat diseases, common bunt caused by *Tilletia tritici* and *T. laevis* was successfully banned from most fields by the invention of seed dressings with hexachlorobenzenes (HCBs) in the 1950s. During the past decades, a continuously increasing area of agricultural land has been converted to organic management, refraining from the use of chemical pesticide applications. Therefore, common bunt as a primarily seed-borne disease is experiencing a come-back since no alternative and equally effective treatments to seed dressings are available. The most sustainable and efficient way to avoid yield and quality losses due to bunt infections is the use of resistant cultivars. Although 17 different resistance genes have been postulated so far, only few have been mapped and are available for applied breeding. In consequence, the development of bunt resistant cultivars is slow and a small number of varieties with high resistance levels are currently available. In this study, we therefore aim to determine how fast breeding lines can be selected that unite bunt resistance and good agronomic performance.

For this purpose, we developed pseudo-back-cross populations with bunt resistance alleles introgressed from exotic donor lines. Resistance QTL in these donors were mapped in previous projects at IFA-Tulln, enabling marker-assisted selection (MAS) via KASP-markers (Muellner *et al*., 2020; 2021). The three resistance donors 'Blizzard', 'Bonneville' (US cultivars registered in the 1990s) and PI 119333 (differential line for the bunt resistance gene *Bt12*) were initially crossed to the susceptible cultivar 'Rainer'. During population development, three back-crossing steps were carried out, each with a different back-crossing parent that was either a variety or an advanced breeding line adapted to Austrian growing conditions. After each back-crossing step, the F_1-progeny was screened for the presence of one to three different resistance QTL inherited from the donors using KASP-markers. In generation BC_3F_1, the number of lines was reduced further by one step of genomics-assisted selection (GAS) based on genomic estimated breeding values (GEBVs), filtering out those lines with promising genetic backgrounds based on genome-wide marker data from genotyping by sequencing (GBS). After the last back-cross, the selected progenies were self-pollinated to generate lines harbouring the resistance QTL fixed in a homozygous allelic state. These lines were identified with another round of MAS. Only the selected homozygous resistant lines were subsequently subjected to field tests for common bunt resistance as well as for yield and quality traits together with a control panel of negatively selected lines. Data from two seasons of common bunt testing in artificially inoculated field trials in Austria and one season of dwarf bunt testing with artificial inoculation in Utah (USA) is available to determine disease resistance levels in the population. In addition, a replicated yield trial was conducted in 2022.

The number of lines undergoing propagation in the greenhouse or field testing was greatly reduced by the MAS and GAS steps. After the individual selection steps in each of the three back-cross generations, 33.6%, 8.8% and 9.1% respectively, of all lines were chosen to be kept in the population. Thereby, not only resources required for field testing were kept low, but also the time from the initial cross to the first homozygous resistant lines in generation BC_3F_2 was reduced by more than 50% compared to a selection scheme based solely on phenotypic selection. Of all lines selected to harbour one or several of the introgressed resistance QTL, 35% (69 lines) were fully or highly resistant (≤5% incidence) to common bunt across two years. Several factors contribute to the fact that almost two thirds of the population showed mild to severe infections: markers applied for MAS were not diagnostic but only flanking the chromosomal regions conferring resistance. The complex pedigrees with five different parents for each line led to a loss of polymorphic markers with each back-crossing step. Individual loci could therefore be selected with only a single marker in some of the lines, leading to low selection accuracies. In addition, some of the resistance loci conferred by the donor lines do not provide full resistance on their own but only in combination with a second locus. As some of the intervals flanked by the applied markers are relatively large, also recombination

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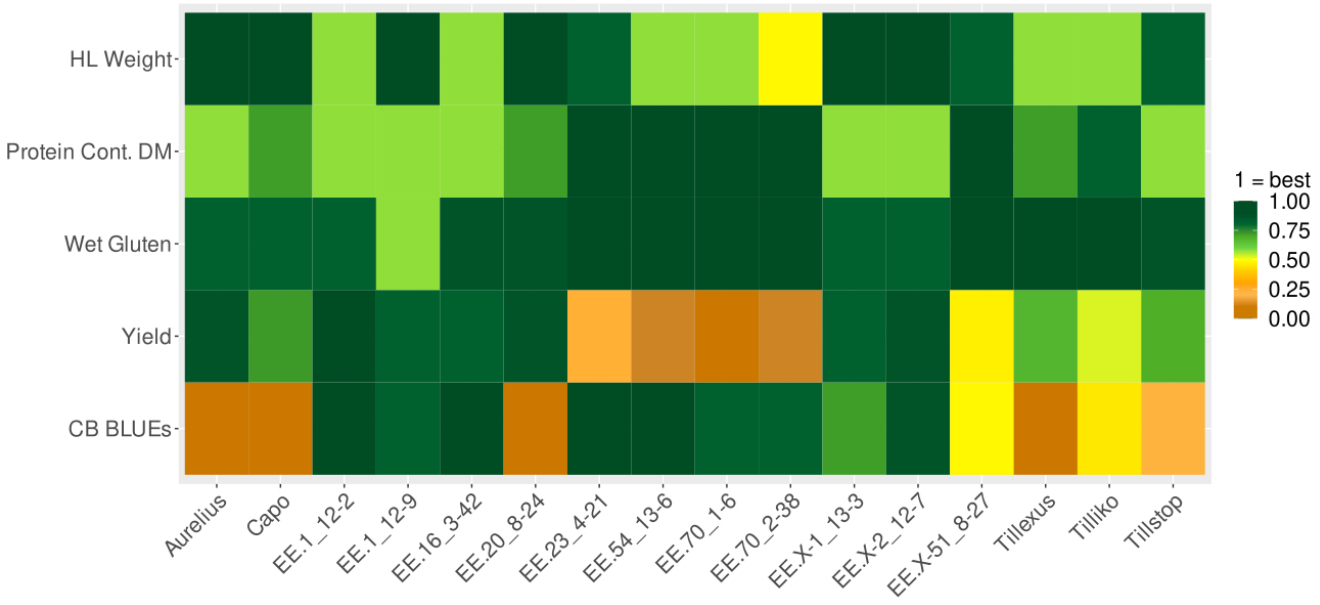

Figure 1 Heatmap showing scores for hectolitre weight, protein content, wet gluten content, yield and common bunt infections across two seasons normalized to a range between 0 and 1 with 1 being the best, desired value (*e.g.*, no bunt infection and high yield both have a score of 1). Scores for quality traits were allocated by considering Austrian thresholds for different wheat quality classes in trading. Scores are given for two bunt-susceptible check cultivars (*i.e.*, 'Aurelius' and 'Capo'), the six best-performing experimental lines in terms of yield and the five best-peforming lines in terms of protein content (genotype names with the prefix "EE"), as well as three cultivars originally registered as bunt-tolerant in Austria (*i.e.*, 'Tillexus', 'Tilliko' and 'Tillstop'). Data on all traits except common bunt is from replicated field trials conducted in Tulln in 2022. Data on common bunt incidence are shown as best linear unbiased estimates (BLUEs) across 2021 and 2022.

events might have occurred in these regions that could not be tracked with the markers and that led to a loss of resistance in positively selected lines.

We also observed that common and dwarf bunt resistance are not conferred by the same genes in our experimental population. While lines harbouring the resistance locus on chromosome 1B showed high resistance against common bunt, they were to a large extent infected by dwarf bunt. The opposite pattern was observed for lines with the *Bt12*-locus on chromosome 7D where most likely recombination events in the chromosomal region were responsible for a loss of resistance against common bunt but not against dwarf bunt. Common bunt incidence was uncorrelated with yield and quality traits. We found experimental lines with complete resistance against common bunt that performed equally well or slightly better in terms of yield and quality than the highly susceptible check cultivars (Fig. 1). Cultivars registered as bunt tolerant in Austria and Germany that were included were moderately to highly infected with common bunt in our trials.

We therefore conclude that MAS is a suitable method to reduce time and resources for the development of bunt resistant and high -performing winter wheat lines. The experimental lines in our population were tested in generation BC_3F_{2n}. Using MAS, it is possible to reach this generation in 2.5 years, while selecting exclusively via phenotypes would take 5.5 years for the same outcome and require a lot of additional resources.

Keywords

Marker-assisted selection · organic agriculture · resistance breeding · seedborne disease · *Tilletia* · *Triticum aestivum*

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IFOAM - Organics International (2017) Position paper: Compatibility of breeding techniques in organic systems. IFOAM, Bonn. [https://www.ifoam.bio/compatibility-breeding-techniques-organic-systems; accessed 21 March 2023]

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7 Discussion

This thesis aims to extend the knowledge about bunt infections and promote bunt resistance breeding. To achieve these aims, bunt resistance loci were identified on different wheat chromosomes and strategies on how to integrate them into breeding programs were explored. The results presented in the publications comprising this cumulative dissertation shall now be evaluated with respect to their relevance for individual research questions.

7.1 Identification of common bunt resistance sources for broadening the genetic diversity available for resistance breeding

Probably due to the reduced breeding activities against common bunt (CB) during the 20th century (Hoffmann and Waldher, 1981), the genetic diversity currently exploited in breeding programs is rather low. In North America, the most widely used resistance factor is *Bt10* (Singh et al., 2016). The few cultivars registered as bunt-resistant or bunt-tolerant in Europe mostly contain *Bt8* and *Bt9* ('Stava', 'Hallfreda') in the Nordic region (Bengtsson et al., 2023) and *BtZ* ('Tilliko') or *Bt5* ('Tillsano') (Oberforster and Plank, 2021) in Central Europe. If single resistance factors are exposed to disease pressure on a comparably large acreage, the potential for resistance break-downs caused by fungal races developing virulence against this factor is high, even for diseases with just one generation per year like CB. It is therefore essential to broaden the genetic diversity for bunt resistance available to breeders and, in consequence, also to farmers.

7.1.1 Geographic origin of bunt resistant wheat accessions

Promising new resistance donors can be found in wheat accessions originating from the Balkan region, Turkey or countries of the Near and Middle East as shown in publication 1. All of the highly CB resistant bread wheat accessions (<1 % incidence) tested in this study came from five countries: Iran, Montenegro, Serbia, Turkey and the United States. However, many of the U.S. accessions were breeding lines resulting from crosses with genotypes originating from the other regions mentioned above (Gordon et al., 2020). Also the two donors used as resistant parents to generate the mapping populations examined in publication 2 can be assigned to Turkey. PI 166910 is a Turkish landrace that was collected near the city of Tokat (Harlan, 1950) and M822123 (PI 554119) is a line developed by crossing this landrace to the susceptible cultivar 'Elgin'. Consequently, all wheat accessions in which new resistance sources were identified in this thesis originate from regions previously described as CB resistance hotspots. These centers of origin for CB resistance largely overlap with centers for dwarf bunt (DB) resistance with the latter being a lot less frequent, though (Bonman et al., 2006). This already raises the question whether resistance against both diseases can be conferred by exactly the same genes as postulated by Metzger and Hoffman (1978) and Goates (1996). If this was the case, the many accessions harbouring CB resistance identified in the Balkan region, Turkey and Iran that Bonman et al. (2006) found among the genotypes comprising the National Small Grains Collections (NSGC) of the United States Department of Agriculture (USDA) should all have been DB resistant, too, which they were not. This discrepancy was not addressed by Bonman et al. (2006) in their publication, but became evident when a subset of the NSGC was tested for DB and CB separately by Gordon et al. (2020) (DB) and in publication 1 of this thesis (CB). Not only were the marker-trait associations (MTAs) identified across the wheat chromosomes completely different for the two diseases. The proportion of lines in the panel showing CB resistance was also a lot higher

(42 %) than those resistant to DB (11.38 %). However, there were not only lines showing CB resistance but susceptibility to DB, also the opposite situation was observed in some genotypes. Therefore, the conclusions seem justified that resistance to both diseases is at least to some extent governed by different genes and that lines which are DB resistant can still be susceptible to CB. This is supported by results obtained by Muellner et al. (2021) who mapped four quantitative trait loci (QTL) in populations with 'Blizzard' and 'Bonneville' as resistance donors. Out of the four QTL, one conferred only DB resistance while another was active only against CB. The results discussed above may, however, also reflect the specific virulence patterns of the few CB and DB isolates used in these studies. In order to properly investigate the question whether or not resistance to DB and CB are essentially conferred by the same loci, a diversity panel of wheat lines with different *Bt* genes would need to be tested with a broader array of races of both DB and CB. Such a study would require considerable resources and is, at least to my knowledge, not available yet. When searching for and selecting genotypes as resistance donors for research or breeding programs, it should consequently be taken into account that bunt resistance genes may not always confer simultaneous resistance to both diseases.

7.1.2 Characterization of genetic loci conferring bunt resistance in donor lines

...through genome-wide association studies

Genome-wide association studies (GWAS) are usually conducted on panels exhibiting high variation in the trait of interest and genetic backgrounds in general, often showing complex types of population structure and relatedness between individuals (Tibbs Cortes et al., 2020). These diversity panels are well suited to detect genotypic variation in form of polymorphisms which are correlated with certain patterns in phenotypic variation (Balding, 2006). GWAS thus enable researchers to identify regions on the genome which have a high probability of being associated with a desired phenotype. Due to the high genetic diversity in the test panel, accessions showing the favourable allele(-combinations) potentially originate from different geographic origins or have different ancestries, thereby broadening the gene pool from which donors can be sourced.

In publication 1, lines showing combined CB and DB resistance originated from at least three main regions (Balkan, Turkey, Iran) since the U.S. breeding lines among them can all be assumed to derive their resistance from accessions coming from one of these regions (Gordon et al., 2020). In a study conducting association mapping in a panel of 330 genotypes which originated from more than 800 different crosses, Mourad et al. (2018) identified 28 lines showing up to 5 % CB infection. Considering this high number of initial crosses and the resulting extensive genetic variation comprised in that panel, the authors suggest that introducing the most resistant lines into breeding programs would lead to beneficial effects on selection gains for CB resistance. However, as GWA mapping constitutes a method to detect (novel) genomic regions associated with bunt resistance, the loci identified in such studies should be validated in experimental populations before their use in applied breeding.

With respect to GWA results such as those obtained by Mourad et al. (2018) who found MTAs for 123 single nucleotide polymorphisms (SNPs) on 14 different chromosomes, validation should first be done for the most promising markers. These could be the ones located in regions already identified to be associated with bunt resistance in other studies. MTAs on chromosomes 2B and 7A found by Mourad et al. (2018) match with results from other mapping studies (Iqbal et al., 2023; Steffan et al., 2017a). Markers significantly associated with CB resistance were also found on these two chromosomes in publication 1. Especially 7A seems promising for the detection of novel bunt

resistance factors since loci on this chromosome were found to influence bunt infection levels in both GWA and QTL mapping studies (Fofana et al., 2008; Bhatta et al., 2018; Mourad et al., 2018; Wang et al., 2019; Muellner et al., 2021).
The same applies for MTAs on chromosomes 1A and 1B detected in publication 1. Physical positions of the significant SNPs overlap with or are in close proximity of chromosomal regions previously identified to harbour QTL for both common (Singh et al., 2016; Muellner et al., 2021) and DB (Chen et al., 2016; Muellner et al., 2020). QTL conferring CB resistance were also mapped to similar regions on 1A and 1B in the populations examined in publication 2. This agreement with the findings of other studies underlines the potential of significant MTAs from publication 1 for their use in the identification of bunt resistant germplasm.

...through bi-parental mapping

Since most of the resistance donors that were identified in studies conducting association mapping for resistance to CB (Bonman et al., 2006; Bhatta et al., 2018; Mourad et al., 2018; Steffan et al., 2022) as well as most lines in the differential set have not been characterized further, investigating the genetic makeup of these genotypes represents another way of extending the pool of genetic resources for resistance breeding. This approach was followed in publication 2. To extend the range of *Bt*-genes from the bunt differential set that can be used in resistance breeding, four mapping populations were developed through crossing either PI 166910 or PI 554119, both harbouring *Bt11* (Goates and Bockelman, 2012; Goates, 2012), with three susceptible winter wheat cultivars. Information about lines containing *Bt11* is scarce, but this genetic factor has been shown to provide full and stable resistance in a range of publications. In European studies, it showed resistance against CB races from countries all across the continent (Blazkova and Bartos, 2002; Liatukas and Ruzgas, 2008; Cadot et al., 2021). Tests against known races of CB and DB identified only two strains of *Tilletia controversa* that were able to overcome *Bt11* resistance (Goates and Bockelman, 2012). Apart from the original donor PI 166910 and the differential line PI 554119, only very few other genotypes indicating *Bt11* are known to date. Some breeding lines originating from crosses between the differential line and semidwarf winter wheat cultivars from Romania which potentially harbour *Bt11* were developed by Oncica and Saulescu (2007) and some of them were tested by Liatukas and Ruzgas (2008). The only other plant introduction identified so far that shows the same reactions to the set of bunt races tested by Goates and Bockelman (2012) is PI 211657. In conclusion, *Bt11* had, despite its extremely stable resistant reactions, neither been exploited for breeding nor had its resistance been characterized in more detail.
Genetic mapping of chromosomal regions associated with CB resistance in the four mapping populations described above identified two QTL on chromosomes 4B and 6D across populations. The main resistance conferring locus was a QTL designated *QBt.ifa-6DL* which mapped to a region co-locating with *Bt9* according to Wang et al. (2019). While co-localization and overlap between QTL positions with known loci could be expected in populations exhibiting quantitative resistance governed by multiple minor effect QTL or deriving their resistance from popular donors like *Bt10*, it was surprising in populations with donors harbouring such a rare and unutilized resistance factor as *Bt11*. Through comparison between haplotype profiles of genotypes postulated to carry either *Bt9* or *Bt11*, respectively, data supporting the assumption of the two genes being distinct resistance factors was obtained. The fact that peak markers for the main QTL on 6D were located in positions of the distal end of the *Bt9* interval or in even more distal regions of the chromosome also underpinned this

hypothesis. Distinction between the locus mapped by Wang et al. (2019) and *QBt.ifa-6DL* was hampered by low mapping resolution on chromosome 6DL. Similar problems were reported for *Bt12* on chromosome 7DS. Chen et al. (2016) and Muellner et al. (2020) both identified a QTL conferring bunt resistance close to the centromere of 7D but whether these two QTL correspond to the same resistance factor (*Bt12*) or are distinct loci could not be unambiguously resolved because of missing marker polymorphisms resulting in large gaps in the linkage maps.
In addition to the major QTL on 6DL, loci with smaller effect sizes were identified in publication 2. The most important one among these was *QBt.ifa-4BS* since it was present in all mapping populations but showed varying effects on CB infection levels. Compared to loci identified in the same chromosomal region in other studies (Singh et al., 2016; Muellner et al., 2020), *QBt.ifa-4BS* on average caused a larger reduction in CB incidence in our mapping populations.
All other QTL identified were only present in single mapping populations and mapped to chromosomes 1A, 1B, 2A and 7B. Among these, the locus on chromosome 1BS could be of special interest since it had a large effect comparable to *QBt.ifa-6DL* in one of the mapping populations. Although the short arm of chromosome 1B has been frequently identified as a source of bunt resistance (Fofana et al., 2008; Dumalasová et al., 2012; Singh et al., 2016; Muellner et al., 2021; Iqbal et al., 2023), it has not yet been determined whether all the QTL found in this region correspond to the same resistance gene(s). Borgen et al. (2023) propose that both *Bt4* and *Bt6* could be located in a region between 16.3 and 28.0 Mbp on 1B. This is in line with earlier reports locating *Bt4*, *Bt5* and *Bt6* on this chromosome (Schmidt et al., 1969; McIntosh et al., 1998).
The major effect QTL on chromosome 6DL corresponding to *Bt11* as well as the QTL on 4BS and 1BS represent highly effective sources of CB resistance that can be introgressed into breeding material. Although the intervals spanned by these loci are rather large (approximately 13-34 Mbp across populations), they could still be selected for via marker-assisted selection (MAS). Multi-parent breeding lines harbouring QTL of similar interval sizes were successfully developed through MAS as described in publication 3.

7.2 Novel common bunt resistance factors and their integration into breeding programs

CB phenotyping is tedious and time-consuming, therefore extensive field trials for screening large populations should be avoided. The key to reduce the number of lines undergoing phenotypic field testing are molecular markers enabling the selection of resistance loci in the lab. MAS can of course only be performed if the chromosomal locations of resistance factors and markers indicative of these locations are known. This information has often been derived through genetic mapping in bi-parental populations resulting from crosses between a resistant and a susceptible parent (Fofana et al., 2008; Dumalasová et al., 2012; Singh et al., 2016; Bokore et al., 2019; Muellner et al., 2021). In fact, all major *Bt*-genes that have been mapped so far were unlocked through bi-parental mapping (Menzies et al., 2006; Steffan et al., 2017b; Wang et al., 2019; Muellner et al., 2020). Following these examples, my co-authors and I identified a major CB resistance locus on chromosome 6D corresponding to the *Bt11*-gene in publication 2.

7.2.1 The *Bt11* resistance factor from the bunt differential set

It is striking that all four *Bt*-genes mapped to date have been found on one of the D-chromosomes in wheat. What is more, three out of these four resistance factors (*Bt9* (Steffan et al., 2017b; Wang

et al., 2019), *Bt10* (Menzies et al., 2006) and *Bt11*) were identified on chromosome 6D. This accumulation of *Bt*-genes on 6D led to complications: according to the results obtained in publication 2, the positions of *Bt9* and *Bt11* are overlapping on the long arm of the chromosome. Even though data support the hypothesis that the two genes are distinct resistance factors, the overlap could not be resolved due to insufficient marker polymorphisms in the region. Genotypic data in publication 2 was obtained from the 25K SNP array for wheat (Gogna et al., 2022). Although this array generally provides a reasonably high number of markers across the wheat chromosomes, one could argue that using an array containing more SNPs would be beneficial. However, Wang et al. (2019) mapped a major QTL for DB resistance corresponding to *Bt9* to 6DL using the 90K SNP iSelect Platform (Wang et al., 2014) having more than 3.5 times the number of markers and encountered the same marker scarcity in the QTL region. The candidate region they identified on chromosome 6DL had a physical size of approximately 1.2 Mbp but comprised just three distinct centimorgan-positions on the genetic map and thereby a lot of co-locating markers (Wang et al., 2019). The problem is not restricted to the 6D chromosome but appears to be common on the D-genome in general as Muellner et al. (2020) also report problems due to a lack of polymorphisms. When mapping *Bt12* to chromosome 7D, they encountered a low number of polymorphic markers and therefore obtained low mapping resolution in the QTL region. Mapping populations in Muellner et al. (2020) and publication 2 of this thesis were derived from crosses between genetically distant genotypes where high allele contrast could usually be expected.

A possible explanation for the failure of both high-density marker data and crossing between genetically distant parents in providing sufficient polymorphic markers on these chromosomes can be found in the evolutionary history of bread wheat. Since the addition of the D-genome from *Aegilops tauschii* to the originally tetraploid (AABB) wheat occurred around 8000 years ago, modern-day hexaploid bread wheat (AABBDD) is comparably young (Cox, 1997; Kihara, 1944). The hybridization even represents a bottleneck in the evolutionary history because it occurred between tetraploid wheat and a single *Ae. tauschii* lineage and was a rare event which might have happened only once or just very few times (Charmet, 2011; Wang et al., 2013). As a consequence, the D genome is characterized by lower diversity and fewer informative markers compared to the A and B genomes (Cox, 1997; Wang et al., 2013).

As a consequence from these challenges in genetic mapping of *Bt11* in publication 2, no universally applicable markers for selection of this locus could be developed. This is in contrast to mapping studies conducted by Wang et al. (2019) and Muellner et al. (2020) who both developed and validated Kompetitive Allele-Specific PCR (KASP) markers indicative for the identified resistance loci. A special situation prohibited these steps in publication 2: the four mapping populations we were using did not share SNP markers in the QTL regions for the 6DL locus. Two of them had the same peak marker, but this marker was not polymorphic in the other two populations. This missing overlap of polymorphisms between the individual populations rendered the data unfit for developing consensus flanking markers for the major resistance locus. However, as we were able to determine a candidate region comprising 2.6 Mbp on the very distal end of chromosome 6DL, researchers and breeders interested in selecting for *Bt11* could identify markers flanking this region which are polymorphic in their populations and use these for MAS.

The major QTL in publication 2 was not the only source of resistance in the mapping population but showed epistatic interactions with other loci on chromosomes 1B, 2A, 4B and 7B. Genotypes harbouring combinations of the 6D QTL with loci on 1B or 4B showed slightly higher resistance

levels compared to those possessing only the 6DL locus. In consequence, introgressing two or more of these loci into breeding material or combining them with other mapped resistance factors would be a promising strategy for the development of lines with high and stable resistance against CB.

7.2.2 Marker-assisted selection for bunt resistance loci in multi-parent breeding lines

Such an approach of stacking multiple resistance loci was followed in publication 3 and the additional contribution of this thesis. Building on plant material originating from the projects by Muellner et al. (2020) and Muellner et al. (2021), multi-parent breeding lines harbouring different combinations of the bunt resistance QTL reported in these two publications were developed through MAS. Starting from three initial resistance donors, the cultivars 'Blizzard' and 'Bonneville' and the differential line for *Bt12* PI 119333, genotypes with complex pedigrees were developed through repeated crossing with different breeding lines and cultivars showing good agronomic properties and adaptation to mid-European growing conditions. Transmission of the desired resistance loci was checked in individual generations using KASP markers flanking the QTL regions. Since phenotypic scoring of bunt resistance in artificially inoculated field trials is time-consuming, resource-intensive and can be done only once per year for winter wheat, selection of bunt resistant lines was done exclusively through MAS in the first generations. Validation of these selections by phenotypic screening in bunt nurseries started in generation $BC_3F_{2:3}$. Through this selection scheme, the advantages of MAS in terms of higher simplicity and speed but also lower costs compared to phenotypic selection were exploited. The approach also unites two traditional breeding methods for which MAS is frequently applied: (i) marker-assisted introgression of resistance loci and (ii) pyramiding of multiple loci (Hospital, 2009). A major advantage of MAS is that selection can be carried out already at the seedling stage (Collard et al., 2005) which enables screening of two generations per year in winter wheat populations instead of one if suitable greenhouse and vernalisation infrastructure is available. Thereby, time from the initial cross to generation BC_3F_2 could be reduced by more than 50 % in the additional contribution of this thesis. Another important factor reducing both time and costs of selection is the reduction of lines entering artificially inoculated field trials. Compared to the number of lines screened during MAS, only around 20 % (publication 3) or even only 10 % (additional contribution) were actually tested in field trials.

The percentage of genotypes among the whole population that were positively selected and had a resistant reaction in field screenings was roughly one third in the works comprised in this thesis. This rather low share was due to several factors which are discussed in more detail in section 7.4.1. Most importantly, the multi-parent breeding lines had complex pedigrees comprising up to ten different genotypes. This limited the choice of molecular markers available for MAS since increasing numbers of parental lines lead to an increasing chance of losing marker polymorphisms. If only a single parental line had the same allele call as the resistance donor at a certain marker position, this marker could no longer be used for selection. While this was a major setback for the selection process, the high genetic diversity in the breeding lines also posed an advantage with respect to practical breeding applications.

Using a different elite parental line in each back-crossing step or even crossing two multi-parent breeding lines which were developed that way resulted in genotypes with high variation in the elite genetic background. The rationale behind this process was to increase chances of finding lines combining favourable agronomic properties with CB resistance and also to ensure high variation for traits which are potential breeding goals in commercial breeding programs. As, for example, Collard

et al. (2005) and Xu and Crouch (2008) critizise, of the multitude of beneficial loci detected in research projects, only very few have made it into practical application in plant breeding. Or, as Bernardo (2008) puts it: "The vast majority of the favorable alleles at these identified QTL reside in journals on library shelves rather than in cultivars that have been improved through the introgression or selection of these favorable QTL alleles." Among several reasons for this phenomenon, these three publications list the lack of thorough validation of the QTL themselves and of markers which can be used to select for these loci. The work on multi-parent breeding lines presented in this thesis is an attempt to validate the QTL and associated markers detected by Muellner et al. (2020) and Muellner et al. (2021) in different genetic backgrounds. Applying MAS for these loci in genetically diverse populations of multi-parent breeding lines mimicks a situation which is, compared to bi-parental mapping populations, more similar to the multiple crosses and resulting small but diverse populations that breeders are dealing with (Hospital, 2009). The results for MAS accuracy in the multi-parent breeding lines confirm the difficulties arising from translation of QTL mapping results into different genetic backgrounds. However, publication 3 and the additional contribution show that introgression of bunt resistance loci into breeding lines and selection of lines combining resistance with desirable agronomic properties can be facilitated and accelerated through MAS. Options for improving this process are discussed in section 7.4.

7.3 Considerations about infection characteristics and phenotyping

Preparing, scoring and analysing artificially inoculated CB trials across a sequence of five years in the course of my PhD project has lead to several observations on and conclusions about the interactions between different environments, inocula and methodologies which will be discussed in the following paragraphs.

7.3.1 Quantitative and qualitative variation in infection levels

...across years

In the publications comprised in this thesis, field trials from six seasons in Austria and two seasons in the Czech Republic were analysed. Especially the multi-year data collected at the experimental station in Tulln, Austria, shows considerable quantitative variation in CB infection levels between seasons. The susceptible check cultivar 'Capo' showed 62.1 % incidence in 2015 but 81.2 % incidence in 2016 (publication 2). These variations in CB severity across years are most likely due to different environmental conditions after sowing (rainfall, temperature) and the resulting effects on colonization of the wheat seedlings described in section 1.1.1. Variations of both quantitative and qualitative nature in bunt experiments carried out over a series of years were also observed by von Kirchner (1916). He highlights the influence of both environmental factors and inoculum application on incidence levels and emphasizes that a resistant reaction of a genotype in a single year is not sufficient to classify it as resistant to CB.

For monitoring purposes, the bunt differential set was screened in the artificial inoculation trials in Tulln each year. Quantitative variation was observed in differentials susceptible to the applied inocula while qualitative variation was small across the years 2015, 2016 and 2019-2021 relevant for the publications in this thesis. Based on results from publications 1 and 2, qualitative differences between incidence levels in the hexaploid differential lines were observed for *Bt8*, *Bt9*, *Bt10*, *Bt13* and *BtP* in these years. It has to be noted, though, that contamination in the seed samples of these

differentials were recognized already in the first seasons and soon afterwards, attempts to clean the samples started (Hermann Buerstmayr, personal communication). However, since also seeds received for comparison from the Germplasm Resources Information Network (GRIN) were not pure, it was not clear which phenotype to choose and experiments were continued with the mixed samples. Varying proportions of the different subtypes in the plots from year to year are therefore the most likely reason for the observed qualitative variation in some differential lines.
The mainly quantitative changes in infection levels from year to year also show that no unintended selection of the race mixture used for inoculation took place. The initial race mix developed at IFA Tulln was a blend of spores collected from naturally infected spikes in three different locations in Austria as described in Muellner et al. (2020). Spore multiplication was initially done on a range of susceptible genotypes like the Austrian cultivars 'Midas', 'Capo' and 'Rainer'. Starting in 2019, infected heads from which spores were collected were not harvested on such highly susceptible genotypes any more but primarily from lines which showed moderate infection levels ranging between 15 % and 50 % incidence. This change was applied to ensure constant levels of virulence of the inoculum and avoid a reduction in aggressiveness over time. If spores were only harvested from highly susceptible cultivars, an unintended selection of the least aggressive races which are able to infect cultivars without any levels of resistance but possibly not genotypes with a certain tolerance might occur. Such a selection would have a negative influence on the validity of studies intended to support resistance breeding since lines with only moderately effective resistance factors would already appear to provide full protection against bunt infections when inoculated with a mildly aggressive race mixture. Gieseke (1929) and Fittschen (1939) already recommended that artificially inoculated trials should be conducted applying a blend of highly aggressive races of bunt at least in the initial tests in order to select only breeding lines with high and stable resistance. To preserve this desired virulence, spore multiplication should not be conducted on a few varieties but rather on a broad range of genotypes (Fittschen, 1939). This is exactly the procedure carried out since 2019 at IFA Tulln. Out of several populations and panels tested in a single year, lines with moderate infection levels are selected and infected ears are harvested as spore sources for trials in the following year.

...across inocula

In addition to the standard race mixture maintained in the described way, a second race mixture has been added to the inocula available at IFA Tulln in 2019. Infected ears of the bunt-tolerant cultivar 'Tilliko' were received and spores were multiplied in the same way as the standard inoculum. Since they proved to be highly virulent against *Bt10* in pre-tests and showed overall high aggressiveness, the new race mix was multiplied and gradually became the standard inoculum for artificial inoculation trials. In publication 3, both trials (2021 and 2022) were conducted with this inoculum and it was also applied on two of the mapping populations of publication 2 in 2022. While no effect of the new race mix on lines with the main resistance QTL on 6DL corresponding to *Bt11* was observed in the latter study, infection levels in lines harbouring only the additional QTL mapped on chromsomes 1B or 4B, respectively, rose considerably compared to the previous standard mix (see ESM 7 of publication 2). As implied by Gieseke (1929) and Fittschen (1939), this testing with a more virulent race mixture would have lead to a stricter selection and the exclusion of more lines if the populations had been screened in the course of a breeding program.
Experiments examining the interactions of these two standard inocula and six more race collections from Austria on a panel of 40 genotypes showed variation in the virulence spectra even though all

bunt populations originated from the comparably small region of three provinces in Eastern Austria (Ritzer et al., 2022; Rabl et al., 2023). Several lines of this panel, including the bunt differential set, were subjected to further inocula tests in publication 3 where artificially inoculated field trials from Austria and the Czech Republic were analysed. Compared to differences observed among virulence patterns of Austrian bunt populations, the variation between the aggressive standard inoculum at IFA Tulln and the bunt population used for inoculation at the Crop Research Institute (CRI) in Prague was much more pronounced. This is in line with early findings in experiments with bunt populations from different geographical origins. Although results between two test years were not consistent for all genotypes, Fittschen (1939) observed high variation in the reactions of a range of cultivars to bunt inocula collected in Cosel (Poland), Zürich (Switzerland), Breslau (Czech Republic) and Halle (Germany). In a more recent test examining virulence reactions of bunt races from many European countries, the United States and Syria on the bunt differential set, high variation between individual races was observed especially on *Bt1*, *Bt4*, *Bt6* and *Bt10* (Blazkova and Bartos, 2002). A local bunt population collected in Lithuania, on the other hand, was shown to be virulent to some extent against all *Bt*-genes except *Bt5*, *Bt8*, *Bt11* and *Bt14* (Liatukas and Ruzgas, 2008). In view of these findings, it is not surprising that the two inocula from Austria and the Czech Republic yielded largely different results in publication 3. The most important implication from such studies for breeding programs is that cultivars with bunt resistance should be developed for specific regions. Knowledge about the local race spectrum and its virulence against known resistance factors is essential to make sure that breeding efforts result in stable resistance against all bunt populations prevalent in the target region or country.

7.3.2 Scoring methods

As outlined above, the effectiveness of genetic factors conferring resistance against bunt infections in experimental populations and breeding programs needs to be validated by multi-year field phenotyping. This is best done in artificially inoculated trials which need to be visually scored for bunt incidence as no automated assessment method has yet been developed. Since infestation with CB can lead to stunting of tillers (Rodenhiser, 1931; Bressman, 1932; Fittschen, 1939) and usually causes changes in shape and colour of wheat ears (Goates, 1996), infection levels could theoretically be determined by visually estimating the percentage of ears with such a modified appearance. Based on observations and experiences made in field trials over the past years, I would, however, strongly advocate against such a scoring method. For breeding purposes, genotypes showing full or at least very high levels of resistance should be selected. These have to show only very rare cases of diseased kernels even under the unnaturally high infection pressure applied in artificially inoculated field trials. Genotypes exhibiting low numbers of infected grains per ear but a larger number of such partially infected ears should be discarded since also partial infections can cause an unacceptably high level of spore contamination. In the experiments conducted by Fittschen (1939), almost one third of all tested varieties showed partial infections and specifically those with higher levels of resistance against CB. Such partial infections are very hard if not impossible to detect by looking at an intact ear since few bunt balls per ear do not cause the typical changes in appearance that can be observed in fully bunted ones. It could therefore easily happen that a plot gets scored as uninfected by visual, "non-invasive" assessment but would reveal incidence levels up to 20 % or more when ears are cut open for scoring. The relatively high frequency of partial infections in Austrian pre-breeding material was shown in publication 3. In these trials, incidence was scored by cutting ears two times, once in

the upper third and once in the lower third. This method provides improved accuracy with respect to partial infections but is more time-consuming and tedious compared to cutting diagonally only once in the middle of the ear. In any case, ears should be cut open when scoring CB incidence since visual scoring by looking at ears cannot provide comparable accuracy if partially infected ears are present.

7.4 Methodology aspects

CB resistance was highly heritable in all trials conducted for the publications comprised in this thesis. The phenotypic variance observed in incidence levels was for the most part due to the genetic component while year effects or genotype-environment interactions explained a relatively small part of the total variance. This is in line with other studies investigating bunt resistance across multiple years (Steffan et al., 2017b; Wang et al., 2019; Muellner et al., 2020, 2021). The high heritability of bunt resistance is important when it comes to selection schemes for breeding programs. As outlined by Löschenberger et al. (2008), highly heritable traits are those which can be selected for in early generations of a program intended for organic breeding before the first yield trials are carried out. Following these suggestions, a breeding scheme run on the multi-parent breeding lines described in publication 3 and the additional contribution could comprise these steps: Throughout line development by crossing, resistance loci are selected by MAS. The selection is subsequently validated in artificially inoculated field trials and only lines with confirmed high levels of bunt resistance would enter preliminary yield trials and undergo further testing and selection.

7.4.1 Considerations on molecular markers for marker-assisted selection

An unexpected obstacle when applying MAS on multi-parent breeding lines was the high ratio of markers which were rendered unsuitable for selection due to missing polymorphisms after the addition of new parental lines to the pedigrees. Because the initial resistance donors, 'Blizzard', 'Bonneville' and PI 119333, were genetically distant to all other parents used for crossing, a high degree of polymorphism was expected. Since this expectation was not met, more thorough testing of crossing partners would have been required before conducting any crosses. To ensure that molecular markers highly indicative for the loci of interest are available for MAS throughout the whole process of line development, markers flanking the QTL region(s) should be chosen and tested for both (i) polymorphisms between the resistance donor(s) and all potential crossing partners and (ii) amplification and discriminative ability during PCR and allele scoring. Selection accuracy in publication 3 and the additional contribution was only around 30 % because markers were not diagnostic for the selected loci but rather flanking QTL regions which were, in some cases, relatively large. In addition, due to the loss of marker polymorphisms in some crosses, several loci were only screened for using markers on one side of the QTL region. When considering that MAS was carried out with non-diagnostic and, in several cases, not even flanking markers, the large potential for improving selection accuracy by using more suitable markers becomes obvious.

7.4.2 Genome-wide association mapping

Two major problems were encountered during genome-wide association mapping in publication 2: (i) low phenotypic variation due to a high number of highly resistant individuals and (ii) confounding effects arising from the genetic background. The latter is a well-known challenge for GWA analyses and has been tackled by applying mixed models which correct for genetic relationship through a

kinship matrix - an approach that actually was initially developed for animal breeding (Korte and Farlow, 2013). Mixed linear models correcting for relatedness between individuals by taking population structure and kinship into account were first proposed by Yu et al. (2006). This approach was applied by Gordon et al. (2020) when conducting GWA mapping for DB in the panel that was investigated in publication 1 of this thesis. However, mixed linear models with simple kinship matrices were not able to sufficiently correct for confounding effects in publication 1. This was presumably due to the additional challenge arising from the very low phenotypic variation in CB incidence levels compared to DB incidence in this diversity panel. Appropriate control of the genetic background while retaining enough variation was achieved by using a mixed linear model with a compressed kinship matrix for which highly similar genotypes were clustered together (Zhang et al., 2010). Several publications (Yu et al., 2006; Zhang et al., 2010; Vilhjalmsson and Nordborg, 2012) have already dealt with handling the complex genetic associations between individual assembled into the diversity panels typically used for GWAS (Tibbs Cortes et al., 2020). The difficulties encountered in publication 1 show that, in addition to population structure and kinship, the phenotypic variation in the trait of interest also needs to be considered when composing a GWA panel. Gordon et al. (2020), who initially assembled the diversity panel from the USDA National Small Grains Collection, optimized it for high variation in DB incidence levels. Taking into account that DB and CB are frequently assumed to be controlled by the same genes (Metzger and Hoffman, 1978; Goates, 1996, 2012), the conclusion seemed valid that GWA mapping for CB should be feasible in the exact same panel. The results have shown that such implications need to be tested and validated, though, since incidence levels can vary substantially between both diseases.

7.4.3 Bi-parental mapping of common bunt resistance loci

One of the main challenges when mapping bunt resistance loci in populations with PI 166910 and M822123 as the donor lines was the low number of polymorphic SNPs on the D-chromosomes. Since the major resistance conferring locus across all four mapping populations was located on the distal end of the long arm of chromosome 6D, the low mapping resolution in this area was especially troublesome. Genotypic data used for linkage map construction in publication 2 was derived from the Illumina Infinium 25 K XT array (Gogna et al., 2022) and it stands to reason that using a genotyping platform with a higher number of markers could possibly solve the problem. As mentioned in section 7.2.1, Wang et al. (2019) genotyped all lines with the 90 K iSelect array (Wang et al., 2014) and mapped a locus in a similar region on 6DL. Since the number of polymorphic markers available for linkage map construction was in a similar range compared to publication 2 in their work, increasing marker density cannot be expected to yield the desired improvements. The low number of polymorphisms available on D-chromosomes was already described in previous studies struggling to determine the precise location of bunt resistance QTL on 7DS (Chen et al., 2016; Muellner et al., 2020, 2021). Since neither a larger number of markers as in Wang et al. (2019) nor the use of genetically highly distant parental genotypes as in Muellner et al. (2020) and publication 2 has proven to effectively increase mapping resolution on the D-genome, the question remains how satisfactory fine-mapping on the respective chromosomes can be achieved.
Another important aspect when conducting bi-parental mapping are the genotypes used to generate the mapping populations. Considering the unexpected challenges arising from the fact that two genetically different sub-lines of PI 166910 were used for crossing in publication 2, it would be advisable to genotype single plants intended as crossing parents before starting to generate

populations. If genotypic data confirms that the parental lines are homogenous, they could be used for crosses. The best option would always be to use single plants for initial crosses for which extensive phenotypic and genotypic data has been obtained in pre-tests.

7.5 Conclusions and implications for future research

The results obtained and published in the course of this thesis extend the current knowledge and toolkit available for CB resistance breeding. My co-authors and I were able to identify genetic resources from a range of geographic regions that are promising targets for (pre-)breeding efforts since they provide resistance against both DB and CB. Chromosomal regions harbouring CB resistance loci were detected using genome-wide association mapping on the one hand and bi-parental mapping on the other hand. Especially a major resistance locus on wheat chromosome 6DL conferring stable and complete resistance against CB infections and corresponding to bunt resistance gene *Bt11* poses an important addition to the set of *Bt*-genes unlocked for practical breeding. The results from this thesis show that lines harbouring combinations of different loci exhibit enhanced levels of resistance and can be identified through MAS. In order to optimize this process and successfully integrate MAS into applied breeding programs, markers used for screening need to be carefully chosen, thoroughly tested and validated in a wide range of genetic backgrounds. Future research projects dealing with bunt resistance in wheat should also take into account that results about DB incidence do not necessarily allow conclusions about the performance of genotypes with respect to CB. Similarly, breeding bunt resistant cultivars should be done with a focus on a certain region as virulence patterns of local bunt populations vary considerably across regions and countries. Taking all these lessons learnt into account, research projects following up on the results presented in this work will deal with ways to optimize the process of selecting breeding lines which unite elite agronomic performance with high and stable resistance against bunt infections. Apart from generating improved information about resistance loci and their integration into breeding schemes via MAS, these future approaches will also evaluate the applicability of genomic selection with respect to bunt research. Preliminary results of a follow-up project extending the set-up outlined in the additional contribution to this thesis show that combining marker-assisted foreground and genomic-assisted background selection could be beneficial for speeding up the development of bunt resistant cultivars. After all, such cultivars are essential to enable economically and environmentally sustainable production of organic or low-input winter wheat.

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