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Determination, characterisation and combination of novel resistance genes to stripe and stem rust in wheat

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Abstract

Triticum aestivum L., commonly known as bread wheat, characterized by a chromosomal composition of 2n = 6x = 42 (AABBDD), is a significant source of dietary protein and daily calorie intake for much of the global population. Stripe rust (*Puccinia striiformis* Westend. f. sp. *tritici* Eriks) and stem rust (*Puccinia graminis* f. sp. *tritici* Erikss & E. Henning) now pose substantial threats to overall wheat production worldwide, since most rust resistance genes in wheat have been overcome by virulent rust fungus races. It is essential to enhance genetic resistance against these devastating diseases.

Wheat acquires crucial reservoirs of new resistance genes through introgressions from derivatives of *Secale cereale*, *Leymus mollis*, *Leymus racemosus*, and *Thinopyrum junceiforme.* This study systematically examined seedling resistance to various stripe rust races to identify new sources of resistance. Six wheat-rye introgression lines (SLU124, SLU125, SLU126, SLU127, SLU128 and SLU129) containing rye chromosomes 4R, 5R, and 6R were identified as carriers of previously undiscovered resistance genes against stripe rust races. Seedling assays confirmed that the stripe rust resistance in line SLU126 was retained over multiple generations. Using genotyping-by-sequencing (GBS) platforms and aligning putative GBS-SNPs with fully annotated rye NLR genes, three Kompetitive Allele-Specific PCR (KASP) markers were designed specifically for a chromosomal region at chromosome 6R, associated with two distinct stripe rust resistance genes. The development and validation of the wheat-rye cryptic translocation 6DS.6DL.6RL.6DL, featuring newfound stripe rust resistance genes, were conducted through seedling resistance assays and molecular analysis. The stripe rust resistance gene in family 29-N3-5 on the rye chromosome 6RL arm was provisionally designated *YrSLU*. Extensive molecular marker analysis and multiple-generation seedling assays revealed that stripe rust resistance in SLU124 is located on the 4RL chromosome arm of rye. Two KASP markers located on the 4RL chromosome were identified as being closely associated with two stripe rust resistance genes in resistant plants of a SLU124 population. Using marker-assisted gene pyramiding, stem rust resistance gene *Sr59* and stripe rust resistance gene *YrSLU* were combined in a single wheat genotype.

Overall, this thesis demonstrated the advantages of marker-assisted gene pyramiding in transferring multiple disease resistance genes within a single genotype. Incorporation of these resistance genes into wheat has expanded the gene pool for combating destructive diseases.

Keywords: Genotyping-by-sequencing (GBS), KASP, *Secale cereale*, marker-assisted gene pyramiding, cryptic translocation, SNP, *YrSLU*

Preface

"**My Lord**! Open my heart, and make my task easy for me, loosen the knot in my tongue, so that they may understand my speech." **(Quran 20:25-29)**

Dedication

This thesis is dedicated to:

My **Father**, in a challenging society, my father defied norms, advocating for my education as a girl.

My **Father-like,** his prayers and love were a constant presence throughout my journey.

My incredible **Mother**, her strength, kindness, and prayers shaped who I am today.

My cherished pillar, my **Husband**, your love, strength, and partnership enrich my life beyond words.

My **Son**, **Muhammad Adam Rizwan Ashraf**, your presence has fuelled my determination to succeed and make you proud.

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List of publications

The foundation of this thesis relies on the content presented in the following papers, referred by Roman numerals in the text:

I. Johansson, E., Henriksson, T., Prieto-Linde, M.L., Andersson, S., Ashraf, R. & Rahmatov, M. (2020). Diverse wheat-alien introgression lines as a basis for durable resistance and quality characteristics in bread wheat. *Frontiers in Plant Science* 11, 1067. https://doi.org/10.3389/fpls.2020.01067

II. Ashraf, R., Johansson, E., Vallenback, P., Steffenson, B.J., Bajgain, P. & Rahmatov, M. (2023). Identification of a small translocation from 6R possessing stripe rust resistance to wheat. *Plant Disease* 107(3), 720-729. https://doi.org/10.1094/PDIS-07-22-1666-RE

III. Ashraf, R., Yazdani, M., Johansson, E., Vallenback, P., Hovmøller, M.S., Patpour, M. & Rahmatov, M. (2023). Marker-assisted gene pyramiding for stem and stripe rust resistance by harnessing rye genes for wheat improvement (submitted for publication).

IV. Ashraf, R., Vallenback, P., Hovmøller, M.S. & Rahmatov, M. (2024). The physical mapping and characterization of the rye 4R chromosome-derived stripe rust resistance gene (manuscript).

Papers I and II are reproduced with the permission of the publishers

Rimsha Ashraf's contributions to the included papers in this thesis are described as follows:

I. Planned and conducted the laboratory work, which involved developing a protocol for SSR analysis and selection of SSR markers to detect rye chromatin in wheat-rye introgression lines, and contributed to writing and editing the final manuscript.

II. Planned the experiment together with the supervisors, conducted the SSR and KASP marker analysis in all generations and wrote the first draft of the manuscript. Contributed to writing and editing the final manuscript.

III. Planned and conducted the experiments, which involved analysis of stripe rust seedlings and markers over several generations, and wrote the manuscript with input from the co-authors.

IV. Designed the study together with the supervisors, conducted the experiments, performed the stripe rust seedling analysis, analysed the data over several generations and wrote the manuscript.

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Abbreviations

BGRI	Borlaug Global Rust Initiative
FISH	Fluorescence in situ hybridisation
GBS	Genotyping-by-sequencing
GBS-SNP	Genotyping-by-sequencing-single nucleotide polymorphism
GP	Gene pool
GRRC	Global Rust Reference Centre
IWGSC	International Wheat Genome Sequencing Consortium
KASP	Kompetitive allele-specific polymerase chain reaction
NLR	Nucleotide-binding and leucine-rich repeat
NLR-GBS	Nucleotide-binding and leucine-rich repeat-genotyping-by-sequencing
PCR	Polymerase chain reaction
SSR	Simple sequence repeat
SNP	Single nucleotide polymorphism
SLU	Swedish University of Agricultural Sciences
RGSCR	Rye genome sequencing consortium reference

1. Introduction

In 2021, global wheat production hit a record high of 770.9 million tons, reaffirming its critical role as a staple food and vital calorie source in the global diet and reflecting its cultural and nutritional significance (FAOSTAT, 2023). In terms of production, mean annual wheat grain production world-wide (752 M ton) is slightly lower than that of rice (768 M ton) and much lower than that of maize (1146 M ton, with some 57% used as animal feed) (Erenstein et al. 2022). Therefore wheat production and trade have immense significance for millions of people who rely on wheat for their fundamental daily sustenance. Today, wheat is the second largest staple crop (after rice) used for human consumption worldwide (FAOSTAT, 2023).

Over the past century, plant breeding has provided high-yielding crops, supporting rising population growth. In the future, utilisation of modern breeding technologies will lead to next-generation crop varieties that meet the demands of further expected population growth. By 2050, the human population is expected to reach over 10 billion and demand for wheat is projected to rise by 60% between 2014 and 2050 (Scott et al. 2021). To meet this demand, breeders and plant scientists need to develop crops and crop varieties with higher yields, enhanced nutrition content, pest and disease resistance and climate adaptability (Hickey et al. 2019).

The process of crop domestication began with field observations by smallholder farmers, evolved to include use of intentional breeding and empirical methods and has been driven by scientific knowledge for over a century (Charmet 2011; Lupton 1987). However, the United Nations Food and Agriculture Organization (FAO) estimates that up to 40% of crop production world-wide is lost to pests, with plant diseases causing economic losses exceeding USD 220 billion and invasive insects accounting for at least USD 70 billion each year (FAO-IPPC Secretariat, 2022). In the specific case

of wheat, diseases and pests cause around 20% production losses globally, amounting in total to 210 million tons of wheat grain per year, which is enough to bake an estimated 290 billion loaves of bread (Savary et al. 2019; Wulff and Krattinger 2022).

World-wide, outbreaks of new races of rust fungi (bio-trophic pathogens) are posing a substantial threat to global food security. There are three main rust diseases distributed globally which cause major yield reductions in wheat. These are: stripe (yellow) rust, caused by *Puccinia striiformis* f. sp. *tritici*; leaf (brown) rust, caused by *Puccinia triticina* Eriks; and stem (black) rust, caused by *Puccinia graminis* f. sp. *tritici* (Bolton et al. 2008; Leonard and Szabo 2005; Liu and Hambleton 2010). In particular, stripe rust and stem rust are highly destructive wheat diseases worldwide, capable of decimating entire crops under favourable conditions (Badebo 2002; Panel 2005; Roelfs 1985a).

To cope with the threat posed by rust diseases, more efforts are required to develop durable cultivars with high-level resistance, in order to slow the spread of the pathogen and reduce yield losses. Integrating cutting-edge technologies with wheat genomic approaches in order to create effective, disease-resistant wheat cultivars can underpin other efforts to meet the challenge of feeding a future global population of 10 billion. Therefore, genomic advances are essential for revealing host-pathogen interactions, pathogen monitoring and resistance breeding (Bouvet et al. 2022).

2. Background

2.1. Stripe rust

A study in Sweden by Jacob Eriksson in the 1890s was the first to classify stripe rust into different *formae speciales* based on its specialisation to different genera of grasses and cereals (Eriksson 1894). The stripe rust fungus belongs to the genus *Puccinia* (about 4000 species) in the family *Pucciniaceae*, order Pucciniales, class Pucciniomycete and division Basidiomycota of the fungi kingdom (Kirk et al. 2008).

Fig.1 *Puccinia striiformis* (Photo: GRRC)

Puccinia striiformis Westend. is the current scientific name of the stripe rust fungus. The wheat stripe rust strain (Fig. 1), formally named *Puccinia striiformis* Westend. f. sp. *tritici* Erikss., is the most economically important *formae speciales* (Chen 2020; Chen and Kang 2017).

2.1.1 Life cycle of stripe rust

Puccinia striiformis is a heteroecious macrocyclic fungus with five spore stages (uredinial, telial, basidial, pycinial and aecial) and requires a cereal or grass plant and an alternate host plant (*e.g*. *Berberis* spp., *Mahonia* spp.) (Fig. 2) for completing its sexual lifecycle (Jin et al. 2010; Wang and Chen 2013). Among the five spore stages, the urediniospore stage is produced in multiple cycles on host plants (cereal or grasses) and is the most significant for persistence of the pathogen and onset of stripe rust disease. This asexual

cycle can repeat many times during the host's growing season, leading to a severe rust epidemic in the cereal crop. During late growth stages, teliospores are produced, have no or very short dormancy and when they germinate, each produces a basidium bearing four basidiospores (Chen 2017). Basidiospores infect the alternate host (*e.g. Berberis* spp.) to complete the sexual cycle, leading to pycniospores and aeciospores on those leaves through fertilization (Kang et al. 2017). Aeciospores infect primary host plants, giving rise to

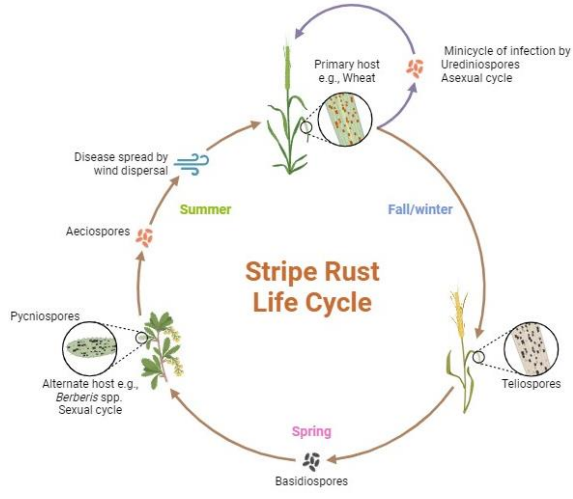

Fig. 2 Life cycle of stripe rust fungus, *Puccinia striiformis* f. sp. *tritici*

production of urediniospores (Jin 2011; Zhao et al. 2016). This complete life cycle was not known until 2010, when it was discovered that the basidiospores of wheat stripe rust fungus are able to infect barberry plants (*Berberis* spp.) and produce aeciospores that in turn are able to infect wheat under controlled conditions (Jin et al. 2010).

2.1.2 Stripe rust in Europe

In 2011, emergence of two new stripe rust races, Warrior (PstS7) and Kranich (PstS8), disrupted the European disease landscape, leading to epidemics in multiple wheat varieties (Hovmøller et al. 2011; Hovmøller et

al. 2016; Hubbard et al. 2015; Rahmatov 2016). Moreover, race PstS10 (known as 'Warrior (-)'), which was first detected in Europe in 2012, has become prevalent in most parts of Europe since 2014. Another lineage consisting of races prevalent on triticale in Northern Europe, named PstS4, has become highly dominant in Europe (Ali et al. 2017; Hovmøller et al. 2011) (www.wheatrust.org).

2.2 Stem rust

Stem rust, caused by *Puccinia graminis* f. sp. *tritici*, (has historically been the major threat to wheat production, causing high yield losses (Singh et al. 2015). The wheat stem rust pathogen is heteroecious, undertaking asexual reproduction on cereals and grasses and requiring an alternate host (*Berberis* spp.) to complete its sexual life cycle (Leonard and Szabo 2005). During the second half of the 20th century, stem rust became less prevalent in Europe as efforts to eradicate common barberry (*Berberis vulgaris*) were introduced (Roelfs 1982; Stakman 1923).

Fig. 3 *Puccinia graminis* (Photo: GRRC)

In 1998, the disease landscape changed when a new highly virulent stem rust race, Ug99 (TTKSK), with virulence to wheat resistance gene *Sr31*, emerged in Uganda and spread across Africa and western Asia (Pretorius et al. 2000). Approximately, 80% of the world's wheat varieties have become vulnerable to the infamous Ug99 race (Singh et al. 2011). Its emergence led to the creation of the Borlaug Global Rust Initiative (BGRI), which now plays a crucial role in developing Ug99-resistant wheat varieties (McIntosh and Pretorius 2011). So far, 13 races of the Ug99 stem rust lineage have been found, affecting wheat production in 13 countries across East Africa and the Middle East (Bhavani et al. 2019). During 20th century, several

notable wheat stem rust epidemics resulted in substantial yield losses of wheat (Roelfs 1985b; Singh et al. 2015).

2.2.1. Stem rust in Europe

In 1994 the barberry eradication laws in Europe were changed, resulting in emergence of new stem rust races, leading to high stem rust disease incidence on wheat and barberry (Berlin et al. 2012). During 2013, wheat stem rust re-emerged in Sweden, Germany, Denmark and the United Kingdom (Saunders et al. 2019). After the occurrence of these 'early warning' infections, in 2016 a significant outbreak of wheat stem rust affected thousands of hectares of both durum and bread wheat in Sicily (Bhattacharya 2017).

Sweden experienced a wheat stem rust outbreak affecting wheat and barley in the growing season of 2017, which was characterised by cool temperatures and an unusually wet growing season. Since then, stem rust has been consistently observed annually on wheat, barley and rye crops in Sweden (Kjellström 2021). The stem rust races TTRTF, TKTTF and TKKTF are highly prevalent races identified in Sweden (Patpour et al. 2022). Furthermore, numerous highly virulent stem rust races have been detected in other European countries, all exhibiting a clear association with barberry (Barnes et al. 2020; Villegas et al. 2022). This increasing vulnerability of European wheat varieties highlights the pressing need for new breeding initiatives to find effective resistance sources.

3. Genetic Resources of Wheat

For almost a century, farmers, breeders and cytogeneticists have utilised the genetic diversity of wild relatives to produce resistant varieties of crops to biotic and abiotic stresses. Many scientists. such as G. O'Mara, Ralph Riley, Ernie Sears and others, have played an important role in wheat improvement through alien introgression by finding disease resistance sources.

3.1 Gene pool

A crop "gene pool concept" has been proposed, based on the crossing ability, taxonomy and genetic relatedness of species (Harlan and de Wet 1971). Drawing upon evolutionary divergence and genomic composition as criteria (Jiang et al. 1993), the concept has been expanded to cover three distinct gene pools: primary, secondary and tertiary.

Following this concept, three main categories have been established for wheat:

The primary gene pool (GP-1) consists of primitive domesticated forms of wheat, landraces and closely related wild species (also called ancestral species) which can be hybridised directly by simple breeding techniques (crossing, selection and backcrossing). These include *Triticum aestivum* (AABBDD), *e.g*. hexaploid spelt (*Triticum spelta* AABBDD), tetraploid *Triticum turgidum* (AABB), diploid *Triticum monococcum* (AA), *Triticum dicoccoides*, *Aegilops tauschii* (DD) and landraces of hexaploid and tetraploid wheat (Feuillet et al. 2008).

The secondary gene pool (GP-2) consists of less closely related *Triticum* and *Aegilops* species sharing at least one homologous genome with wheat. Conventional breeding and manipulative methods can be used, with *Ae. speltoides* (SS genome) and *Triticum timopheevii* (AAGG) being part of this pool. Apart from some allelic and structural variations, the genome of species belonging to the primary and secondary gene pools is homologous to that of wheat (Moskal et al. 2021).

The tertiary gene pool (GP-3) includes diploid and polyploid species from the tribe *Triticeae*, direct hybridisation with which is not possible due to their homoelogous genome (related but not identical) to that of wheat. There are a large number of such species from numerous different genera, *e.g. Agropyron, Pseudoroegneria*, *Psathyrostachys*, *Thinopyrum, Elymus, Secale cereale, Hordeum vulgare* and *Leymus* species (King et al. 2022).

3.2 Rye for wheat resistance breeding

Rye (*Secale cereale* 2n=2x=14, RR) has been commonly used as a source of elite genes in wheat improvement programmes for more than 60 years. High collinearity of the genome of these two cereal species allows interspecies chromosomal translocations and substitutions. Therefore, rye chromatin has been used to transfer several genes for resistance to biotic and abiotic stresses into the wheat genome (Moskal et al. 2021) (Table 1).

In addition, wheat is hexaploid and can easily tolerate important changes in its genome. This has led to the development of various genetic stocks with different types of modified lines, *e.g.* some forms of this chromosomal modification have been used to produce monosomic, telocentric, deletion and nullisomic lines (Lukaszewski 2015). Plant materials of this kind have played a pivotal role in genetic investigations aimed at developing wheat cultivars resistant to diseases.

3.3 Tapping rye chromosomes

A key introgression in the history of wheat was the 1RS-1BL chromosomal translocation from rye created in the early 20th century. It increased wheat yield and tolerance to biotic and abiotic stresses and is still present in several important cultivars currently in use.

Some useful stripe rust and stem rust resistance genes have been successfully transferred from *Secale* spp. (Table 1). New sources of genetic diversity are essential for improving wheat yield, adaptation to climate change and resistance to biotic stresses.

Table 1. *Currently identified and temporarily designated resistance genes to the stripe rust and stem rust pathogens in wheat-rye and triticale stocks*

Gene symbol	**Chrom -osome**	***Secale cereale* (rye) cultivar**	**Translocation**	**Reference**s
*Yr*9	1RS	Petkus	1BL.1RS	(Rahmatov et al. 2017; Shi et al. 2001)
*Yr*CN17	1RS	Petkus-L155	1BL.1RS	(Ren et al. 2009; Tang et al. 2008)
*Yr*R212	1RS	L155, R12	1BL.1RS	(Luo et al. 2008)
yrCH45-1	1RS	SW1862	1BL.1RS	(Yang et al. 2016)
*Yr*83	6RL	T-701a	6R(6D)	(Li et al. 2020a)
*Sr*31	1RS	Petkus	1BL.1RS	(Rahmatov et al. 2016c)
*Sr*50 (SrR)	1RS	Imperial	1DL.1RS	(Mago et al. 2002; Mago et al. 2004; Mago et al. 2015; Yu et al. 2014)
*Sr*1RSAmigo	1RS	Insave	1AL.1RS	(Friebe et al. 1996; Liu et al. 2014; Mourad et al. 2019)
*Sr*7b	1RS		1DL.1RS,3DL.3 RS	(Rahmatov et al. 2016c)
*Sr*36	1RS		1R(1D)	(Rahmatov et al. 2016c)
Sr59	2RL	VT828041a	2DS.2RL	(Rahmatov et al. 2016a)
SrBj, SrJ	2R	×*Triticosecale*		(Adhikari and McIntosh 1998; Olivera et al. 2013)
SrNin	2R	×*Triticosecale*		(Adhikari and McIntosh 1998)
SrVen	2R	×*Triticosecale*		(Tyrka and Chelkowski 2004)
*Sr*27	3RS	Imperial	3AS.3RS, 3AL.3RS, 3BL.3RS	(Marais 2001; Marais and Marais 1994)
SrSatu	3R	×*Triticosecale*	3R(3D)	(Rahmatov et al. 2016c)
SrLa1, SrLa2	3R	×*Triticosecale*		(Adhikari and McIntosh 1998)

4. Aims and Objectives of the Research

The main aims of the research presented in this thesis were to: (i) identify new resistance sources against stripe rust in various wheat-alien introgression lines; (ii) confirm presence of stripe rust resistance genes in Swedish wheat-rye introgression lines; (iii) characterise the genetic basis of resistance from these sources, using conventional and novel genomic tools; (iv) apply marker-assisted gene pyramiding for stripe rust and stem rust resistance; and (v) integrate specific resistance genes for stripe rust and stem rust into locally adapted wheat cultivars, thereby enhancing sustainability in wheat production.

Specific objectives of the work presented in Papers I- IV were to:

- Evaluate wheat-alien introgression derivatives from *Secale cereale*, *Leymus mollis*, *Leymus racemosus* and *Thinopyrum junceiforme*, for the purpose of identifying new sources of resistance to improve production and quality (Paper I)
- Identify and characterise stripe-rust resistance genes in wheat-rye introgression lines, using conventional methods and novel genomic tools (Paper II and IV)
- Develop KASP markers using genotype-by-sequencing for these resistance genes, in order to facilitate their use in future breeding programmes (Paper II and IV)
- Transfer stripe rust resistance genes to wheat and identify molecular markers associated with resistance gene (Paper II and IV)
- Apply marker-assisted gene pyramiding for stripe rust and stem rust resistance genes (Paper III)
- Perform molecular and cytogenetic validation of wheat-rye introgression lines (Paper II and IV)

5. Materials and Methods

5.1 Plant material resources

Numerous winter wheat-alien introgression lines have been developed by crossing Swedish winter triticale lines SV856003, SV876012, SV876032 and AD99 wheat-Leymus hybrid with winter hexaploid wheat cultivars ('Goerzen,' 'Holme' and 'Kraka') (Forsström and Merker 2001). A large number of spring wheat-rye hybrid lines have also been developed by crossing the Mexican spring hexaploid triticale cultivars 'Beagle' and 'Drira' with the well-known Swedish spring hexaploid wheat cultivars 'Drabant', 'Prins', 'Sonett' and line SV77328 (Merker and Rogalska 1984).

Using embryo culture techniques, several wheat-*Leymus* hybrids have been developed, *e.g*. wheat with *Leymus mollis*, wheat with *Leymus racemosus* and wheat with *Thinopyrum junceiforme*. These hybrids have been subjected to a single backcross with both Hpph 5RL·5BS wheat-rye translocation line and *Triticum turgidum* var. *carthlicum* (Merker and Lantai 1997).

The lines resulting from these different crosses possess rye chromosomes 1R, 2R, 3R, 4R, 5R and 6R as individual disomic substitutions. Moreover, some lines contain wheat-rye translocations such as 1DL·1RS, 1BL·1RS, 2RL·2BS, 3DL·3RS and 5AL·5RS, while other lines feature various combinations of rye chromosome substitutions, such as 1R+2R, 1R+3R, 1R+6R, 5R+4R+7R and 1R+6R+4R+7R (Merker 1979; Merker and Rogalska 1984). The material used in this thesis comprised: wheat-rye introgression lines, which were used for seedling analysis against several races of stripe rust in Papers I-IV; wheat lines with introgressed chromatin

from *Leymus mollis*, *Leymus racemosus* and *Thinopyrum junceiforme* (Ellneskog-Staam and Merker 2001, 2002), which were used in Paper I; and spring wheat-rye introgression lines SLU124, SLU125, SLU126, SLU127, SLU128 and SLU129, with their origin in crosses of hexaploid triticale line SV876012 with winter wheat cultivar Holme (Forsström and Merker 2001), which were used in Papers I-IV. The lines SLU124 and SLU126 were crossed with the 'Chinese Spring' (CS) *ph1b* mutant in order to induce meiotic recombination between 6R and 6D homoeologous regions (Papers II and IV).

5.2 Marker-assisted gene pyramiding

Line TA5095, containing the *Sr*59 gene conveying resistance to stem rust races (Rahmatov et al. 2016a), line #392 from SLU126 containing the *YrSLU* genes conveying resistance to the major stripe rust races (PaperII), and three commercial varieties were crossed in Paper III. Previously developed KASP Markers for genes *Sr59* and *YrSLU* were used to select plants in each generation.

5.3 Seedling resistance evaluation to stripe and stem rust

Stripe rust seedling resistance tests were conducted at the University of Minnesota in St. Paul, USA. Six wheat genotypes (SLU124 to SLU129) were evaluated together with five USA stripe rust races (named PSTv-14, PSTv-37, PSTv-40, PSTv-218 and PSTv-221) (Papers II and IV). Seedling analysis was carried out at the Global Rust Reference Center (GRRC), Aarhus University, Flakkebjerg, Denmark, against stripe rust races DK229_19, ET58/21, DK09_11sp and DK69/15 according to Sørensen *et al*. (2016) (Papers III and IV). Using a similar protocol, seedling analysis of wheat-alien introgression lines against a wide array of stripe rust races was also carried out at GRCC (Paper I). *Puccinia striiformis* was evaluated using a scale ranging from 0 to 9, with the scoring procedure conducted 16 days post-inoculation, in accordance with the methodology described by McNeal et al. (1971) (Papers I-IV).

Stem rust seedling resistance tests against the stem rust races TTKSK, TTRTF and TTTTF were conducted at GRRC Aarhus. To characterise

severity of seedling infection with *Puccinia graminis*, a scale of 0 to 4 was used, with scoring performed 14 days after inoculation as described by Stakman *et al*. (1962) (Paper III).

5.4 Marker-assisted selection

The line SLU126 was crossed with the '*CS' ph1b* mutant to induce meiotic recombination between the 6R and 6D homoeologous regions. The resistant plants of the BC_1F_1 generation were assessed using the touchdown molecular markers Xpsr128, Xpsr574 and Xawjl3 to detect homozygous *ph1b* plants (Roberts et al. 1999). In addition, previously developed polymorphic SSR markers for the 4R, 5R and 6R rye chromosomes (Khlestkina et al. 2004; Li et al. 2013; Li et al. 2018; Martis et al. 2013) were used in this thesis to confirm presence of rye chromosomes in resistant plants of BC_1F_1 and BC_1F_2 (Papers I, II and IV). The BC_1F_2 population obtained (selfed from BC_1F_1 plants) and plants of the BC_1F_1 (backcrossed to CSA and SLU820) were phenotyped at the seedling stage with the mixture of stripe rust races (PSTv-14+PSTv-37). Later, the $F_{3:4}$ populations were also phenotyped with additional stripe rust races, namely PSTv-40, PSTv-218 and PSTv-221. These were genotyped with molecular markers for the presence of the 6RL chromosome (Paper II).

5.5 Genotyping-by-sequencing (GBS) of parental lines

Cytogenetic fluorescence *in situ* hybridisation (FISH) analysis was unable to detect the translocation in the selected material in this thesis, because of the very small size of the translocation from rye to wheat. In order to achieve small alien transfers in wheat without encountering linkage drag, it is imperative to employ high-density molecular markers like single nucleotide polymorphism (SNP) obtained from genotype-by-sequencing (GBS) data. This enables precise detection of even the tiniest translocation or introgression breakpoints within the wheat relatives. Genotype-by-sequencing has been applied previously at the Genomic Center, University of Minnesota, with parental lines CS *ph1b*, CSA, Holme, Sv876012, SLU820, SLU124 and SLU126 (Poland et al. 2012), by aligning with wheat

(International Wheat Genome Sequencing et al. 2018) and rye reference genomes (Rabanus-Wallace et al. 2021).

Following filtration of SNP markers by using the Burrow–Wheelers Alignment tool (BWA) v0.7.15 (Li and Durbin 2009), a total of 12,195 SNPs on chromosome 4R, 12,660 on chromosome 5R and 10,675 on chromosome 6R were retained for subsequent analysis.

5.6 Development of KASP markers

By aligning the GBS reads to the rye nucleotide-binding and leucine-rich repeat (NLR) genes, the physical locations of SNPs associated with NLR genes on the 4R, 5R, and 6R chromosomes in the parental lines were identified. KASP primers were designed using a 120-base pair flanking sequence (with 60 bases upstream and 60 bases downstream) surrounding the NLR-GBS position. This approach enabled conversion of chromosome-specific NLR-GBS markers into KASP primers. Next, 13 (4R), nine (5R) and 38 (6R) KASP primers with two allele-specific forward primers with FAM- (5´GAAGGTGACCAAGTTCATGCT3´) and HEX-(5´GAAGGTCGGAGTCAACGGATT3´) compatible tails and one common reverse primer were developed and screened for polymorphisms between two parents, the SLU126 line and the *CS ph1b* mutant. Finally, the KASP markers were used to analyse the F_4-resistant plants (Paper II). Furthermore, MapChart (https://www.wur.nl/en/show/mapchart.htm) was used to draw a physical map (Papers II and IV). Existing KASP markers for genes *Sr59* (Rahmatov et al. 2016a) and *YrSLU* (Paper II) were validated for gene pyramiding in Paper III.

6. Results and Discussion

6.1 Stripe rust seedling analysis

Six wheat-rye introgression lines (SLU124, SLU125, SLU126, SLU127, SLU128 and SLU129) containing rye chromosomes 4R 5R and 6R were identified as possessing broad-spectrum resistance against 30 stripe rust races at both seedling and adult stage (Papers I, II and IV). These lines proved to be resistant to five North American stripe rust races (PSTv-14, PSTv-37, PSTv-40, PSTv-218 and PSTv-221), as well as the Warrior and Kranich races (dominant in Europe), confirming presence of novel stripe rust resistance genes (Papers II and IV).

Fig.4 Reaction to *P. striiformis* f. sp. *tritici* (*Pst*) race PSTv-14+PSTv-37 of SLU126 and Family 29N3-5(right side), reaction of *Pst* race DK09_11sp of SLU124 and family #16 (left side).

Segregation distortion of stripe rust resistance against a mixture of stripe rust races (PSTv-14+PSTv-37 Fig. 4) in the BC_1F_1 population (CSph1b x SLU126 x CSph1b) and BC_2F_1 population (29-N3-5 x CSA and 29-N3-5 x

SLU820) was observed (Paper II). Similarly, a significant segregation distortion was observed against stripe rust races (PSTv-14, PSTv-37 and DK09_11sp) in the BC_1F_1 population (CSph1b x SLU124 x CSph1b), suggesting the presence of additional gene(s) (Paper IV).

Stably resistant plants of family 29-N3-5 at BC_1F_4 were checked against additional races (PSTv-40, PSTv-218 and PSTv-221), which resulted in a similar resistance response to PSTv-40 and to the mix PSTv-14+PSTv-37, while clear segregation (IT 23/78) in resistance response was noted to the PSTv-218 and PSTv-221 races. The segregation pattern for the stripe rust races used in evaluations indicated possible presence of more than one resistance gene in the BC_1F_4 lines (Paper II).

Moreover, lines 16-8-1, 16-8-2, 16-8-3 and 16-8-4 of the BC_1F_4 generation exhibited IT 23/56 against the DK09_11sp stripe rust race (Fig. 4), indicating the potential presence of additional resistance genes (Paper IV). However, lines carrying genes *Sr59* and *YrSLU* showed varied stripe rust resistance in seedling assays, with higher susceptibility to race DK09/11sp and segregation observed for races DK229_19, ET58/21 and DK69/15, with most families susceptible to DK09/11sp.

Stripe rust and powdery mildew resistance genes have been identified on rye chromosomes 4R, 5R and 6R in several previous studies, with the findings indicating that 4R and 6R in particular are potential sources of stripe rust resistance (An et al. 2019; An et al. 2015; Li et al. 2020b; Schneider et al. 2016; Xi et al. 2019). Segregation distortion is a prevalent phenomenon observed on crossing wheat with wild relatives (Marais et al. 2010).

Segregation distortion has also been reported for a novel 2DS·2RL Robertsonian translocation developed using CS *ph1b* mutant lines (Rahmatov et al. 2016a). The emergence and spread of new virulent races have profoundly impacted stripe rust epidemics on a global scale (Ali et al. 2017). Hence, it is imperative to identify novel stripe rust resistance genes for integration into the national wheat breeding programme, is a cost-effective and eco-friendly approach to developing resistant lines.

6.2 Evaluation of stem rust

The wheat-rye disomic substitution lines and wheat-*T. junceiforme* lines were found to carry resistance genes against a wide array of stem rust races

at seedling stage (TTKSK, TTKST, TTTSK, TRTTF, TTTTF, TPMKC, QTHJC, RKQQC, TKTTF, MCCFC) and to some stem rust races at adult plant stage (TTKSK + TTKST and TKTTF). In the evaluation of stem rust seedlings, certain wheat–rye substitution lines and wheat-*T. junceiforme* lines were pinpointed as potential carriers of new stem rust-resistance gene/s. (Paper I).

The founder line TA5095 (*Sr59*) used for gene pyramiding showed resistance at seedling stage to two African stem rust races (TTKSK and TTRTF) and one North American race (TTTTF) (Paper III). The results also showed that the lines developed contributed to pyramiding of genes, resulting most likely in strong resistance to most stem rust races, as has previously been reported for lines carrying gene *Sr59* (Rahmatov et al. 2016a), while the resistance to stripe rust from *YrSLU* is strong for some races, but less strong for others (Paper II). Several effective resistance genes to stem rust have been transferred from the secondary and tertiary gene pools and are now being used in wheat breeding (Singh et al. 2015). One of the most successful stem rust resistance genes, *Sr31*, has been extensively utilised, delivering long-lasting protection against stem rust races in agriculture for over three decades (Singh et al. 2008). Stem rust resistance gene *Sr59* confers a high level of resistance to several stem rust races, including Ug99 (Rahmatov et al. 2016a). Presence of several resistance genes, directed at either similar or different diseases, improves the durability and effectiveness of cultivar (Ellis et al. 2014; Paillard et al. 2012). Therefore, resistance gene pyramiding may contribute to increased life span of each of the resistance genes through a synergistic effect of the genes (Klymiuk et al. 2019).

6.3 Wheat-alien introgression lines

The work in this thesis demonstrated that wheat-alien introgression lines derivatives from *S. cereale*, *L. mollis*, *L. racemosus* and *T. junceiforme* are potential sources of resistance to stripe rust, stem rust, powdery mildew and Fusarium head blight disease. In addition, these lines were found to exhibit high yield, good agronomic performance and high allelopathic potential. Further analysis showed that these wheat-alien introgression lines are a good

source of iron and zinc, while the low cadmium concentration makes them a promising potential genetic resource for wheat improvement (Paper I).

Wild relatives of wheat are a rich source of micronutrients and show high allelopathic potential (Bertholdsson et al. 2012; Borrill et al. 2014). A number of stripe rust and stem rust resistance genes, such as *Sr59, Yr83* and many more, have been identified using wheat relatives (Borrill et al. 2014; Li et al. 2020b; Rahmatov et al. 2016b).

6.4 Empirical verification of molecular markers

Simple sequence repeat (SSR) molecular markers were used specifically for targeting presence/absence of rye chromosomes, *i.e*. 4R, 5R and 6R. Swedish wheat-rye introgression lines, identified through SSR analysis as having chromosomes 4R, 5R and 6R, were found to possess novel genes conferring resistance to stripe rust (Papers I and IV). The SSR markers used were unable to detect rye chromatin in the wheat-rye introgression lines, due to the very small translocation (Paper II). However, presence of rye chromosome 4R in the SLU124 population over several generations was confirmed using these SSR markers (Paper IV).

Polymerase chain reaction (PCR)-based molecular markers are useful for detecting rye chromatin within wheat-rye introgression lines (Khlestkina et al. 2004; Li et al. 2013; Li et al. 2018; Martis et al. 2013). Cytological methods like FISH are widely used for identifying wheat-alien introgression lines, but have proven to be unsuitable for small chromosomal translocations (Tiwari et al. 2014). Therefore, molecular markers are essential for detecting small alien chromosome segments in the wheat genome.

6.5 FISH analysis

FISH analysis was performed to detect *Secale cereale* chromosomes in the wheat genome of parental line SLU126. However, it was not detected in resistant plants in later BC_1F_4 and BC_2F_3 generations of the SLU126 population, due to the very small size of the rye chromosome translocation (Paper II). Therefore, it was necessary to develop molecular markers to detect the cryptic translocation (Paper II). Similarly, cytogenetic analyses

were unable to detect cryptic translocations in previous studies, necessitating the use of molecular marker assays for their identification (Caceres et al. 2012; Fu et al. 2013).

6.6 GBS analysis

In parental lines SLU126 (Paper II) and SLU124 (Paper IV), 13 NLRs were detected on rye chromosome 4R, nine on chromosome 5R and 38 on chromosome 6R using fully annotated rye NLR genes to GBS reads. The GBS approach reveals high density polymorphisms among parental lines, which gives a high-density physical map. Additionally, a total of 25 NLR genes were physically mapped and identified within the 6RL segment from 645,846,469 to 879,975,498 bp (Paper II) and 12 NLR genes within the 4RL segment (Paper IV). Selected SNPs surrounding NLR gene positions on the 6RL and 4RL chromosomes were used to develop KASP markers.

The NLR protein family plays an important role in activating the plant immune system by detecting specific microbial effectors (Kourelis and Van Der Hoorn 2018). There are more stripe rust resistance genes on rye chromosome 6R than on 4R and 5R, but 4R is also a potential source of rust resistance genes (An et al. 2019; Li et al. 2016). Therefore, rye chromosomes 6R and 4R were used in this thesis to identify novel stripe rust resistance genes. A previous study by Tiwari *et al*. (2014) used GBS technology for high-throughput SNP markers, facilitating mapping and cloning of novel genes from wild wheat relatives.

6.7 Development and characterisation of a new cryptic translocation (6DS.6DL.6RL.6DL)

Seedling resistance screening showed that lines with a cryptic translocation, possibly of the form 6DS.6DL.6RL.6DL, exhibited a highly resistant response to all diverse stripe rust races against which were tested (Paper II). In Paper II, transfer of a small wheat-rye translocation containing a stripe rust resistance gene was induced by the CS *ph1b* mutant into the wheat genome.

Presence of the resistance gene at the distal part of 6RL was verified with physical mapping and molecular markers (KASP), and the gene was

designated *YrSLU* (Paper II). The cryptic translocation 6DS.6DL.6RL.6DL is undetectable by cytogenetic analysis due to very small size, meaning that molecular markers are necessary, and therefore KASP markers were developed and validated. Transfer of small alien chromatin fragments minimises linkage drag and undesired traits, enhancing breeder-friendly cultivar development.

The *Ph1* gene in wheat controls homologous chromosome pairing, inhibiting recombination between homoeologous chromosomes (Roberts et al. 1999). The CS *ph1b* mutant markedly decreases the size of chromosome segments introgressed with resistance genes, including *Sr39, Sr43, Sr59* and *Yr83* (Li et al. 2020b; Niu et al. 2011; Niu et al. 2014; Rahmatov et al. 2016a). Several studies have highlighted the importance of molecular markers for detection of cryptic introgressions, such as those from *Aegilops geniculate*, *Thinopyrum intermedium and S. cereale* (Dong et al. 2004; Fu et al. 2014; Kuraparthy et al. 2007). This thesis identified the novel resistance gene *YrSLU*, which is effective against diverse stripe rust races through a very small translocation of rye chromosome within wheat.

6.8 Development of KASP markers

KASP assays, designed with SNP sequences from GBS data, were first validated in parental lines SLU124, SLU126 and the CS *ph1b* mutant. Among the 38 KASP markers employed, three successfully distinguished the CS *ph1b* mutant from SLU126 (Paper II). Thereafter, resistant plants in the BC_1F_4 and BC_2F_3 generation were validated by the three KASP markers, but the CS *ph1b* mutant and susceptible plants were not detected.

The 375C1 KASP marker is at 868,123,650 bp, while markers 387C2 and 392C1 are at 873,285,080 bp and 873,285,112 bp, respectively. This 5,161,430 bp gap indicates two individual genes found to co-segregate (Paper II).

Among the KASP primers with 12 NLR-GBS markers from 4RL, three (77C1, 90C1, 46C1) were identified as distinguishing SLU124 from lines CS *ph1b* and CSA. Two of these primers (77C1 and 46C1) effectively distinguished resistant and susceptible BC_1F_3/F_4 plants, suggesting that rye chromosome 4R is the sole source of observed resistance. Further seedling

and cytogenetic analyses are needed for population advancement (Paper IV). The KASP markers and stripe rust resistance genes used in this thesis can be stacked with others, enabling the development of wheat lines with multiple resistance genes. Molecular markers and disease screening assays have been useful previously in transferring resistance genes such as *Lr57/Yr40* and *Pm21* (Kuraparthy et al. 2007; Xing et al. 2018).

The KASP markers developed in this thesis are likely to be valuable for pyramiding the stripe rust resistance gene in rye chromosomes 4R and 6R, along with other resistance genes, into commercial wheat cultivars.

6.9 Marker-assisted gene pyramiding

In Paper III, gene pyramiding of stripe rust and stem rust resistance genes was achieved through KASP marker assays with speed breeding techniques. KASP markers precisely monitored the presence of *Sr59* and *YrSLU* resistance genes in successive progeny. The selection process in each generation was effectively achieved using speed breeding under normal green-house conditions (Paper III).

The marker-assisted gene pyramiding approach used in this thesis can enable a notable reduction in pesticide use by targeting resistance to multiple diseases within the same genotype. Previous studies have shown that marker-assisted selection in plant breeding can enhance efficiency and precision in achieving breeding objectives (Collard and Mackill 2008; Hasan et al. 2021). This thesis demonstrated its potential for developing wheat varieties that are both disease-resistant and economically viable.

7. Conclusions

This thesis precisely identified novel stripe rust resistance sources within wheat-rye introgression derivatives originating from *Secale cereale*. These findings highlight the specific potential of *Secale cereale* as a genetic resource for improving wheat traits.

Moreover, gene pyramiding using molecular markers for resistance to stem rust (gene *Sr59*) and stripe rust (gene *YrSLU*) helped to transfer both these disease resistance genes efficiently into a single cultivar.

Ongoing emergence of new virulent races is placing strong demands on plant breeders and geneticists to develop durable rust-resistant wheat varieties through marker-assisted breeding or gene pyramiding.

Based on the results obtained in Papers I-IV, the following conclusions were drawn:

- Wheat-alien introgression lines possess a distinct potential for precise contributions to wheat improvement, presenting significant opportunities to enhance production, resistance, and quality traits.
- Wheat-rye multiple disomic substitution lines SLU124 and SLU126 4R (4D), 5R (5D), and 6R (7D), introgressed from rye (*Secale cereale*), possess resistance to 30 races of stripe rust.
- New wheat-rye small translocation line N3-5 is resistant to five American *Puccinia striiformis* races, induced by a Chinese Spring *ph1b* crossing. It features a reduced rye segment, providing potential for wheat improvement.
- Cryptic translocation 6DS.6DL.6RL.6DL (SLU126) temporarily designated *YrSLU*, contains two individual stripe resistance genes within the 6RL segment and confers a high level of resistance to several stripe rust races.
- Through aligning putative GBS-SNPs with annotated rye NLR genes in parental lines (CS *ph1b*, SLU124, SLU126, CSA and SLU820), the precise location of 25 NLR genes on chromosome

6RL, 12 NLR genes on chromosome 4RL and nine NLR genes on chromosomes 5R was identified.

- An efficient KASP marker assay was validated for marker-assisted selection for the *YrSLU* genes transferred from 4R and 6R rye chromosomes.
- Through seedling and marker analysis, it was determined that in wheat line SLU124, the 4RL chromosome arm of rye is the source of stripe rust resistance.
- Use of marker-assisted gene pyramiding in wheat breeding can provide significant and durable disease resistance against stem rust and stripe rust.
- The combination of phenotype (seedling resistance) and genotype (GBS and KASP markers) analysis used in this thesis was effective in identifying the source of resistance to broad-spectrum stripe rust races in wheat-rye introgression lines.

8. Exploring Future Horizons: Potential Directions for Further Research

- The transfer of *YrSLU* resistance genes into adapted wheat cultivars, followed by a comprehensive assessment of the agronomic performance of these cultivars.
- Gene designation is required for identification and characterisation of resistance genes.
- The novel line with the 6DS.6DL.6RL.6DL wheat-rye cryptic translocation should be tested against additional Asian stripe rust races and against other diseases and pests.
- Evaluation for susceptibility or resistance to leaf rust, fusarium head blight, powdery mildew, and tan spot disease is essential for the wheat-rye introgression lines.
- The nutritional potential of wheat-rye introgression lines should be evaluated, to determine impacts on health attributable to elevated levels of macro- and micronutrients.
- The novel resistance genes identified in this thesis should be pyramided into Swedish wheat for durable and long-term resistance.

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Popular science summary

Wheat consumption in Sweden is predicted to rise to 970,000 metric tons by 2026, representing an annual average increase of 0.8% since 2021. Winter wheat, averaging an annual harvest of 2.8 million tons, constitutes 41% of the total cereal harvest in Sweden, while spring wheat comprises only 7%. The main concern regarding future wheat production is the current vulnerability of wheat to fungal diseases, such as stripe rust and stem rust, which cause substantial yield losses. Therefore, wheat lines which are resistant to stripe and stem rust diseases are needed. Novel wheat-rye introgression lines possessing such resistance were developed in this thesis. The primary emphasis in the work was on stripe rust (caused by the fungus *Puccinia striiformis* f.sp. *tritici*), which poses a significant challenge for wheat breeders worldwide. Stripe rust is categorised as a cool-season disease, thriving optimally in temperatures between 10 and 18°C (50-64°F), along with intermittent rain or dew events. The previously less damaging stem rust disease (caused by *Puccinia graminis* f.sp. *tritici*) has become a significant disease in Europe since 2016 and is causing major wheat yield losses. Thus, farmers are being forced to invest significant resources in purchasing and applying fungicides for chemical control of stripe and stem rust, with associated potential environmental challenges. However, developing disease-resistant cultivars is a more cost-effective and environmentally friendly method for agricultural disease control.

In this thesis, new Swedish wheat-rye introgression lines that are stably resistant to broad-spectrum stripe rust races were developed. The rye chromosomal segment conferring resistance and improved wheat yield in these lines is very small, which minimises co-transfer of undesired traits. Seedling analysis was used to select resistant plants at an early stage and support breeding for resistance.

New genetic markers for stripe rust resistance genes from rye chromosomes that have been transferred into wheat were developed in this thesis. These markers can help breeders to verify the presence of rye chromatin behind the resistance in wheat-rye introgression lines.

New wheat genotypes were also developed by transferring both stripe rust and stem rust resistance genes, as confirmed with the help of molecular markers. The plants obtained were subjected to speed breeding under normal greenhouse conditions, which facilitated efficient plant growth and harvesting within six weeks. This provides an effective management strategy to obtain faster advance to the next selection and evaluation stages.

Stripe rust resistance was confirmed under controlled conditions in this thesis. If this resistance persists within the field, the material can be very useful for breeders and farmers, as an invaluable genetic resource for creation of resistant cultivars. This will facilitate sustainable wheat cultivation for farmers, ensuring sufficient food production and mitigating challenges associated with disease outbreaks.

Populärvetenskaplig sammanfattning

Konsumtion av vete i Sverige förutspås att öka till 970 000 ton till år 2026, vilket representerar en årlig ökning av 0,8 % sedan 2021. Höstvete, med en genomsnittlig skörd per år på 2,8 miljoner ton, utgör 41 % av den totala sädesskörden i Sverige medan vårvete endast utgör 7 %. Det största oron när det gäller framtida veteproduktion är vetets nuvarande sårbarhet för svampsjukdomar, såsom gulrost och svartrost, vilka orsakar betydande skördeförluster. Därför behövs vetelinjer som är resistenta mot dessa svampsjukdomar. Nya vete-råg-introgressionslinjer med sådan resistens utvecklades i denna avhandling. Den primära tyngdpunkten i detta arbete låg på gulrost (orsakad av svampen *Puccinia striiformis f.sp. tritici*), vilket utgör en betydande utmaning för veteuppfödare världen över. Gulrost kategoriseras som en svalsäsongssjukdom, som frodas optimalt i temperaturer mellan 10 till 18°C,(50-64°F), tillsammans med perioder av regn och dagg. Den tidigare mindre skadliga svampsjukdomen svartrost (orsakad av *Puccinia graminis f.sp. tritici)* har blivit en betydande sjukdom i Europa sedan 2016 och orsakar stora förluster av veteskörden. Därmed tvingas bönder investera betydande resurser i inköp och applicering av fungicider för kemisk bekämpning av gul- och svartrost, med de potentiella miljöutmaningar som detta innebär. Att utveckla sjukdomsresistenta sorter är dock en mer kostnadseffektiv och miljövänlig metod för sjukdomsbekämpning i jordbruket.

I denna avhandling har nya svenska vete-råg-introgressionslinjer utvecklats som är stabilt resistenta mot bredspektrum gulrostraser. De kromosomala segment från råg som ger resistens och förbättrad avkastning hos vete i dessa linjer är mycket små, vilket minimerar samtidig överföring av oönskade egenskaper. Plantanalys användes för att välja resistenta plantor i ett tidigt skede och främja förädling för resistens.

Nya genetiska markörer utvecklades i denna avhandling för resistensgener mot gulrost som har överförts till vete från rågkromosomer. Dessa markörer kan hjälpa växtförädlare att verifiera närvaron av rågkromatin bakom resistensen i vete-råg-introgressionslinjer. Nya genotyper av vete utvecklades också genom att överföra gener för resistans mot gulrost och svartrost, vilket bekräftats med hjälp av molekylära markörer. De erhållna plantorna utsattes för snabbförädling under normala växthusförhållanden, vilket underlättade effektiv planttillväxt och skörd inom sex veckor. Detta ger en effektiv förvaltningsstrategi för att snabbare gå vidare till nästa urvals- och utvärderingssteg.

Resistans mot gulrost bekräftades under kontrollerade förhållanden i denna avhandling. Om denna resistans kvarstår på fältet kan materialet vara mycket användbart för växtförädlare och lantbrukare, som en ovärderlig genetisk resurs för att skapa resistenta sorter. Detta kommer att underlätta för en hållbar veteodling för jordbrukare, säkerställa tillräcklig livsmedelsproduktion och mildra utmaningar i samband med sjukdomsutbrott.

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Diverse Wheat-Alien Introgression Lines as a Basis for Durable Resistance and Quality Characteristics in Bread Wheat

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Wheat productivity has been significantly improved worldwide through the incorporation of novel genes from various gene pools, not least from wild relatives of wheat, into the commonly cultivated bread and durum wheat. Here, we present and summarize results obtained from a diverse set of wheat-alien introgression lines with mainly introgressions of rye, but also of *Leymus* spp. and *Thinopyrum junceiforme* into bread-wheat (*Triticum aestivum* L.). From this material, lines carrying 2RL were found with good agronomic performance and multiple resistance not least towards several races of powdery mildew. A novel resistance gene, one of few showing resistance towards all today identified stem rust races, designated *Sr59*, was also found originating from 2RL. Lines with multiple introgressions from 4R, 5R, and 6R were found resistant towards the majority of the stripe rust races known today. Due to lack of agricultural adaptation in these lines, transfer of useful genes into more adapted wheat material is a necessity, work which is also in progress through crosses with the CS*ph1b* mutant, to be able to only transfer small chromosome segments that carry the target gene. Furthermore, resistance towards Russian wheat aphid was found in lines having a substitution of 1R (1D) and translocations of 3DL.3RS and 5AL.5RS. The rye chromosomes 1R, 2R, and 6R were found responsible for resistance towards the Syrian Hessian fly. High levels of especially zinc was found in several lines obtained from crosses with *Leymus racemosus* and *Leymus mollis*, while also some lines with 1R, 2R, or 5R showed increased levels of minerals and in particular of iron and zinc. Moreover, lines with 1R, 2R, 3R, and *Leymus* spp. introgressions were also found to have a combination of high iron and zinc and low cadmium concentrations. High variation was found both in grain protein concentration and gluten strength, measured as %UPP, within the lines, indicating large variation in bread-making quality. Thus, our study emphasizes the impact that wheat-alien introgression lines can contribute to current wheat lines and shows large opportunities both to improve production, resistance, and quality. To obtain such improvements, novel plant breeding tools, as discussed in this

paper, opens unique opportunities, to transfer suitable genes into the modern and adapted wheat cultivars.

Keywords: agronomic performance, baking quality, breeding, disease and pest resistance, *Leymus* spp., *Secale cereale* L., *Triticum aestivum* L.

INTRODUCTION

Wheat is one of the three major crops of importance for food security worldwide, the other two being rice and maize (FAO, 2016). Bread wheat (*Triticum aestivum* L.) is a hexaploid and the most commonly cultivated species of wheat (95%), belonging to the tribe Triticeae and the family Poaceae (McFadden and Sears, 1946; Dubcovsky and Dvorak, 2007). The second most commonly cultivated form of wheat is durum wheat (*Triticum durum* L.), contributing 5% to the total production (Dubcovsky and Dvorak, 2007). In total, wheat contributes 20% of the total calories and proteins consumed by the human population, thereby contributing to a higher total protein intake than the whole total meat consumption summed (Shewry and Hey, 2015).

Due to the high contribution of wheat to the daily human food intake, human food security is highly vulnerable to the increasing threats to wheat production from climate change, including global warming (Steenwerth et al., 2014). Wheat yield is also negatively affected by abiotic and biotic stresses resulting in economic losses to farmers (Husenov et al., 2020). The population growth predicted to be more than 9 billion people worldwide in 2050, result in additional demand on food production, simultaneously bringing an increasing competition for arable land for food production (FAO, 2016). To meet these challenges, novel wheat cultivars are urgently needed, adapted to contribute high yield under sustainable and demanding cultivation conditions (Shiferaw et al., 2013). For this purpose, novel plant breeding methodologies have to be developed in order to most beneficially use available genetic resources and smart and rapid plant development to produce the needed wheat materials in time to cope with needs and challenges.

Plant breeding to obtain sustainable, high resistance and high-quality crops are dependent on suitable genes for the wanted traits. For many traits, such genes are available within the breeding material in on-going breeding programs for the crop and will be easily transferred by breeders through ordinary crossing schemes. However, domestication and breeding practices have reduced the presence of rare and favorable allelic variation to biotic and abiotic stresses and environmental changes originally found in the wild relatives (Tanksley and McCouch, 1997; Singh et al., 2018). Therefore, wild relatives, landraces, and close relatives of wheat are a unique source of novel genetic variations for introgression into modern cultivars (Molnár-Láng et al., 2015). For wheat, several useful transfers of genes from landraces have been reported including e.g. the *Rht* dwarfing genes, the powdery mildew resistance gene *Pm24*, and several biotic and abiotic stress resistance genes (Kihara, 1983; Zeven, 1998; Huang and Röder, 2011; Cavanagh et al., 2013; Singh et al., 2018). Also, genes have been transferred to wheat from non-*Triticum* (alien) species, where transfers from e.g. rye (*Secale cereale*) have resulted in widely cultivated wheat cultivars (McIntosh et al., 1995; Friebe et al., 1996). The most successful alien transfer into the wheat genome is that of the 1RS chromosome segment, in the form of 1AL.1RS, 1BL.1RS, and 1DL.1RS translocations (Rabinovich, 1998; Mago et al., 2015), contributing several resistance genes for powdery mildew, leaf, stripe and stem rusts. Out of them, the 1BL.1RS wheat-rye translocation has contributed immensely to global wheat production as a source of resistance genes (*Sr31*/*Yr9*/*Lr26*/*Pm9*) to wheat fungal diseases (Schlegel, 2020), but it is also known to contribute weak and sticky dough (Dhaliwal et al., 1988). Rye is a unique source of many important traits for wheat improvement, e.g. the resistance genes *Sr27*, *Sr50*, *Sr1RS*^{Amigo}, *Lr25*, *Lr45*, *Pm7*, etc. have been identified from rye (The et al., 1991; Marais and Marais, 1994; McIntosh et al., 1995; Friebe et al., 1996), although these genes have contributed limitedly to agricultural production until now. More recently, some novel resistance genes from rye i.e. *Sr59*, *Yr83*, and *Pm56* have been introgressed into wheat (Rahmatov et al., 2016a; Hao et al., 2018; Li et al., 2020), which may be used as durable sources against fungal diseases. Herbicide-resistant evolution is challenging weed management; therefore, the allelopathic potential is a good solution to mitigate weed management in crop production. Bertholdsson et al. (2012), reported that rye is an excellent source of allelopathic potential that can be used for wheat breeding. In addition, Iron (Fe) and Zinc (Zn) deficiency are severely affecting human health, causing several physiological disorders, symptomatic anemia, stunting, etc., and therefore high content in staple crops such as wheat are of outmost importance (Johansson et al., 2014; Johansson et al., 2020). The recent great advancements in genomic and cytogenetic tools open opportunities to transfer alien resistance genes to wheat, simultaneously avoiding linkage drag issues.

The present paper is focusing on opportunities and challenges of the use of a diverse set of wheat-alien introgression lines with mainly introgressions of rye, but also of *Leymus* spp. and *Thinopyrum junceiforme* into bread-wheat (*T. aestivum* L.). This provides useful insight into the identification and characterization of wheat-alien introgression lines based on several studies through diseases and pests screening, agronomic performances and molecular markers. Resistances and quality characteristics of wheat within this material, connections to introgressed chromosomes, localization of genes, and status for transfer of these genes are described here. Finally, a short overview is given as to the impact of novel breeding strategies for the use of alien germplasm in modern breeding.

MATERIALS AND METHODS

Plant Materials

A set of winter and spring wheat-alien introgression lines maintained at the Plant Breeding Department at the Swedish

University of Agricultural Sciences were used in different part of the hereby presented studies. These lines were developed by crossing and backcrossing strategies during 1980 to 2000 by the late Professor Arnulf Merker at the Swedish University of Agricultural Sciences (**Table 1**). The wheat-alien introgression lines used for the present paper contained rye chromosomes with 1R, 2R, 3R, 4R, 5R and 6R in the form of a single disomic substitution wheat–rye translocations such as 1DL.1RS, 1BL.1RS, 2BS.2RL, 3DL.3RS and 5AL.5RS, lines with multiple combinations of rye chromosome substitutions such as 1R + 2R, 1R + 3R, 1R + 6R, 5R + 4R + 7R and 1R + 6R + 4R + 7R (Merker, 1979; Merker and Rogalska, 1984), and lines with introgressed chromatin from *Leymus mollis*, *Leymus racemosus*, and *T. junceiforme* (Ellneskog-Staam and Merker, 2001; Ellneskog-Staam and Merker, 2002). The full material used has previously been completely described in Rahmatov (2016) and Rahmatov et al. (2017).

Field Trials

A total of 180 of the winter wheat lines and 57 of the spring wheat lines were evaluated by field trials for multiple resistance and agronomic performance during two executive seasons, 2014 and 2015, in Svalöv, Sweden and in Harzhof and Laberweinting in Germany. During these seasons, the lines were continuously evaluated and scored (scale 1–9) for lodging (winter wheat) and presences of diseases (spring and winter wheat). Comparisons of presence of diseases and alien material were carried out (Andersson et al., 2016).

Diseases Screening

Stem rust seedling resistance assays with ten *Pgt* races and adult plant responses with three *Pgt* races (TTKSK + TTKST, TKTTF and MCCFC), were carried out on 185 and 94 of the winter and spring wheat-alien introgression lines under field conditions following the procedure described in Hysing et al. (2007) and Rahmatov et al. (2015); Rahmatov et al. (2016a, b). For the stripe rust evaluations, 189 of the winter and 73 of the spring wheat-alien introgression lines were tested in the seedling and adult plant stages. Twelve stripe rust races with different virulence/avirulence combinations and geographic origins were used for screening at the seedling stage along with adult plant evaluations in the field according to Rahmatov et al. (2017). Hysing et al. (2007), evaluated a set of 2BS.2RL wheat-rye translocation lines against stripe rust, leaf rust, and powdery mildew races.

Hessian Fly and Russian Wheat Aphid Screenings

A total of 57 spring and 185 winter wheat-alien introgression lines were evaluated in 2011 and 2012 at the seedling stage against Hessian fly (HF) and the Russian wheat aphid (RWA) in collaborations with ICARDA. In brief, the rearing rooms for HF experiments were kept at 20°C, Rh 70–80%, and the cycle of 16/8 h light/dark was used. Six or ten seeds per wheat accession were sown in hill plots in metal flats 55 × 45 × 10 cm, in total 48 accessions per box plus controls, in a mixture of soil:sand:peat (2:1:1). After 5–6 days, at the one-leaf stage, infestation by HF was done with about 30 females and 10 males under net for 3–4 days (El Bouhssini et al., 2013). The scoring took place 20 days after infestation, with the number of resistant and susceptible plants per accession. The first screening was conducted in the spring and winter materials in 2011, and a second screening was only conducted in the winter materials in 2012. Based on these two screenings, lines with 100% resistance reaction to HF were selected for further confirmations in four separate screenings.

The RWA biotype was collected from Tel Hadya, Syria, and thereafter reared on the susceptible wheat cultivar (Andersson et al., 2015). The experiments were carried out in a greenhouse at 19–20°C, with light/dark photoperiod 16/8 h and relative humidity of about 60%. The accessions were planted in a

TABLE 1 | Wheat-alien introgression lines and respective parents evaluated in this study.

Cross/Pedigree	Plant habit	No. of lines	Type	Reference
Triticale[a]	Spring and winter	5	×*Triticosecale*	Forsstrom and Merker, 2001
Wheat[a]	Spring and winter	8	*Triticum aestivum* and *Tr. carthlicum*	Forsstrom et al., 2002
Sv 876012 x H	Winter	37	Wheat–rye introgressions	Forsstrom and Merker, 2001
Sv 876032 x H x K	Winter	54	Wheat–rye introgressions	Forsstrom and Merker, 2001
Sv 856003 x H	Winter	6	Wheat–rye introgressions	Forsstrom and Merker, 2001
Sub 1R + 2R	Winter	42	Wheat–rye introgressions	Forsstrom and Merker, 2001
Malysh	Winter	6	Wheat–rye introgressions	Merker, 1984
Starke × Otello	Winter	7	Wheat–rye introgressions	Merker, 1984
Uno × Holme	Winter	8	Wheat–rye introgressions	Merker, 1984
Triticale VT828041	Spring	6	Wheat–rye introgressions	Merker, 1984
Triticale Drira	Spring	23	Wheat–rye introgressions	Merker, 1984
Triticale Beagle	Spring	12	Wheat–rye introgressions	Merker, 1984
Triticale VT83 615	Spring	2	Wheat–rye introgressions	Merker, 1984
Triticale VT83 591	Spring	4	Wheat–rye introgressions	Merker, 1984
Triticale VT 82 8039	Spring	5	Wheat–rye introgressions	Merker, 1984
3R BB14 (Cimmyt 1974)	Spring	4	Wheat–rye introgressions	Merker, 1984
Leymus mollis	Winter	42	Wheat–*L. mollis*introgressions	Merker and Lantai, 1997
Leymus racemosus	Spring	22	Wheat–*L. racemosus* introgressions	Merker and Lantai, 1997
Th. junceiforme	Spring	16	Wheat–*T. junceiforme* introgressions	Merker and Lantai, 1997
[a]Parental cultivars and Lines	309	TOTAL		

randomized (alpha design) order together with susceptible and resistant controls in each planting tray, in a mixture of soil, sand, and peat (2:1:1). An evaluation was done when symptoms were seen on susceptible checks, using the ICARDA RWA damage scale with a 1–3 scale for leaf rolling (LR) and 1–6 scale for leaf chlorosis (LC) (El Bouhssini et al., 2011). In the second advanced screening, selected accessions from the first screening results were repeated at four separate times (Andersson et al., 2015).

Allelopathic Potential of Wheat-Alien Introgression Lines

Allelopathic potential of the wheat–rye introgression lines were tested according to Bertholdsson et al. (2012). In this study, seeds of *Chenopodium alba*, *Lolium perenne*, *Brassica napus*, *Lactuca sativa*, *Eruca sativa*, *Sinapis indicum* and *Sinapis alba* were used to find high root growth inhibition when grown together with rye. In this investigation, four pregerminated cereal seedlings were planted along the wall of 400-ml Phytotech tissue culture vials (bottom diameter 75 mm) filled with 20 ml 0.35% water agar, and eight pregerminated mustard seedlings (*S. alba* cv. Medicus) were planted in a circle in the center of the vials. The experiment was tested in four replicates, and the dry weight of the shoot and root were measured (Bertholdsson et al., 2012).

Analysis of Grain Samples for Micronutrients Concentration and Protein Composition

A total of 40 of the lines were evaluated for micronutrients (e.g. Iron, Zinc, and Cadmium) content with Inductively Coupled Plasma Mass Spectrometry (ICPMS) at the University of Minnesota, similarly as described in Hussain et al. (2010) and Moreira-Ascarrunz et al. (2016). Briefly, all samples were ashed in a muffle furnace for 12 h at 485°C. Then, the ash was dissolved in 5 ml of 20% HCl followed by dilution with 5 ml of deionized water. The ICPMS provides concentration assays for several microelements, including zinc, iron, and cadmium in mg/Kg.

The complete set of winter wheat alien translocation lines were analysed with SE-HPLC according to Johansson et al. (2001) to evaluate the total amount of SDS-extractable proteins (TOTE) and percentage of unextractable polymeric protein in total polymeric protein (%UPP). A high correlation is known to exist between TOTE and grain protein concentration and between %UPP and gluten strength (Malik et al., 2011; Malik et al., 2013) and thereby this methodology can be used to understand relationships with bread-making quality (Hussain et al., 2012; Hussain et al., 2013; Vazquez et al., 2019).

Statistical Analyses

The statistical software SAS 9.3 (SAS, 2011) was used for principal component analyses (PCA) calculations to understand relationships between minerals and protein factors with evaluated wheat-alien introgression lines. In order to understand and visualize the distribution and relationship between variables and factors evaluated, principal component analysis (PCA) can be applied to orthogonally represent the variables in a data matrix vector. PCA is known to show the distribution of dependent variables and independent factors, in a loading and score plot, respectively (Wold et al., 1987). Values of content of Iron, Zinc and Cadmium were calculated by mini tab for wheat, Triticale, wheat–rye and wheat-*Leymus* lines and presented as boxplots with lowest and highest observations as well as lower and upper quartile and median.

RESULTS

Multiple Resistance and Agronomic Performance

The lines showed varying agronomic performance, with some lines being almost comparable to currently grown wheat in Sweden while others differed largely. Large variation was found in the material both for lodging and presence of diseases (**Figure 1**). However, the majority of the winter wheat lines had strong stem and with limited lodging, thus making them of interest as a source of lodging resistance (**Figure 1B**). Presence of 1R, 2R, 3R, 5R, 1R + 6R and *L. racemosus* correlated with decreased levels of infections with powdery mildew, *Zymoseptoria tritici (*causal agent of *Septoria triticae* blotch) and Fusarium head blight during field conditions. Lower levels of leaf, stem and stripe rusts infection responses were found in lines with 1R, 2R, 3R, 1R + 3R, 1R + 6R, and *L. racemosus*, respectively.

Rusts Screenings

From the stem rust seedling evaluation, eleven 2R (2B), three 2R (2D), and three 3R (3D) wheat–rye disomic substitution lines, and seven wheat-*T. junceiforme* were found to carry potentially new stem rust resistant gene/s (**Table 2**). Based on the ten *Pgt* races, known resistance genes could not be postulated because their reactions did not correspond to the avirulence/virulence profile of the races tested. All lines that were resistant at the seedling stage remained resistant at the adult plant stage against races TTKSK + TTKST in Kenya and TKTTF in Turkey. Trace resistance was found in several of the lines tested at St. Paul, Minnesota, against the race MCCFC (**Table 2**), although only a few number of lines were tested due to winter type of the material and limited seed available.

The wheat-alien introgression lines showed high variability in resistance/susceptibility reactions against the twelve stripe rust isolates applied to screen for resistance genes (**Table 3**). The screening resulted in 149 lines (57% of the lines), postulated to contain a combination of known *Yr* genes e.g. *Yr1*, *Yr2*, *Yr9*, and *Yr32*. However, six of the multiple wheat–rye introgression lines with 4R, 5R and 6R were identified as highly resistant against a total of 25 stripe rust races, including the twelve used for the full material (**Table 3**). Thus, these six lines might possess a new stripe rust resistance gene/s. Molecular cytogenetic analysis showed that the 4R, 5R and 6R rye chromosomes substituted 4D, 5D and 6D wheat chromosomes. Further studies are going on for determining the underlying genetic basis of these resistance gene/s.

Aphid and Hessian Fly Resistance

Among the total of 242 evaluated lines, 235 germinated and showed a high variation in resistance to RWA (**Figure 2**). A total

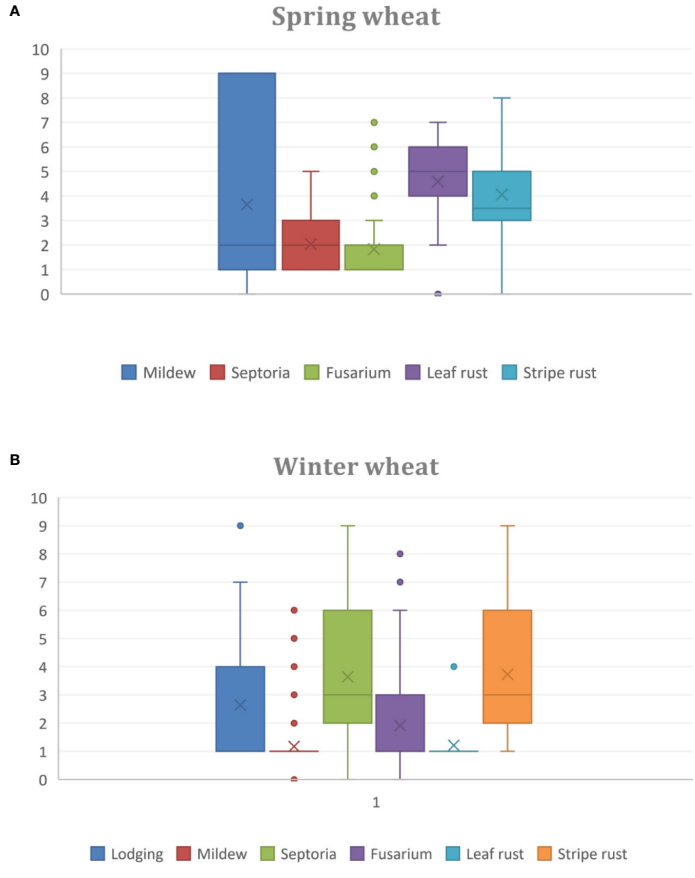

FIGURE 1 | Boxplots showing variation in lodging and various diseases based on scoring of the material from 0 to 9, in wheat alien introgression lines of **(A)** Spring wheat, and **(B)** Winter wheat, from field trials during two years in Sweden and Germany. In each boxplot, five bars are represented, indicating smallest observation, lower quartile, median, upper quartile, and largest observation, respectively. X marks the mean value.

of 23 accessions were identified as resistant against the RWA. Resistance was found to RWA, particularly in accessions having substitutions of 1R instead of 1D [1R (1D) or 1R (1D) + 6R (6D)], in translocations to 3D or 5A (3DL.3RS and 5AL.5RS) and accessions with introgressions of *L. mollis*.

The first screening (242 lines) for HF resistance showed 11 winter and two spring wheat accessions with 100% resistance, while in the second screening, nine of the 11 winter wheat accessions were proofed with 100% resistance, which also holds true for the additional four repeated screenings (**Table 4**). These fully resistant winter wheat accessions contained 1R, 1R + 6R, 1RS + 2RL, 1RL + 2RL, 2RL, and 2R translocations or substitutions. The presence of these genes in our alien wheat material might be one explanation for the HF resistance found although the presence of full resistance in accessions with the substitution 1R.1D in winter wheat and the translocation 1RS.1DL in spring wheat indicate the presence of additional unknown resistance genes in the present material. Besides, high and partial levels of resistance with the presence of 1R, 1RS, 2R, 3R, 3RS, 4R, 5R, 6RL, and *L. racemosus* and *L. mollis* substitutions and translocations were found promising sources against HF.

Nutritional Benefits

Principal component analyses indicated high levels of Cadmium (Cd) in the winter wheat lines as compared to the rest of the evaluated lines, while *Leymus* spp. was indicated as containing high levels of Iron (Fe) and Zinc (Zn; **Figure 3**). Mean values of

TABLE 2 | Stem rust seedling and adult plant resistance tests in the wheat–rye and wheat–*T. Junceiforme* introgression lines with potential sources of new stem rust resistance gene/s.

#	Chromosome	Seedling Resistance Test										Adult Plant Resistance		
		TTKSK, 1 Rep.	TTKSK, 2 Rep.	TPMKC	TTTTF	QTHJC	RKQQC	TTKST	TRTTF	TTTSK	TKTTF	MCCFC	TTKSK+ TTKST	TKTTF	MCCFC
SLU73	2R susbstituted 2B	2+	2	3+	;1	;01/3+	11+	2/2+	1+2/3+4	22+	1/2+	4	20MR	10MR	–
SLU74	2R susbstituted 2B	2+	2+	1 3+ Z	;1/1	;-1	;1	;11+	1+2/3+	22+	1/2+	4	30MR	10MR	–
SLU75	2R susbstituted 2B	2	2	3+	0;/0; 3+	;-1	;1	11+	33+	22+	11+	4	20MR	10MR	–
SLU76	2R susbstituted 2B	2	2	3+	;1	;1/2-	;11+	;11+	11+3	22+	3+	4	30MRMS	10MS	–
SLU77	2R susbstituted 2B	2	2	3+	2+3/4/;1	;1/1+3	;11+/2	;11+/2/3+	33+	22+	3+	4	30MRMS	10MRMS	–
SLU78	2R susbstituted 2B	2+	2+	3+	;/3-	1+3-	;11+3-	;11+2+	22+3+	22+	3+	4	30MRMS	10MS	–
SLU79	2R susbstituted 2B	2	2	3+	;/3-	;13-	;13-/3	;12+	11+/3+	22+	1	4	30MRMS	10MR	–
SLU80	2R susbstituted 2B	2+	2+	33+	3+	33+/;13-	33+/;13-	;11+/3+	1+2/3+	22+	3+	4	30MRMS	10MS	–
SLU81	2R susbstituted 2B	2+	2+	3+	3-/1;	11+	11+	1+3-	33+	22+	3+	4	40MRMS	10MS	–
SLU82	2R susbstituted 2B	2+	2+	3+	; 1 3-/4	;13-/3+	;13-/3+	;1/3	3+	22+	3+	4	40MRMS	10MS	–
SLU83	2R susbstituted 2B	2+	2+	4	; 1 3-	13-/3+	13-/3+	11+/3+	33+	22+/3+	3+	4	40MRMS	10MS	–
SLU210	2R susbstituted 2D	0;	0;	0;	1+	0;	;1	0;	;12	;1	1	0;	20RMR	5RMR	TR
SLU214	3R substituted 3D	0;	0;	11+	4	0;	0;	0;	;11+	3+	3+	0;	20R	10MRMS	TR
SLU219	3R susbstituted 3D	0;	0;	0;	0;	0;	0;	0;	0;	0;	0;	0;	5TR	5R	TR
SLU222	3R susbstituted 3D	0;	0;	33+	4	1+2/3-	3	0;	22+	;	33+	11+	10RMR	70S	10R
SLU238	2R susbstituted 2D	1	1	2	22-	12-	;1	;11+	;01	;1	1-	;01-	10R	10RMR	TR
SLU239	2R susbstituted 2D	1	1-	2	22-	12-	;1	;01	;01	;1	1-	;01-	20RMR	5RMR	TR
SLU251	Th.-Wheat	0;	0;	3+	4	33+	3+	0;1	3+	0;	3+	4	–	–	–
SLU252	Th.-Wheat	0;	0;	33+	3+	33+	3+	0;	3+4	0;	3+	3+	–	–	–
SLU253	Th.-Wheat	1	0;	33+	3+	33+	3+	1+3-	2+3-	0;	3+	3+	–	–	–
SLU255	Th.-Wheat	0;	0;	3+	4	33+	3+	0,1	3+	0;	3+	3+	–	–	–
SLU256	Th.-Wheat	0;	0;	3+	3+	33+	3+	0;	3+4	0;	3+	3+	–	–	–
SLU274	Th.-Wheat	0;	0;	3+	4	33+	3+	0,1	3+	0;	3+	3+	–	–	5MS
SLU275	Th.-Wheat	0;	0;	33+	3+	33+	3+	0;	3+4	0;	3+	3+	–	–	30MS

Seedling infection types observed based on 0–4 scale (Stakman et al., 1962). The lines with;0–2+ types considered as resistant. The lines with 3–4 types considered as susceptible. Adult plant response was evaluated based on the Cobb Scale (Peterson et al., 1948) and host response to infection based on pustule type and size (Roelfs et al., 1992). TR, Trace Resistance; R, Resistance; MR, Moderately Resistance; MRMS, Moderately Resistance to Moderately Susceptible; and MS, Moderately Susceptible. A total of 94 lines of the total material were screened for adult plant resistance, explaining the lack of data for some of the lines presented here.

TABLE 3 | Resistance(R)/susceptibility(S) of wheat-alien introgression lines to isolates of *Puccinia striiformis tritici*.

Isolates	6 lines fromSv 876012 × H with 4R + 5R + 6R	256 lines			
		R	MR	MS	S
SE 205/12	R	12	23	102	119
UK 94/519	R	49	2	24	182
DK 66/02	R	84	4	29	140
Taj 01a/10	R	17	34	87	119
ER 02/03	R	174	15	54	14
DK 11/09	R	178	11	46	21
DK 71/93	R	66	34	99	56
AF 87/12	R	207	10	22	16
DK09/11	R	39	14	104	98
DK 122/09	R	6	15	126	109
SE 100/09	R	220	8	16	12
TR 34/11	R	170	41	8	32

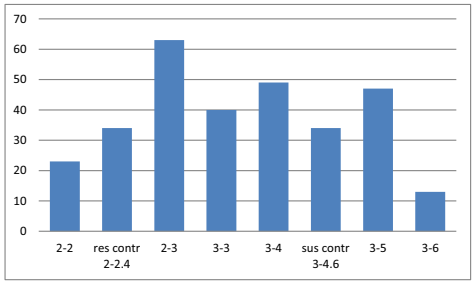

FIGURE 2 | Evaluation of 235 wheat alien introgression lines for resistance against Russian wheat aphid (RWA), resulting in number of lines with different scales of resistance. Scale: leaf rolling (LR)-leaf chlorosis (LC); 1-1 and 1-2 = highly resistant, 2-2 = resistant, 2-3 = moderately resistant, 3-3 moderately susceptible, 3-4, 3-5, 3-6 = susceptible. For the susceptible and resistant controls, mean values of 34 lines are used.

minerals content in the different types of material (Wheat–rye introgressions, *Leymus* spp. introgressions, wheat, and triticale) verified the high content of Zn in the *Leymus* spp. introgression lines and the high Cd content in the wheat lines (**Table 5**). A relatively high Fe content was found in two of the parental wheat lines used in the present study; Sonett (57.0 mg/kg) and Prins (60.6 mg/kg). Furthermore, the triticale parents, Drira (51.8 mg/kg) and Beagle (63.3 mg/kg), were observed to contain a high level of Zn (**Table 5**). Overall, the minimum 22.7 mg/kg and maximum 64.2 mg/kg for Fe concentrations were observed in the wheat–rye introgression lines with 1R, 2R, 3R, 5R, and 6R rye chromosomes (**Table 5**). The minimum and maximum Zn concentrations produced by these wheat–rye introgression lines were 32.9 mg/kg and 89.3 mg/kg, respectively. The overall grain Cd concentration ranged from 0.02 to 0.13 mg/kg, in which the lines with low Cd concentration were observed to be 0.015 to 0.017 mg/kg in the wheat–rye introgression 1R (1D), and the lines with *L. mollis* and *L. racemosus* chromosomes. Interestingly, nine of the lines with a high combination of Fe (ranged from 47.4 to 64.2 mg/kg) and Zn (ranged from 53.7 to 83.4 mg/kg) concentration and low Cadmium concentration (ranged from 0.02 to 0.07 mg/kg) were detected in the wheat-rye 1R (1D), 2R (2D), 2R (2B), 3R (3B), and *L. mollis* and *L. racemosus* intogression lines (**Table 5**).

Baking Quality

The evaluated alien introgression lines showed a high level of variability both in grain protein concentration and gluten strength (**Figure 4**). A total of 40% of the lines showed a higher grain protein concentration than the standard cultivar, Dragon, while 8% of the lines showed higher gluten strength than the standard. The 10% of the evaluated lines with the highest grain protein concentration (TOTE), were all found to have either addition of chromosome 1R, 2R, 4R, and 6R or a 1R/1D translocation (**Table 6**). Several of the high grain protein concentration lines also had additions of 1R and 6R. The lines with high gluten strength (% UPP) were found either to have introgressions from *Leymus* or additions of either 1R + 2R or 1R + 4R (**Table 6**).

TABLE 4 | Accessions of Swedish winter wheat with rye substitutions and translocations showing resistance for Hessian fly at separate screenings.

Acc.No.	Subs./transl.	1st screen		2nd screen		Mean 4 screens	
		Tot pl	% inf	Tot pl	% inf	Tot pl	% inf
Kr 08-59	1R.1D	5	0	10	0	9.25	0
Kr 08-60	1R.1D + 6R.6D	5	0	10	0	8.75	0
Kr 08-76	T1RS.1BL + T2BS.2RL	2	0	10	0	9.5	0
Kr 08-79	2R.2B	5	0	10	0	9.25	0
Kr 08-89	T1RL.1BS + T2BS.2RL	5	0	10	0	9.5	0
Kr 08-90	T1RL.1BS + T1BS.2RL	2	0	10	0	9.5	0
Kr 08-91	T2RL.2BS	5	0	10	0	9	0
Kr 08-94	T2RL.2BS	5	0	10	0	10	0
Kr 08-95	T2RL.2BS	5	0	10	0	9	0
Res Cont	6RL	5	0	10	0	10	0
Sus Cont	-	5	100	10	100	10	100

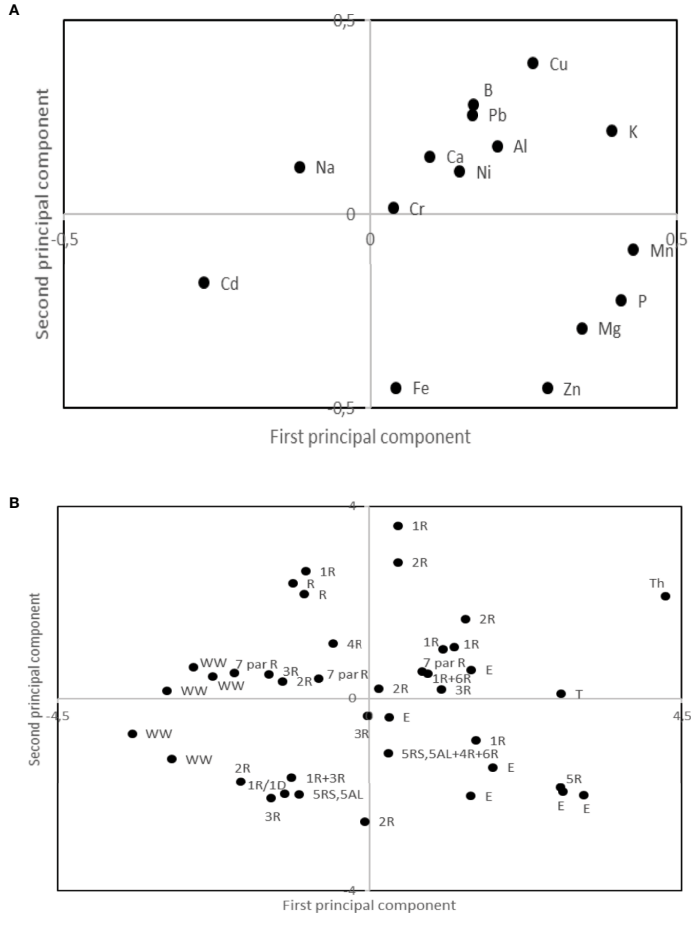

FIGURE 3 | Loading **(A)** and score **(B)** plot from principal component analyses of mineral composition in winter wheat (WW), Triticale (T), Rye (R), and alien substitution and translocation lines with rye introgressions (given as R and a what type of), Thinopyrum (Th) and Leymus (E) introgressions. The first and second principal component explained 23.0 and 17.0% of the variation, respectively.

DISCUSSION

New sources of genetic diversity are essential to improve yield, root growth, stand establishment, adaptation to climate change, nitrogen use efficiency, water use efficiency, resistance to abiotic and biotic stresses, biomass, photosynthetic potential, nutritional and end-use quality. In this paper, results from studies over a range of years are compiled to highlight the importance of wheat-alien introgression lines as a potential source of several important traits for wheat improvement. Our studies proved that these wheat-alien introgression lines carry various genetic variation e.g. resistance to diseases (rusts, powdery mildew, *S. triticae*, and Fusarium head blight), pests (Hessian fly and aphids), agronomic performance, weed competition, yield potential, microelements (Fe, Zn, Cd, etc.), fertility, alpha amylase activity, and positive end-use quality.

The evaluated 2R (2B) and 2R (2D) substitution lines showed resistance to all stem rust races at both the seedling and adult plant stages. Additionally, three of the 3R (3D) (SLU214, SLU219, and SLU222) substitution lines and seven of the wheat-*T. junceiforme* were found as potential sources of stem rust resistance genes. From the screening of a collection of wheat-alien introgression lines, the line SLU238 [2R (2D) wheat-rye disomic substitution] possessed resistance to many

TABLE 5 | Mean values of zinc, iron and cadmium concentrations in wheat, triticale, *Leymus* spp., wheat–rye introgression and wheat–*Leymus* spp. introgression lines.

Plant lines	Fe (mg/kg)		Zn (mg/kg)		Cd (mg/kg)	
	Mean	Range	Mean	Range	Mean	Range
Wheat (n = 5)	45.0	31.0–60.6	39.5	34.5–48.7	0.09	0.07–0.12
Rye (n = 2)	39.7	38.1–41.2	35.2	33.8–36.6	0.00	0.00–0.00
Triticale (n = 5)	37.9	29.5–45.0	48.9	38.7–63.3	0.09	0.07–0.15
Leymus **spp.** (n = 3)	49.4	41.4–59.1	75.8	62.4–83.4	0.02	0.00–0.02
Wheat–rye introgression (n = 22)	38.9	22.7–64.2	54.8	32.9–89.3	0.05	0.00–0.10
Wheat–*Leymus* **spp.** introgression (n = 3)	47.5	43.0–51.9	63.6	53.1–69.1	0.04	0.02–0.06

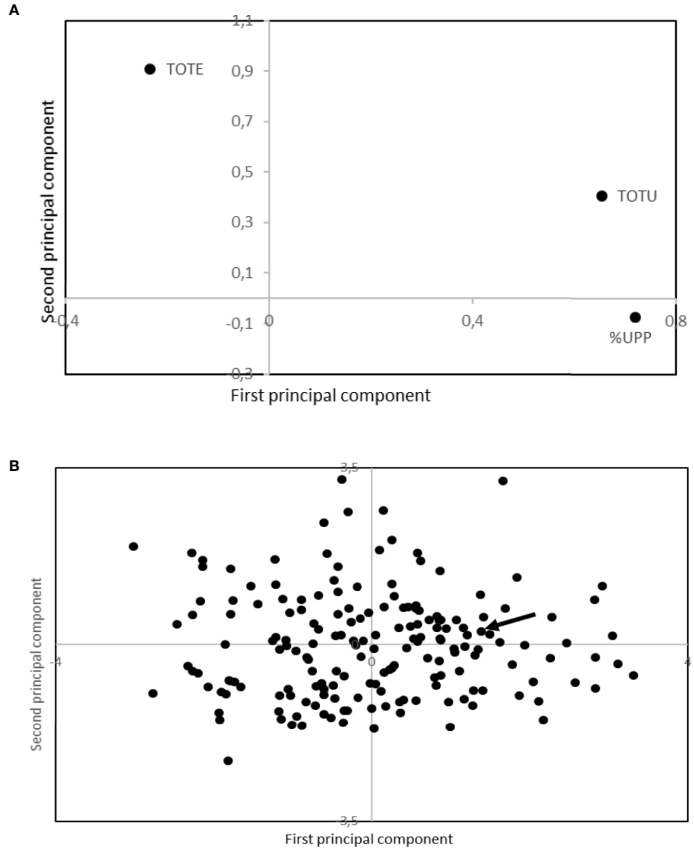

FIGURE 4 | Loading **(A)** and score **(B)** plot from principal component analyses of storage protein composition from SE-HPLC. The arrow is indicating the Swedish spring wheat line, used as a standard within the analyses. TOTE, total amount of SDS-extractable proteins; TOTU, total amount of SDS-unextractable proteins; and % UPP, percentage of unextractable polymeric protein in total polymeric protein. The first and second principal component explained 58.8 and 35.6% of the variation, respectively.

TABLE 6 | Accessions of Swedish winter wheat with substitutions and translocations (rye = R, Leymus) showing high (in descending order) total amount of SDS-extractable protein (TOTE—correlating to grain protein concentration) and percentage of unextractable polymeric protein in total polymeric protein (%UPP—correlating with gluten strength).

TOTE			%UPP		
Acc.No.	**Subs./transl.**	**Rel. values**	**Acc.No.**	**Subs./transl.**	**Values**
Kr 08-10	1R, 4R, 6R, 7R	1.74	Kr 08-109	*Leymus*	86.4
Kr 08-54	1R/1D	1.70	Kr 08-107	*Leymus*	85.7
Kr 08-16	1R, 4R, 6R, 7R	1.64	Kr 08-111	*Leymus*	85.1
Kr 08-57	1R/1D	1.63	Kr 08-104	*Leymus*	84.3
Kr 08-15	1R, 4R, 6R, 7R	1.60	Kr 08-100	2RL/2BS	81.9
Kr 08-08	1R, 4R, 6R, 7R	1.60	Kr 08-28	1R + 6R	81.6
Kr 08-55	1R/1D	1.59	Kr 08-79	1R + 2R	81.0
Kr 08-09	1R, 4R, 6R, 7R	1.59	Kr 08-80	1R + 2R	80.3
Kr 08-53	1R/1D	1.54	Kr 08-110	*Leymus*	79.3
Kr 08-30	IR + 6R	1.53	Kr 08-04	1R + 4R	79.2
Kr 08-143	5R/5A	1.53	Kr 08-108	*Leymus*	78.6
Kr 08-63	1R + 6R	1.52	Kr 08-77	1R + 2R	78.4
Kr 08-75	1RS + 2RL	1.50	Kr 08-106	*Leymus*	76.6
Kr 08-76	1RS + 2RL	1.50	Kr 08-01	1R + 4R	75.3
Kr 08-82	1R + 2R	1.47	Kr 08-95	1R + 2R	75.0
Kr 08-60	IR + 6R	1.47	Dragon		74.9
Kr 08-84	1R + 2R	1.45			
Kr 08-156	1BS/1RL	1.45			
Kr 08-52	1R/1D	1.45			
Kr08-20	IR+6R	1.44			
Dragon		1.22			

For TOTE the 20 accessions with highest value and their corresponding alien segments are shown while for %UPP, those higher than the standard (Dragon = Swedish winter wheat).

races of *Pgt*, including the widely virulent race TTKSK (Rahmatov et al., 2016a). In previous studies, Rahmatov et al. (2016b), reported that by the crossing of SLU238 and CS *ph1b* mutant, a new wheat-rye Robertsonian translocation 2DS·2RL was developed as the source of the gene *Sr59*. To this date, no stem rust resistance genes have been reported from the 2R chromosome, but chromosome 2R from different rye sources has been described as a source of resistance to various diseases and insects and also various agronomic traits. Previously, the resistance genes to leaf rust *Lr25* and *Lr45*, powdery mildew *Pm7* and Hessian fly resistance gene *H21* have been reported from the 2R chromosome (Friebe et al., 1996; Friebe et al., 1999). Furthermore, the resistance genes *Sr27*, *Sr31*/*Yr9*/*Lr26*, *Sr50*, *Sr1RS*^{Amigo} and, *SrSatu* have been described, originating from the rye chromosomes 1R and 3R, and these have been found to be effective against many of all the three rusts races (Marais and Marais, 1994; Mago et al., 2002; Singh et al., 2011; Olivera et al., 2013). Out of these resistance genes, *Sr31* has been deployed widely and provided durable resistance against stem rust races for over 30 years in agriculture (Singh et al., 2008).

Agronomic performances of some of the alien-wheat introgression lines were similar to wheat for grain yield, straw length, lodging, grain volume weight, 1000-kernel weight, fertility, grain a-amylase activity, and end-use quality (Hysing et al., 2007; Andersson et al., 2016) while some of the lines showed large variation in agronomic performance. Field studies indicated a correlation between the presence of rye (1R, 2R, 3R, 5R, 1R + 6R) and *L. racemosus* chromosomes, with low level of powdery mildew, *S. triticae* and Fusarium head blight infections (Andersson et al., 2016). Previous studies have reported an *Fhb3* resistance gene to Fusarium head blight derived from *L. racemosus* (Qi et al., 2008), which might also be present in our wheat—*L. racemosus* introgression lines. Therefore, future evaluation of these lines to other powdery mildew and *Z. triticae* isolates at seedling and adult plant stages are needed. Hysing et al. (2007), reported that red coleoptile color was correlated to the presence of the 2BS.2RL translocation allowing this character to be used as a morphological marker. Furthermore, lines with the 2BS.2RL translocation were demonstrated a high level of resistance against leaf rust and powdery mildew at the seedling stage (Merker and Forsström, 2000; Hysing et al., 2007) and adult plant resistance to TTKSK (Ug99; Rahmatov et al., 2015), thus indicating presence of uncharacterized resistance gene/s. Valuable rye chromosomes harboring beneficial genes from 4R, 5R, and 6R have also been identified (Rahmatov et al., 2017). These lines containing 4R, 5R, and 6R chromosomes are pointed out here as useful due to the fact that they are possessing novel stripe rust resistance genes. Further investigations are needed to understand the underlying genetic basis of this resistance. In various studies, stripe rust and powdery mildew resistance genes have reported on the 4R, 5R, and 6R chromosomes (An et al., 2015; Schneider et al., 2016; Xi et al., 2019), in which *Yr83* was mapped on the 6RL (Li et al., 2020). Besides this, chromosomes 4R and 6R have been demonstrated to contribute increased protein content and also to be associated with good pollinator traits (Nguyen et al., 2015; Schneider et al., 2016). Thus, there is a need to further exploit these wheat-alien introgression lines with various chromosome constitutions for wheat improvement.

High levels of resistance were identified in lines with the 1R, 3RS, 1R + 6R, 5R, and *L. mollis* chromosome introgressions against RWA. Resistances to RWA obtained from the wheat-alien introgression lines particularly lines with the 3R, 5R and *L. mollis* chromosomes have not previously been reported (Andersson et al., 2015). Previously, *Dn7*, *Gb2*, and *Gb6* resistance genes to cereal aphids have been reported on chromosome arm 1R (Friebe et al., 1996; Friebe et al., 1999; Anderson et al., 2003). Also, 1RSam.1AL and MA1S.1RLe(1B), 1Re(1D) wheat–rye translocation, and substitution lines were shown with a high level of resistance against HF and RWA, and these lines are now used in the international wheat breeding programs (Crespo-Herrera et al., 2019). The wheat-alien introgression lines with the presence of 1R, 1RS, 2R, 3R, 3RS, 4R, 5R, 6RL, and *L. racemosus* and *L. mollis* chromosomes provides resistance to the Syrian HF biotype. Previous studies have verified alien germplasm to contribute HF resistance in wheat through the *H21* and *H25* resistance genes from rye, located on 2R and 6R, respectively (Friebe et al., 1999). Hysing et al. (2007), reported that lines with the T2BS.2RL were susceptible to the HF biotypes thus this indicates different rye sources used for developing Swedish wheat-alien introgression lines. Host resistance to these insects is the most effective way of control, and various resistance genes have been derived from

alien species. The resistances to RWA and HF reported here originating from alien material have not previously been described and can, therefore, be useful to widen the pool of resistance genes in wheat breeding for resistance to RWA and HF.

The wheat-rye introgression lines displayed a good source of allelopathic potential, while lines with *L. mollis* chromosome showed a low level of allelopathic potential and the bread wheat genotypes showed no allelopathic activity. These wheat-alien lines can be used as a source of allelopathic potential and weed competitiveness in breeding programs to improve weed suppression ability for wheat improvement. Bertholdsson et al. (2012a, b), showed that the highest allelopathic potential was found in lines with 1R and 2R chromosomes. Moreover, some lines with multiple rye chromosomes (1R + 6R and 1R + 4R + 6R + 7R) were also showed high allelopathic activity (Bertholdsson et al., 2012). Previous studies have identified lines with 1R substitution showing early vigour, which can be positive for the root exudation of allelochemicals (Ehdaie et al., 2003). Breeding efforts for the allelopathic potential is considered as a complex trait (Bertholdsson, 2007), although successful examples are present on rice (Kong et al., 2006) and spring wheat (Bertholdsson, 2010). Quantitative trait loci (QTLs) linked to allelopathic traits have found on wheat chromosomes (Wu et al., 2003), thus, this indicates that allelopathic traits inherited quantitatively. The lines with high allelopathic potential identified in this study may be worthwhile for the breeding of allelopathic wheat, particularly for the purpose of organic wheat.

Various zinc, iron, and cadmium concentrations were identified in these lines. Wild relatives of wheat represent a reach source of micronutrient benefits because they have a huge and deep rooting system during its vegetation period that most efficiently uptake micronutrient if they are available in the soil (Borill et al., 2014). This has been proved by using natural genetic diversity for micronutrient uptake that can increase the nutrient content in wheat through genetic improvement (Velu et al., 2014). For instance, studies have indicated high levels of Fe and Zn to be encoded by a *Gpc-B1* locus, present in particular in wild emmer wheat (Uauy et al., 2006; Johansson et al., 2020). Thereby, genetic biofortification in wheat can be enhanced using these wheat-alien introgression lines as a source of natural genetic diversity.

Plant breeding is mostly targeting traits that improve yield potential, i.e. resistance to biotic and abiotic stresses, although for wheat improved baking and bread-making quality is also of outmost importance (Helguera et al., 2020). Wheat flour has, in particularly due to its unique protein properties, qualities which makes it outstanding for end-uses for daily food products such as bread, pastries, biscuits, porridge, cookies, etc. (Johansson et al., 2013). The gluten proteins, the gliadins, and the glutenins, encoded on group 1 and group 6 of the wheat chromosomes, are to a high extent responsible for the impact on the baking quality of wheat (Johansson et al., 2013). Alien introgressions into the wheat genome have often resulted in negative effects on the baking quality, e.g. the *Sec-1*, *Sec-2*, and *Sec-3* genes from rye instead of corresponding wheat genes at the group 1 chromosome of wheat (Kim et al., 2005). However, introgressions of rye from other parts of the genome than from the group 1 chromosomes might have less tremendous effects on the baking quality. Thus, previous results have indicated that 2BS.2RL wheat–rye translocations only had minor effects on baking quality (Hysing et al., 2007). These authors indicated that there were not any significant differences between the translocation and non-translocation groups like for grain a-amylase activity, grain starch, protein content, and other agronomic performances. Bread-making quality is known to be determined to a large extent by the gluten proteins, their amount and distribution (Johansson et al., 2013). Thus, the grain proteins concentration, the specific protein composition, the amount of specific proteins, and the amount and size distribution of polymeric protein are all factors of relevance for the bread-making quality (Finney and Barmore, 1948; Johansson et al., 2002; Johansson et al., 2003; Johansson et al., 2005; Johansson et al., 2008; Johansson et al., 2013). The evaluated alien introgression lines showed a high level of variability in both grain protein concentration and gluten strength. Thus, the alien material evaluated here, seems to have also interesting properties when it comes to specific quality breeding. Introgressions of *Leymus* seem to be able to contribute both high nutrition and high gluten strength to the material.

Alien Breeding Through Novel Tools

Introgression of desired genes from wild relatives into the bread wheat has become widely recognized as diversifying genetic diversity. However, wheat-alien chromosome additions often contribute negatively to the agricultural value of the line, therefore, desired gene/s has to be transferred into the wheat genome. Such transfers are normally blocked by the presence of a *Ph1* (*Pairing homoeologous*) allele, which strictly controls homologous chromosome pairing across the hexaploid genome to prevent hybridization between wheat and an alien species (Riley and Chapman, 1958). Anyhow, alien chromosome segments carrying gene/s of interest have been widely transferred into the wheat genome using the CS *ph1b* homoeologous recombination, radiation, and embryo culture techniques (Sears, 1977; Sears, 1993; Chen et al., 1994; Merker and Lantai, 1997). These approaches in a combination of molecular and cytogenetic manipulations were used to facilitate the introgression of *Sr26* and *Lr19* from *Thinopyrum ponticum*, *Sr39* from *Aegilops speltoides*, *Sr59* from *S. cereale*, etc. with small alien chromosome segments (Sharma and Knott, 1966; Merker and Lantai, 1997; Niu et al., 2011; Rahmatov et al., 2016a; Rahmatov et al., 2016b). More recently, reference genomes have been made available for wheat, (IWGSC, 2014), rye (Bauer et al., 2017), barley (IBGSC, 2012), rice (IRGSP, 2005), and Brachypodium (IBI, 2010), greatly facilitating the forward and reverse genetics in crops. Various high-throughput genotyping platforms such as the 9K and 90K Illumina Infinium SNP arrays and the 35K and 820K Affymetrix Axiom arrays have been developed for gene and QTL mapping (Wang et al., 2014; Winfield et al., 2016; Allen et al., 2017). In addition, genotyping-by-sequencing and exome capture sequencing opens

up more opportunities for markers development and gene isolation (Poland et al., 2012; Krasileva et al., 2017). All these genotyping platforms provide tremendous tools to assess the genetic diversity and allelic variation across plant genomes. However, a low level of SNP polymorphism between hexaploid wheat and wild relatives has been reported which negatively impact the use of the mentioned platforms (Winfield et al., 2016). Therefore, Tiwari et al. (2014) suggested the use of flow cytometric chromosome sorting to develop unique SNP markers for the mapping of alien genes to overcome these challenges. Whole-genome shotgun sequencing is becoming another valuable breeding tool in terms of time and cost, which are already used in major crops such as wheat (Brenchley et al., 2012), maize (Hufford et al., 2012), rice (Huang et al., 2012), and soybean (Fang et al., 2017). However, the transfer of desired alien gene/s remains a challenge, although some advances have been made in transferring resistance genes. Jupe et al. (2013) developed an exome capture and sequencing of nucleotide-binding leucine-rich repeat (NLR) genes in potato. Such resistance gene enrichment sequencing (RenSeq) allowed a rapid cloning of the *Sr22* and *Sr45* resistance genes through mutational genomics (Steuernagel et al., 2016). Another approach, MutChromSeq, has been applied through mutational genomics, chromosome flow sorting and sequencing that has resulted in the cloning of the *Pm2* resistance gene (Sánchez-Martín et al., 2016). Interestingly, another cloning approach suggested a combination of association genetics and R gene enrichment sequencing, which rapidly identified stem rust resistance genes for cloning (Arora et al., 2019). Besides, a combination of cisgenesis and genome editing tools may accelerate the plant breeding process (Cardi, 2016). Also, the use of speed breeding may significantly accelerate the generation times and breeding cycles (Watson et al., 2018). Therefore, integration of high-throughput genotyping and precise phenotyping tools may efficiently assist in transferring the introgression of small alien chromatin segments to develop new genetic diversity for wheat improvement. For example, the development of synthetic wheat and 1RS chromosome arm has made a great contribution to sustainable wheat production. Evidently, for the development of superior crop cultivars requires new genetic variation that meets sustainable agriculture and food security needs.

CONCLUSIONS—ALIEN GENES INTO MODERN WHEAT—FUTURE PERSPECTIVES

Every day, the human population is growing, and with that the demand of food from sustainable and healthy crop production. To adequately meet the global food demand required by 2050, there is a need to increase wheat yield annually. These can be achieved through the two unique opportunities; plant breeding and improved agronomic practices. Importantly, to meet projected food demand, the breeding programs need to broaden the existing genetic base, in particular by the use of alien species with the potential to improve yield, resistance to biotic and abiotic stresses and quality. Several of our studies have identified new sources of resistance to fungal diseases and insects in the wheat-alien introgression derivatives from *S. cereale*, *L. mollis*, *L. racemosus* and *T. junceiforme*. Also, these lines exhibiting good agronomic performances, high allelopathic potential, and superior end-use quality traits. Our results suggest that some of the lines could be used as a source of high Iron and Zinc and low Cadmium concentrations. These findings show that the wheat-alien introgressions are a potentially useful genetic resource for wheat improvement. The introgression of large alien chromosomes usually challenges researchers and breeders by causing linkage drag that can negatively effect on yield and quality properties. Fortunately, with the presence of high-throughput genotyping and phenotyping tools, opportunities increase to transfer desired gene/s with a small alien chromosome segment. Consequently, research is currently underway to transfer stem and stripe rust resistance genes into the elite wheat background to be used by breeders to develop superior wheat cultivars with new resistance genes. Further, additional research is also in progress for characterization and transferring of useful traits such as micronutrients (Zn, Fe, and Cd), allelopathic potential, diseases, and insect resistance as well as stable baking quality.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

EJ, TH, SA and MR planned various parts of the study, the hypothesis, and the objectives. TH, MP-L, SA, RA carried out various parts of the field and lab work. All authors contributed to compiling various parts of the results. EJ and MR planned the writing of this paper and did the first draft. All authors contributed to the article and approved the submitted version.

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II

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Identification of a Small Translocation from 6R Possessing Stripe Rust Resistance to Wheat

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Abstract

Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* Eriks. & E. Henn, is the most devastating fungal disease of bread wheat. Here, a wheat-rye multiple disomic substitution line, SLU126 4R (4D), 5R (5D), and 6R (7D), possessing resistance against 25 races of *P. striiformis* f. sp. *tritici*, was used and crossed with Chinese Spring *ph1b* to induce homeologous recombination to produce introgressions with a reduced rye chromosome segment. Seedling assays confirmed that the stripe rust resistance from SLU126 was retained over multiple generations. Through genotyping-by-sequencing (GBS) platforms and aligning the putative GBS-single-nucleotide polymorphism (SNPs) to the full-length annotated rye nucleotide-binding leucine-rich repeat (NLR) genes in the parental lines (CS *ph1b*, SLU126, CSA, and SLU820), we identified the physical position of 26, 13, and 9 NLR genes on chromosomes 6R, 4R, and 5R, respectively. The physical positions of 25 NLR genes on chromosome 6R were identified from 568,460,437 bp to 879,958,268 bp in the 6RL chromosome segment. Based on these NLR positions on the 6RL chromosome segment, the three linked SNPs (868,123,650 to 873,285,112 bp) were validated through kompetitive allele-specific PCR (KASP) assays in SLU126 and resistance plants in the family 29-N3-5. Using these KASP markers, we identified a small piece of the rye translocation (i.e., as a possible 6DS.6DL.6RL.6DL) containing the stripe resistance gene, temporary designated *YrSLU*, within the 6RL segment. This new stripe rust resistance gene provides an additional asset for wheat improvement to mitigate yield losses caused by stripe rust.

Keywords: genotyping-by-sequencing, KASP markers, NLR genes, physical mapping, translocation

Wheat (*Triticum aestivum* L.) is a major staple food crop, cultivated in diverse climatic conditions around the globe, and provides 20% of the daily protein and calories to the human diet (The International Wheat Genome Sequencing Consortium (IWGSC) et al. 2018). The contribution of wheat to food security is significant, especially for poor farmers in West and Central Asia and North Africa, where wheat is a critical part of the daily diet (Husenov et al. 2021). However, in the Western world (e.g., the United States and Europe) as well as in Russia and China, wheat has a significant impact on socioeconomic development (https://www.fao.org/faostat/en/#data).

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* Eriks. & E. Henn, is one of the major diseases of wheat, causing severe yield losses of approximately 5 million tons annually with a value of $1 billion (Beddow et al. 2015; Wellings 2011). The frequent emergence of new stripe rust races threatens global wheat production, because such novel races can overcome the current resistance present in wheat cultivars, thereby challenging resistance breeding of wheat (Hovmøller et al. 2021). Also, due to the constant evolution and mutation of *P. striiformis* f. sp. *tritici* races, the majority of the resistance genes originating from the primary gene pool have become ineffective within a relatively short period of time. Thus, a continuous search for novel resistance genes that can protect against the disease is essential for the development of novel broad-spectrum resistant genotypes. Thus, wild relatives of wheat are considered a rich source of valuable genes for wheat improvement (e.g., contributing to crop productivity, nutritional value, end-use quality traits, and yield potential). When it comes to stripe rust, 21 of the 83 currently described *Yr* resistance genes have been derived from the secondary and tertiary genepools, and most of them have also been utilized to improve resistance in wheat (McIntosh et al. 2017; Wang and Chen 2017). Examples of introgressions from such genepools are *Yr8*, *Yr17*, *Yr28*, *Yr37*, *Yr38*, *Yr40*, *Yr42*, and *Yr70* from *Aegilops* spp. and *Yr50* and *Yr69* from *Thinopyrum* spp. (Wang and Chen 2017). In addition to the currently described *Yr* resistance genes, more than 15 other *Yr* resistance genes have been identified and given a temporary designation (McIntosh et al. 2017). To contribute durable and broad-spectrum resistance to stripe rust in wheat lines, the pyramiding of *Yr* resistance genes, including those from different wild relatives, has been suggested as a sound strategy (Johnson 1984). Using that strategy, the combination of *Yr5* (*T. spelta* var. Album), *Yr10* (*T. aestivum*/*T. spelta*/*T. vavilovii*), and *Yr15* (*T. dicoccoides* accession) have been identified to contribute broad-spectrum and durable resistance against most of the emerging stripe rust races globally (Klymiuk et al. 2018; Marchal et al. 2018). Most plant resistance genes encode nucleotide-binding leucine-rich repeat (NLR) proteins, which are widely used in plant breeding for disease resistance (Jones and Dangl 2006; Kourelis and van der Hoorn 2018). Hence, the availability of the rye reference genome (Rabanus-Wallace et al. 2021) allows for a detailed study of its NLR complement by applying the NLR-Annotator tool to the reference genome (Steuernagel et al. 2020).

Aside from being a crop in its own right, rye (*Secale cereale* L., $2n = 2x = 14$, RR) is also considered a member of the tertiary gene pool of wheat, contributing genes useful for bread wheat improvement against both abiotic and biotic stresses (Moskal et al. 2021). Introgression of rye genes into wheat has recently gained a renewed interest because such introgressions will increase the diversity of genes that can contribute to wheat improvement in terms of disease resistance but also for other traits. Examples of stripe rust resistance genes that have been introgressed from rye into wheat are *Yr9*,

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YrCn17, *YrR212*, and *yrCH45-1* from 1R, and *Yr83* from 6R (J. Li et al. 2020; Luo et al. 2008; McIntosh et al. 2017; Ren et al. 2009; Yang et al. 2016). Moreover, introgressions of 1R, 2R, 4R, 5R, and 6R, carrying genes for *Yr* resistance, have been derived from *S. cereale*, and *S. africanum* (An et al. 2019; Lei et al. 2011, 2012, 2013; Li et al. 2016; Xi et al. 2019). Thus, rye is an important source of valuable genes conferring resistance to diseases and pests (Friebe et al. 1996; Johansson et al. 2020; Moskal et al. 2021; Rahmatov et al. 2016b), which still is largely untapped and inaccessible to wheat breeders.

Homologous chromosome pairing in wheat with chromosomes originating from the tertiary gene pool is strictly hindered by the *pairing homeologous* (*Ph*)*1* and *Ph2* alleles residing on chromosomes 5BL and 3DS, respectively (Mello-Sampayo 1971; Riley and Chapman 1958). However, a deletion of the *Ph1* locus (*ph1b* in hexaploid and *ph1c* in tetraploid wheat) allows homeologous chromosome pairing to take place between wheat and alien chromosomes, thereby contributing opportunities for meiotic recombination of DNA (Giorgi and Barbera 1981; Sears 1977). Therefore, a Chinese Spring (CS) *ph1b* mutant has been used most effectively to induce recombination between wheat and rye chromosomes, as well as between chromosomes of wheat and other wild relatives of wheat. Examples of successful transfer of genes, facilitated by the CS *Ph1b* mutant, are the introgression of *Sr32* from *Aegilops speltoides* (Mago et al. 2013), *Sr39* from *A. speltoides* (Niu et al. 2011), *Sr43* from *Thinopyrum ponticum* (Niu et al. 2014), *Sr47* from *A. speltoides* (Klindworth et al. 2012), *Sr53* from *A. geniculata* (Liu et al. 2011), *Sr59* from *S. cereale* (Rahmatov et al. 2016a), *Yr83* from *S. cereale* (J. Li et al. 2020), and rust resistance genes from *A. sharonensis* (Khazan et al. 2020). Furthermore, the CS *Ph1b* mutant contributed to improvements of end-use quality by eliminating the secalin (*Sec-1*) allele when 1R was introgressed to wheat (Lukaszewski 2000). In most cases, cytogenetic analyses such as fluorescent in situ hybridization (FISH) and genomic in situ hybridization (GISH) have been applied for identifying introgression breakpoints, although resolution of the breakpoint has been low in rare cases. To detect small introgressions in the wheat genome, where FISH and GISH may be limited, molecular markers are essential tools. Indeed, molecular markers have revealed the presence of *A. geniculata* chromatin in the wheat genome linked with the *Yr40*/*Lr57* resistance genes (Kuraparthy et al. 2007), cryptic introgression of *Dasypyrum villosum* (Caceres et al. 2012), and cryptic rye 2R and 5R chromatin contributing genetic and epigenetic variations (Fu et al. 2013). Transfer of resistance genes often results in additional linked genes being transferred with the introgression, leading to linkage drag which often has a negative impact on end-use quality or yields from the additional alien transfer (Zeven et al. 1983). Therefore, the inclusion of only a small chromosome segment to reduce linkage drag is the best approach, combined with detection of the transferred resistance gene by applying high-density marker platforms; that is, genotyping-by-sequencing (GBS), DArTseq, kompetitive allele-specific PCR (KASP) assays, and so on.

A large number of wheat-rye introgression lines have been developed by crossing and backcrossing by the late Professor Arnulf Merker at the Swedish University of Agricultural Sciences (Sveriges lantbruksuniversitet [SLU]) (Forsström and Merker 2001; Merker 1984). From field and greenhouse evaluations, we identified line SLU126 [4R (4D), 5R (5D), and 6R (7D) multiple wheat-rye disomic substitutions] with a broad-spectrum resistance to a wide-array of *P. striiformis* f. sp. *tritici* races ($n = 25$) (Rahmatov et al. 2017), including the present study. The present study aimed to identify and characterize the resistance genes against stripe rust present in this highly resistant winter wheat-rye introgression line, by utilization of a combination of traditional methods and novel genomic tools. A second aim was to develop KASP markers for these resistance genes in order to facilitate their use in future breeding programs.

Materials and Methods

Plant materials

In previous work at SLU, the winter wheat-rye multiple disomic substitution lines SLU124 to SLU129 with 4R (4D), 5R (5D), and 6R (7D) have been developed from crosses between the hexaploid triticale breeding line Sv876012 and the winter wheat cultivar Holme (Forsström and Merker 2001). The CS *ph1b* mutant line (Sears 1977) was kindly provided by Dr. Steven Xu, United States Department of Agriculture–Agricultural Research Service. Initially, all of the abovementioned wheat genotypes (except SLU820) were tested against 13 *P. striiformis* f. sp. *tritici* races at the seedling stage in the greenhouse, and an additional field assessment was carried out at the adult stage against two *P. striiformis* f. sp. *tritici* races (Rahmatov et al. 2017). In this study, the CS *ph1b* was crossed with the SLU126 (as a representative of the winter wheat-rye multiple disomic substitution lines SLU124 to SLU129) and the resulting F_1 was backcrossed to CS *ph1b*, producing 190 BC_1F_1 seeds. Selected (based on two mixes of PSTv-14 and PSTv-37 races and *Xpsr128*, *Xpsr574*, and *Xawjl3* markers) BC_1F_1 plants were selfed and produced approximately 500 BC_1F_2 seeds. Furthermore, 240 BC_2F_1 seeds were produced by backcrossing selected BC_1F_1 plants with two wheat genotypes, Chinese Spring (CSA) and SLU820. We assessed the seedling response of 376 BC_1F_2 plants and 240 BC_2F_1 plants to a mixture of PSTv-14 and PSTv-37 races.

Stripe rust seedling evaluation

Five *P. striiformis* f. sp. *tritici* races, available at the University of Minnesota, St. Paul, MN, U.S.A. were used in this study: PSTv-14, PSTv-37, PSTv-40, PSTv-218, and PSTv-221 (Wan et al. 2017). Stripe rust seedling response was evaluated at BC_1F_1 to BC_1F_4 and BC_2F_1 to BC_2F_3 generations using the PSTv-14 and PSTv-37 races in a 50:50 mixture (spores weighed equally and inoculum prepared with Soltrol). The PSTv-14 and PSTv-37 races are prevalent in the United States and have a broad virulence spectrum; thus, they were expected to contribute a high infection pressure on our lines, which were expected to contain multiple resistance genes. Additionally, the PSTv-40, PSTv-218, and PSTv-221 races were used in later generations (BC_1F_4 and BC_2F_3) to evaluate the stripe rust seedling resistance toward more races. Five to seven seeds were sown in SC10 cell containers (4 cm in diameter by 21 cm in height) containing a 50:50 mixture of steam-sterilized native soil and Sunshine MVP mix (Sungro Horticulture Distributors Inc.), a growing medium containing vermiculite, Canadian sphagnum peat moss, coarse perlite, starter nutrient charge, gypsum, and dolomitic limestone. The plants were maintained until full emergence of the second leaves (14 days) in the greenhouse at a day and night temperature of 22 and 19°C, respectively, 40% humidity, and a 14-h photoperiod (supplemented by 400-W high-pressure sodium lamps emitting photons at a minimum of 300 μmol s^{-1} m^{-2}). Plants were fertilized with slow-release pellets (Osmocote 14- 14-14; Scott's Company) at 0.3 g/pot. Stored urediniospores were removed from a –80°C freezer, immediately heat-shocked for 10 min, and then placed in a rehydration chamber (at approximately 80% relative humidity) for 2 to 4 h until inoculation. A mix of the *P. striiformis* f. sp. *tritici* races PSTv-14 and PST-v37 was suspended in a lightweight mineral oil (Soltrol 170; Phillips Petroleum), which was then inoculated onto the plants. Inoculated plants were placed in a dark chamber at 10°C and 100% relative humidity for 24 h. Plants were then transferred to the growth chamber for 18 h at 20 ± 2°C and 6 h at 18 ± 2°C with a photoperiod 16 h of light and 8 h of darkness. Seedling infection types (ITs) on the first and second leaves of plants were scored separately at 14 to 16 days postinoculation using a 0-to-9 scale, where 0 to 2 = immune to resistant, 3 to 4 = moderately resistant, 5 to 6 = moderately susceptible, and 7 to 9 = susceptible to very susceptible (McNeal et al. 1971).

Molecular marker analyses

Genomic DNA was isolated from young leaf tissue according to Edwards et al. (1991), with some slight modifications. The DNA from BC_1F_1 plants was analyzed with the touchdown molecular markers *Xpsr128*, *Xpsr574*, and *Xawjl3* to detect homozygous *ph1b* plants (Roberts et al. 1999). Additionally, 34 (4R), 30 (5R), and 74 (6R) molecular markers (in total, 138 markers) were utilized in the present study, and all were previously mapped in the corresponding rye chromosomes (Khlestkina et al. 2004; Li et al. 2013, 2018; Martis et al. 2013). These polymorphic markers were used to select plants

with the desired haplotypes (i.e., containing rye chromatin from the specific chromosomes of interest: 4R, 5R, and 6R) from the BC_1F_1 and BC_1F_2 generations. The PCR master mix for all markers and the cycling conditions of the PCR were based on specific primer sets for correct product amplification (Khlestkina et al. 2004; Li et al. 2013, 2018; Martis et al. 2013). The PCR products were resolved on 2 to 4% agarose gels and visualized under UV light after GelRed staining.

GBS of parental lines and cytogenetic analyses

The parental lines (SLU126, Sv876012, Holme, CS *ph1b*, CSA, and SLU820) were genotyped by GBS (Poland et al. 2012) at the Genomic Center, University of Minnesota. From all parental lines, 10- to 12-cm-long segments of young leaf tissue were collected for DNA isolation. Genomic DNA was isolated using the BioSprint 96 DNA Plant Kit (Qiagen, Valencia, CA, U.S.A.) and, thereafter, quantified with PicoGreen for GBS. Obtained sequence information was aligned to the wheat reference genome (The International Wheat Genome Sequencing Consortium (IWGSC) et al. 2018), and rye reference genome (Rabanus-Wallace et al. 2021) using the Burrow-Wheelers Alignment tool (v0.7.15) (Li and Durbin 2009). Additionally, NLR sequences on chromosomes 4R, 5R, and 6R (described in the next section) were obtained, which were converted to a "pseudo" reference genome to align the obtained reads. Variant calling (single-nucleotide polymorphisms [SNPs], primarily) was accomplished using Samtools+Bcftools (Li 2011) with default parameters. SNPs with minor allele frequency of less than 5% and more than 20% missing data were removed. After filtering the SNP markers, 12,195 (4R), 12,660 (5R), and 10,675 (6R) SNP markers were retained for further analysis. FISH or GISH was performed on lines from the BC_1F_4 and BC_2F_3 families fixed for resistance to a mixture of PSTv-14 and PSTv-37 races to visualize wheat-rye introgression events, following previously described methods (Rahmatov et al. 2016a, b).

Development of KASP markers

The full-length annotated rye NLR genes (272 NLR for 4R, 83 NLR for 5R, and 184 NLR for 6R) were kindly provided by Prof. Nils Stein, Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, and Dr. Brande Wulff and Dr. Burkhard Steuernagel, John Innes Centre (Rabanus-Wallace et al. 2021). These full-length annotated rye NLR genes were aligned to the GBS reads using BLASTN search (Zhang et al. 2000) to identify putative NLR-associated SNPs for the 4R, 5R, and 6R chromosomes in the parental lines (SLU126, Sv876012, Holme, CS *ph1b*, CSA, and SLU820). Match criteria were set to 100% identity, with no mismatch or gaps allowed with a minimum E-value of 1E-10 (threshold value for BLAST). Thus, after aligning the GBS reads to the rye NLR genes, physical positions of NLR-associated SNPs were detected for the 4R, 5R, and 6R chromosomes in the parental lines. The 120-bp flanking sequence (60 bp of each upstream and downstream) of the NLR-GBS position were used for developing KASP primers. The specific chromosome-linked NLR-GBS markers were converted into KASP primers according to Rahmatov et al. (2016a), which were thereafter validated (Tables 1 and 2). MapChart (https://www.wur.nl/en/show/mapchart.htm) was used to draw the physical map.

Table 1. List of kompetitive allele-specific PCR (KASP) markers used in the study to detect 4R, 5R, and 6R chromosomes (Chr) in SLU126, CS *ph1b*, CSA, and SLU820

Type of marker[a]	Primers	Markers tested	Chr	Polymorphic markers in the SLU126
GBS-SNP	KASP	13	4R	3
GBS-SNP	KASP	9	5R	0
GBS-SNP	KASP	38	6R	9

[a] Genotyping-by-sequencing single-nucleotide polymorphism (GBS-SNP).

Results

Stripe rust seedling resistance test, cytogenetics, and molecular markers in parental lines

The lines SLU124, SLU125, SLU126, SLU127, SLU128, and SLU129, as well as Sv876012, exhibited broad-spectrum resistance against 12 *P. striiformis* f. sp. *tritici* races at the seedling stage, whereas CS *ph1b*, CSA, and SLU820 were all highly susceptible (Table 3). A GISH analysis detected multiple disomic substitutions at mitotic metaphase in SLU126 with the presence of 42 chromosomes (Table 2; Supplementary Fig. S1). We aligned the raw GBS reads against the IWGSC and RGSCR reference genomes to remove perfect-match reads. The GBS resulted in 12,195 SNPs for 4R, 12,665 SNPs for 5R, and 10,675 SNPs for 6R for the lines SLU124 to SLU129. In line Sv876012, no D chromosomes from wheat were observed; instead, seven rye chromosomes were present (Table 3). Also, chromosomes 4D, 5D, and 7D were absent in the SLU124 to SLU129 lines (Table 3). Thus, the GBS analysis identified the SLU124 to SLU129 lines as having multiple wheat-rye disomic substitutions of 4R (4D), 5R (5D), and 6R (7D) (Table 3), corresponding also with the results of the GISH analysis (Supplementary Fig. S1). In total, five polymorphic rye-specific simple-sequence repeat (SSR) markers for chromosome 4R, seven for chromosome 5R, and four for chromosome 6R were identified, allowing for the specific detection of 4R, 5R, and 6R, respectively, in SLU126 and their absence in CS *ph1b*, CSA, Holme, and SLU820 (Table 4).

Identifying stripe rust resistance gene in BC_1F_1 (CSph1b × SLU126 × CSph1b)

In total, 190 BC_1F_1 seeds were derived from the cross between CS *ph1b* and SLU126 and, when they were tested at the seedling stage to a mix of the *P. striiformis* f. sp. *tritici* races (PSTv-14 + PSTv-37), a segregation of 162 resistant plants and 28 susceptible plants was obtained. This segregation did not fit a 1:1 ratio ($\chi^2 = 94.5$, $P \leq 2.4$), indicating a possible complementary gene action with segregation distortion. Analyses using agarose gels and *ph1b* markers (*Xpsr128*, *Xpsr574*, and *Xawjl3*) showed that 39 of the 162 resistant plants were identified as homozygous to the *ph1b* allele. In total, 16 polymorphic SSR markers, which all were able to identify the presence and absence of 4R, 5R, and 6R in the utilized parental lines in the present study, were used for identification of these chromosomes in the 39 plants showing resistance in seedling resistance tests and being

Table 2. Kompetitive allele-specific PCR (KASP) markers and their physical positions in the 6RL chromosome

SNP ID[a]	Primer name	Primer sequence	Physical position of SNP in 6RL (bp)
375	KASP_6RL_375A1	CAGGCCTGCCTCCCCGG	868,123,650
	KASP_6RL_375A2	AACAGGCCTGCCTCCCCGA	–
	KASP_6RL_375C1	GGCCGGGCCGAGCAGCGT	–
387	KASP_6RL_387A1	GAATCCGGTGCACGTCGC	873,285,080
	KASP_6RL_387A2	CTGAATCCGGTGCACGTCGG	–
	KASP_6RL_387C2	CTATCACTTTCAGGCGGACGAGTT	–
392	KASP_6RL_392A1	CGTCCGCCTGAAAGTGATAGCTA	873,285,112
	KASP_6RL_392A2	GTCCGCCTGAAAGTGATAGCTG	–
	KASP_6RL_392C1	GGAAAAATCAGAGGTCACGGCTGAT	–

[a] SNP = single-nucleotide polymorphism.

homozygous for the presence of the *ph1b* allele. However, only 11 of these 39 plants were identified to possess the 4R, 5R, and 6R chromosomes, when assessed with the 16 polymorphic SSR markers (Table 4). In all, 5 of the 39 plants (5-N2-2, 15-N7-6, 29-N3-5, 44-N4-5, and 46-N4-7) did not show any positive response on any of the 16 SSR markers used for evaluation of the presence of rye chromatin (Table 4). The fact that these five plants showed a resistance pattern similar to that of their resistant parent indicates that they contain the resistance genes, and the fact that the rye chromatin was not detectable with the utilized markers indicates that the rye chromatin in the plants no longer contains the part recognized by the marker (i.e., the size of the segment of the rye chromatin has been decreased). The 11 plants identified to contain 4R, 5R, and 6R with the markers and the 5 plants being resistant and not recognized as having rye chromatin by any of the markers were selfed to obtain BC_1F_2 families, which were tested to a mix of the *P. striiformis* f. sp. *tritici* races (PSTv-14 + PSTv-37). These seedling resistance tests resulted in 11 families showing segregation (resistant or susceptible), while 5 families (13-N7-4, 25-N3-1, 32-N4-1, 37-N3-4, and 46-N4-7) were found to be highly susceptible to the PSTv-14 + PSTv-37 mix of *P. striiformis* f. sp. *tritici* races. In total, 376 BC_1F_2 plants were analyzed with the same 16 SSR markers as described above, although the presence of 4R, 5R, and 6R chromosomes verified by these markers did not correspond with resistance patterns of the families. Again, the lack of correlation between resistance reactions and marker identification indicates that the rye segment in the lines that contributed resistance has, in some of the cases, lost the part recognized by the markers, due to the fact that it is short. For the BC_1F_2 families 15-N7-6, 29-N3-5, and 44-N4-5, none of the 16 SSR markers used could identify any presence of rye alleles, thereby corresponding to the findings of lack of such identification in their corresponding BC_1F_1 families. The fact that the utilized 16 SSR markers were not able to detect the rye chromatin responsible for resistance reactions made the utilized 16 SSR markers not useful for further detection of resistance genes present in the wheat-introgression lines analyzed. Based on the fact that the families 15-N7-6, 29-N3-5, and 44-N4-5 most likely contained only a small fragment of rye chromatin (carrying the resistance gene although not being identified by the 16 used markers), and plants from BC_1F_2 of these families were selfed to receive BC_1F_3 generation. The BC_1F_3 plants were again tested with a mix of the PSTv-14+PSTv-37 *P. striiformis* f. sp. *tritici* races, so that resistant plants could be selected and self-crossed to produce BC_1F_4. Nine BC_1F_4 lines from the 29-N3-5 family were identified as stably resistant to PSTv-14+PSTv-37 (Table 5; Fig. 1). In total, six BC_1F_4 lines (numbers 119, 124, 383, 391, 392, and 484) of the 29-N3-5 family were analyzed by GISH or FISH, although no rye chromosome segment was found with these methods despite the fact that all families (except 119 and 124 to PSTv-221) showed seedling resistance to all *P. striiformis* f. sp. *tritici* races (Table 5). Based on GISH or FISH analysis, all BC_1F_4 families possessed 42 chromosomes (Supplementary Fig. S1). Thus, these results indicated that the introgression events were so small that rye chromatin was not detectable with GISH or FISH. The nine BC_1F_4 lines identified as stable were tested with additional *P. striiformis* f. sp. *tritici* races (namely, PSTv-40, PSTv-218, and PSTv-221), which resulted in a similar (except line 391) resistance response on PSTv-40 as for the mix of the PSTv-14 + PSTv-37 *P. striiformis* f. sp. *tritici* races, while a clear segregation in resistance response was noted to the PSTv-218 and PSTv-221 races (Table 5). Thus, all nine BC_1F_4 lines showed a segregating (IT 2-3 or 7-8) resistance response (i.e., some plants showed resistance IT 2-3 and some plants showed susceptible reaction IT 7-8) to the PSTv-218 race (Table 5). The resistance response to the PSTv-221 race was more variable, with lines 383 and 391 (BC_1F_4) identified as stably resistant (IT 2-3), indicating the presence of homozygous resistance genes, whereas lines 484 and 488 segregated with IT 2-3 or 7-8 (Table 5). Additionally, two of the lines (386 and 392), showed IT 2-3 or 4-5, and lines 120 and 124 showed IT 4-5 or 7-8, while family 119 showed IT 4-5 (Table 5). The segregation pattern toward the evaluated *P. striiformis* f. sp. *tritici* races indicates a possible presence of more than one resistance gene in the BC_1F_4 lines.

Backcrossing into CSA and SLU820

Plants of the BC_1F_1 family 29-N3-5 was backcrossed to CSA and SLU820, and the obtained BC_2F_1 progeny was assessed to the PSTv-14 + PSTv-37 mix of *P. striiformis* f. sp. *tritici* races at the seedling stage. The observed segregation pattern of both the backcrossing (29-N3-5 × CSA and 29-N3-5 × SLU820) populations did not fit into the 1:1 or 15:1 ratios, indicating a significant segregation distortion in both populations (Table 6). All of the BC_2F_1 resistant and susceptible plants were assessed with the 16 SSR markers, although none of the SSR markers detected rye segments present corresponding to resistance reactions. The 32 (BC_1F_1 29-N3-5 × CSA) and 36 (BC_1F_1 29-N3-5 × SLU820) resistant plants from each backcrossing were transplanted to obtain BC_2F_2 families. Among the 386 BC_2F_2 plants in the 29-N3-5 × CSA population, 170 were found to be resistant, twhile 224 plants were identified as resistant to the PSTv-14 + PSTv-37 mix of *P. striiformis* f. sp. *tritici* races in the 29-N3-5 × SLU820 population (Table 6). Three of the evaluated BC_2F_3 lines (129, 136, and 322), all from the backcross with CSA, were found homozygous for resistance to races PSTv-14 + PSTv-37 and PSTv-40. Two other lines (128 backcross to CSA and 325 backcross to SLU820) were segregated for resistance to the race PSTv-40 (Table 5). Line 356 was found highly susceptible to the PSTv-14 + PSTv-37 mix of *P. striiformis* f. sp. *tritici* races, with an IT of 8-9 (Table 5). The evaluated BC_2F_3 lines all displayed segregating or susceptibility responses to PSTv-218 and PSTv-221 (Table 5; Fig. 1). Two of the evaluated lines (325 and 354) showed a fixed resistance to race PSTv-221, whereas the

Table 3. Seedling resistance in parental lines to different *Yr* races[a]

Lines	DK169/16	DK214/12	DK127/16	DK46/14	DK09/11sp	SE141/11	DK111/02	Taj14a/10	PSTv-14	PSTv-37	PSTv-40	PSTv-218	PSTv-221	GBS
CS*ph1bM*	7-7	7-8	7-8	8-8	7-8	7-8	7-7	7-8	8-8	7-8	8-8	8-8	8-8	AABBDD
CSA	7-7	7-8	7-8	7-8	7-8	7-8	7-7	7-7	8-8	8-8	7-8	7-8	8-8	AABBDD
Holme	2-3	7-8	7-8	7-8	7-8	7-8	7-8	7-8	7-7	7-8	7-7	7-7	7-8	AABBDD
Sv876012	2-3	2-3	3-3	3-3	2-3	2-3	2-3	2-3	1-2	2-3	1-2	2-3	2-3	AABBRR
SLU820	–	–	–	–	–	–	–	–	7-8	8-8	7-8	7-8	8-8	AABBDD
SLU124	2-3	2-3	3-3	3-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	$AABBD_RD_R$
SLU125	2-3	2-3	2-3	3-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	$AABBD_RD_R$
SLU126	2-3	3-3	2-3	3-3	2-3	2-3	2-3	3-3	2-3	2-3	2-3	2-3	2-3	$AABBD_RD_R$
SLU127	3-3	2-3	2-3	2-3	2-3	2-3	3-3	2-3	2-3	2-3	2-3	2-3	2-3	$AABBD_RD_R$
SLU128	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	2-3	$AABBD_RD_R$
SLU129	3-3	2-3	2-3	3-3	2-3	2-3	2-3	3-3	2-3	2-3	2-3	2-3	2-3	$AABBD_RD_R$

[a] GBS = Genotyping-by-sequencing. Infection types (ITs) were observed based on a 0-to-9 scale (McNeal et al. 1971). Plants with ITs of 1-2 to 2-3 were considered resistant and plants with ITs of 7-7 to 8-8 were considered susceptible to highly susceptible.

other two lines, 322 (IT 2-3 or 7-8) and 354 (IT 2-3 or 4-5), showed a segregation in resistance to race PSTv-221 (Table 5). These segregation patterns in resistance reactions indicate the presence of more than one resistance gene in the populations. GISH or FISH was used to determine whether BC_2F_3 families (129, 322, 325, and 354) have a rye chromosomal constitution but no rye chromosome segments were detected (Table 5; Supplementary Fig. S1). Similar results were observed in the two backcrossing populations. Thus, most likely, a small rye chromosomal segment with stripe rust resistance was introgressed into wheat but was not possible to detect with GISH or FISH.

GBS in parental line, physical mapping, and developing KASP markers

CS *ph1b*, CSA, Holme, Sv876012, SLU820, and SLU126 were sequenced using the GBS platform. Obtained GBS datasets were aligned to both the wheat and the rye reference genomes, where the alignment to the rye reference genome was used to map the putative SNPs for the 4R, 5R, and 6R chromosomes. A physical position of the 12,195 SNPs for 4R (82,6531 bp 906,392,438 bp), 12,665 SNPs for 5R (128,895 to 875,792,828 bp), and 10,675 SNPs for 6R (147,224 to 885,150,592 bp) were identified. Moreover, the physical position of a total of 13 NLRs in 4R, 9 NLRs in 5R, and 32 NLRs in 6R was detected in the SLU126 line, using the full-length annotated rye NLR genes. In total, 25 NLR genes were identified in the 6RL segment starting from 645,846,469 to 879,975,498 bp (Fig. 2). The harboring SNPs around the NLR gene positions in the 6RL chromosome were selected and utilized for the development of KASP markers. By evaluating the 38 KASP markers in the 6RL chromosomal segment, 9 were identified (158C2, 180C2, 344C1, 375C1, 387C2, 390C1, 392C1, 393C1, and 398C1) that were able to clearly distinguish CS *ph1b*, CSA, and SLU820 from SLU126 (Table 4). Of these nine KASP markers, three (375C1, 387C2, and 392C1) were amplified in the BC_1F_1 family 29-N3-5 (Table 4). The physical position of the 375C1 KASP marker is located in the 868,123,650-bp region, whereas the physical positions of the 387C2 and 392C1 KASP markers are located in the 873,285,080- and 873,285,112-bp regions, respectively. Thus the distance of 5,161,430 bp between the 375C1 KASP marker and the 387C2 and 392C1 KASP markers indicates the presence of two individual genes (Fig. 3), found to cosegregate (Table 5). The KASP marker 375C1 detected the line 44-N4-5, whereas none of the three KASP markers were detected in the other four lines (5-N2-2, 15-N7-6, 44-N4-5, and 46-N4-7) (Table 4). The KASP markers 375C1, 387C2, and 392C1 were subsequently used to analyze BC_1F_2 (29-N3-5) and BC_2F_1 (29-N3-5 × CSA and 29-N3-5 × SLU820) resistant and susceptible plants after the seedling assay (PSTv-14 + PSTv-37). Subsequent analysis of the BC_1F_2 and BC_2F_1 plants with these three KASP markers identified the SLU126 allele in the resistant BC_1F_2 and BC_2F_1 plants; however, these markers failed to amplify any signal in the susceptible plants (Table 5; Fig. 3). After the seedling assay with the PSTv-14 + PSTv-37 mix of *P. striiformis* f. sp. *tritici* races, the 375C1, 387C2, and 392C1 KASP markers were used in the BC_1F_3 (346 plants) and BC_2F_2 (394 plants) resistant plants to confirm the presence of the rye allele. Also, the presence of 375C1, 387C2, and 392C1 alleles was confirmed in the BC_1F_4 and BC_2F_3 plants after the stripe rust seedling tests with all five *P. striiformis* f. sp. *tritici* races: PSTv-14 + PSTv-37, PSTv-40, PSTv-218, and PSTv-221 (Table 5). By the use of these three KASP markers, we were able to confirm a cosegregation of the resistant phenotype with the rye alleles, indicating that stripe rust resistance is conferred by rye chromatin. However, line SLU126 might also possess an additional independent resistance gene other than the 375C1, 387C2, and 392C1 resistant alleles identified here that explains the various infection types to all *P. striiformis* f. sp. *tritici* races tested (Table 5). In this study, a very small piece of the 6RL chromosome was introgressed into the wheat genome, only detectable with specific KASP markers.

Identification of small translocation for stripe rust in the 6R chromosome

In total, 16 SSR polymorphic markers in the BC_1F_1, BC_1F_2, and BC_2F_1 progenies (from the 29-N3-5 family) were not able to detect the presence of any wheat-rye translocation or rye chromatin

Table 4. Presence of rye alleles from different chromosomes in BC_1F_1 and BC_1F_2 populations and its parental lines as detected by polymorphic rye-specific simple-sequence repeat (SSR) and kompetitive allele-specific PCR (KASP) markers[a]

		SSR markers to 4R					SSR markers to 5R						
Lines	IT[b]	Xglk-030	Xglk-201	TNAC1464	Xglk.ssrR4-213	Xglk.ssrR4-201	TNAC1497	Xglk.ssrR5-211	Xglk.ssrR5-193	Xglk.ssrR5-003	XREMS1218	XREMS1237	TNAC1541
CSph1b	8-8	–	–	–	–	–	–	–	–	–	–	–	–
CSA	8-8	–	–	–	–	–	–	–	–	–	–	–	–
SLU820	8-8	–	–	–	–	–	–	–	–	–	–	–	–
Holme	7-8	–	–	–	–	–	–	–	–	–	–	–	–
SLU126	2-3	+	+	+	+	+	+	+	+	+	+	+	+
Sv876012	2-3	+	+	+	+	+	+	+	+	+	+	+	+
5-N2-2	2-3	–	–	–	–	–	–	–	–	–	–	–	–
7-N2-3	2-3	–	–	–	–	–	–	–	–	–	–	–	–
13-N7-4	2-3	–	–	–	–	–	–	+	+	+	+	+	+
15-N7-6	2-3	–	–	–	–	–	–	–	–	–	–	–	–
16-N8-1	1+6	+	+	+	+	+	–	–	–	–	–	–	–
25-N3-1	2-3	–	–	–	–	–	+	+	+	–	+	+	+
26-N3-2	2-3	+	–	–	–	–	+	+	+	+	+	+	+
29-N3-5	2-3	–	–	–	–	–	–	–	–	–	–	–	–
30-N3-6	2-3	+	+	+	+	+	+	+	+	+	+	+	+
32-N4-1	2-3	+	+	+	+	+	+	+	+	+	+	+	+
33-N8-1	2+6	–	–	–	–	–	+	+	+	+	+	+	+
35-N3-2	2-3	–	–	–	–	–	+	+	+	+	+	+	+
37-N3-4	2-3	–	–	–	–	–	+	+	+	+	+	+	+
44-N4-5	2-3	–	–	–	–	–	–	–	–	–	–	–	–
46-N4-7	2-3	–	–	–	–	–	–	–	–	–	–	–	–
54-N7-2	2-3	–	+	+	+	+	+	+	+	+	+	+	+
174 (29-N3-5)	2-3	–	–	–	–	–	–	–	–	–	–	–	–
180 (29-N3-5)	2-3	–	–	–	–	–	–	–	–	–	–	–	–
214 (29-N3-5)	2-3	–	–	–	–	–	–	–	–	–	–	–	–
245 (29-N3-5)	2-3	–	–	–	–	–	–	–	–	–	–	–	–
336 (29-N3-5)	2-3	–	–	–	–	–	–	–	–	–	–	–	–
340 (29-N3-5)	2-3	–	–	–	–	–	–	–	–	–	–	–	–
349 (29-N3-5)	2-3	–	–	–	–	–	–	–	–	–	–	–	–

(*Continued on next page*)

[a] Symbols "+" and "–" indicate presence and absence, respectively, of the corresponding alleles.
[b] Infection type (IT) on a scale of 1 to 9 for PSTv-14 PSTv-37.

segment in the lines evaluated (Table 4). However, the KASP markers 375C1, 387C2, and 392C1 were able to detect rye chromatin in the BC_1F_1 family 29-N3-5 (Table 4). The family 29-N3-5 was identified as possessing a putative wheat-6R homeologous recombination due to a short segment of rye chromatin introgressed. The three KASP markers (375C1, 387C2, and 392C1) developed could successfully detect resistant plants that were derived and obtained from the family 29-N3-5 (Table 5). The cryptic translocation possibly occurred in the form of 6DS.6DL.6RL.6DL, due to the fact that the rye chromosome is syntenic to the wheat chromosome 6, although more research is needed to verify the form of this translocation.

Discussion

The present study clearly demonstrates the development of a new wheat-rye small translocation line with resistance to five *P. striiformis* f. sp. *tritici* races: PSTv-14, PSTv-37, PSTv-40, PSTv-218, and PSTv-221. We transferred the new stripe rust resistance gene, *YrSLU,* from *S. cereale* into wheat as a 6DS.6DL.6RL.6DL small translocation using the CS *ph1b* mutant. As in previous studies that reported cryptic introgressions from *A. geniculata* (Kuraparthy et al. 2007), *D. villosum* (Caceres et al. 2012), *T. intermedium* (Dong et al. 2004), and *S. cereale* (Fu et al. 2013), these were not detectable with cytogenetic analyses, while molecular marker assays were a necessity for their detection. There were no suitable markers present that were able to detect the small translocation (6DS.6DL.6RL.6DL) of this study; therefore, we developed KASP markers 375C1, 387C2, and 392C1 to verify the presence of the rye chromatin behind the resistance. Then, the KASP assay was able to identify the small rye chromatin segment in the BC_1F_1 (29-N3-5 family), BC_2F_1 (29-N3-5 × CSA and 29-N3-5 × SLU820), BC_1F_4, and BC_2F_3 that corresponded with the resistance. The chromosomal location to which the KASP markers (375C1 387C2, and 392C1) are connected is on the 6RL chromosome between 868,123,650 bp (375C1) and 873,285,080 bp (387C2 and 392C1), which is a 5,161,430-bp difference. These 6RL chromosome regions were undetectable by the SSR analysis because of the small translocation.

The rye genome is known to be highly rearranged compared with that of wheat (Devos et al. 1993). Different parts of chromosome 6R have been translocated from different rye genotypes into the wheat genome as, for example, 6RS.6DL and 6DS.6RL (G. Li et al. 2020), wheat-rye 6R disomic addition line (An et al. 2015), T6BS.6BL-6RL (Mukai et al. 1993), and 6AL·6RS (Hao et al. 2018). Recently, a new stripe rust resistance gene, *Yr83,* has been introgressed from rye as T6AL·6RL and T6AS·6RL (J. Li et al. 2020) using the CS *ph1b* mutant. The rye chromosome 6R is syntenic to wheat chromosome 6 and, thus, the small translocation possibly occurred in the 6DL arm of wheat (6DS.6DL.6RL.6DL). The CS *ph1b* mutant induces meiotic pairing and recombination between homeologous chromosomes (Riley and Chapman 1958; Sears 1977). Previous studies have also shown that the use of the CS *ph1b* mutant significantly shortens the chromosome segment with resistance genes introgressed (e.g., as for *Sr39*, *Sr43*, *Sr59*, and *Yr83*) (J. Li et al. 2020; Niu et al. 2011, 2014; Rahmatov et al. 2016a). Thus, the CS *ph1b* mutant has an important role in inducing homeologous meiotic recombination between the chromosomes of wheat and wild relatives and introgression of suitable genes. In this study, the transfer of a small wheat-rye translocation containing a stripe rust resistance gene is induced by the CS *ph1b* mutant into the wheat genome. The transfer of small pieces of alien chromatin is favorable to avoid linkage drag and unwanted traits and is more breeder friendly for cultivar development. Also, in previous work, resistance genes such as *Lr57*/*Yr40* and *Pm21* have been transferred through molecular marker assays and disease screening (Kuraparthy et al. 2007; Xing et al. 2018). Fu et al. (2013) reported a cryptic introgression of rye chromatin into the wheat genome, which was obtained by inducing the wheat-rye 2R and 5R monosomic addition lines. A wheat-*D. villosum* cryptic translocation has impacted several phenotypic performances as compared with CSA (e.g., early flowering, low genotype-environment interaction, higher grain yield per spike, resistance to powdery mildew, higher protein content, and flour with high sodium dodecyl sulfate-sedimentation value) (Caceres et al. 2012). Chromosome 6R has been identified to contribute

Table 4. (*continued from previous page*)

SSR markers to 6R				KASP markers to 6RL									
Xglk.ssrR6-032	Xglk.ssrR6-121	Xglk.ssrR6-159	Xglk.ssrR6-041	158C2	180C2	344C1	375C1	387C2	390C1	392C1	393C1	398C1	Generation
−	−	−	−	−	−	−	−	−	−	−	−	−	Parental
−	−	−	−	−	−	−	−	−	−	−	−	−	Parental
−	−	−	−	−	−	−	−	−	−	−	−	−	Parental
−	−	−	−	−	−	−	−	−	−	−	−	−	Parental
+	+	+	+	+	+	+	+	+	+	+	+	+	Parental
+	+	+	+	+	+	+	+	+	+	+	+	+	Parental
−	−	−	−	−	−	+	−	−	−	−	−	+	BC_1F_1
+	+	−	+	−	−	−	−	−	−	−	−	−	BC_1F_1
−	−	+	−	−	−	−	−	−	−	−	−	−	BC_1F_1
−	−	−	−	−	−	−	−	−	−	−	−	−	BC_1F_1
+	−	+	−	+	−	−	−	−	−	−	−	−	BC_1F_1
−	−	−	−	−	−	−	−	−	−	−	−	−	BC_1F_1
+	−	+	−	−	−	−	−	−	−	−	−	−	BC_1F_1
−	−	−	−	−	−	−	+	+	−	+	−	−	BC_1F_1
+	−	+	−	−	−	−	−	−	−	−	−	−	BC_1F_1
+	−	−	+	−	−	−	−	−	−	−	−	−	BC_1F_1
+	−	+	−	−	−	−	−	−	−	−	−	−	BC_1F_1
+	+	+	+	−	−	−	−	−	−	−	−	−	BC_1F_1
−	−	−	−	−	−	−	−	−	−	−	−	−	BC_1F_1
−	−	−	−	−	−	−	+	−	−	−	−	−	BC_1F_1
−	−	−	−	−	−	−	−	−	−	−	−	−	BC_1F_1
+	−	+	−	+	−	−	−	−	−	−	−	−	BC_1F_1
−	−	−	−	−	−	−	+	−	−	−	−	−	BC_1F_2
−	−	−	−	−	−	−	+	−	−	−	−	−	BC_1F_2
−	−	−	−	−	−	−	+	+	−	+	−	−	BC_1F_2
−	−	−	−	−	−	−	+	+	−	+	−	−	BC_1F_2
−	−	−	−	−	−	−	+	+	−	+	−	−	BC_1F_2
−	−	−	−	−	−	−	−	+	−	+	−	−	BC_1F_2
−	−	−	−	−	−	−	−	−	−	−	−	−	BC_1F_2

Table 5. *Yr* seedling infection types (ITs), kompetitive allele-specific PCR (KASP) marker identifications, and genomic in situ hybridization (GISH) or fluorescent in situ hybridization (FISH) determination of genomes present in BC_1F_4 and BC_2F_3 lines and their parental lines[a]

		Yr seedling test				KASP markers				
Line	**Family and cross**	**PSTv-14 PSTv-37**	**PSTv-40**	**PSTv-218**	**PSTv-221**	**375C1**	**387C2**	**392C1**	**GISH or FISH**	**Note**
CS *ph1b*	Parental	8-8	8-8	8-8	8-8	–	–	–	AABBDD	Parental
CSA	Parental	8-8	8-8	8-8	8-8	–	–	–	AABBDD	Parental
Holme	Parental	7-8	7-8	7-8	7-8	–	–	–	AABBDD	Parental
Sv876012	Parental	1-2	1-2	1-2	1-2	+	+	+	Not tested	Parental
SLU126	Parental	1-2	1-2	1-2	1-2	+	+	+	$AABBD_RD_R$	Parental
SLU820	Parental	7-8	8-8	8-8	7-8	–	–	–	AABBDD	BC_1F_4
119	29-N3-5	2-3	1-2	2-3/7-8	4-5	–	+	+	AABBDD	BC_1F_4
119 (IT 4-5)	29-N3-5	–	–	–	4-5	–	+	+	Not tested	BC_1F_4
120	29-N3-5	2-3	1-2	2-3/7-8	4-5/7-8	–	+	+	Not tested	BC_1F_4
120 (IT 7-8)	29-N3-5	–	–	–	7-8	–	–	–	Not tested	BC_1F_4
124	29-N3-5	2-3	1-2	2-3/7-8	4-5/7-8	–	+	+	AABBDD	BC_1F_4
383	29-N3-5	2-3	2-3	2-3/7-8	2-3	+	+	+	AABBDD	BC_1F_4
386	29-N3-5	2-3	2-3	2-3/7-8	2-3/4-5	+	+	+	Not tested	BC_1F_4
386 (IT 7-8)	29-N3-5	–	–	7-8	–	–	–	–	Not tested	BC_1F_4
391	29-N3-5	2-3	2-3/4-5	2-3/7-8	2-3	+	+	+	AABBDD	BC_1F_4
391 (IT 7-8)	29-N3-5	–	–	7-8	–	–	–	–	Not tested	BC_1F_4
392	29-N3-5	2-3	23	2-3/7-8	2-3/4-5	+	+	+	AABBDD	BC_1F_4
484	29-N3-5	2-3	23	2-3/7-8	2-3/7-8	+	+	+	AABBDD	BC_1F_4
484 (IT 7-8)	29-N3-5	–	–	–	7-8	–	–	–	Not tested	BC_1F_4
488	29-N3-5	2-3	23	2-3/7-8	2-3/7-8	+	+	+	Not tested	BC_1F_4
128	29-N3-5 × CSA	2-3	2-3/4-5	7-7	7-8	–	–	–	Not tested	BC_2F_3
129	29-N3-5 × CSA	2-3	23	2-3/4-5	7-8	–	–	–	AABBDD	BC_2F_3
136	29-N3-5 × CSA	2-3	23	2-3/4-5	7-8	–	–	–	Not tested	BC_2F_3
322	29-N3-5 × CSA	2-3	23	7-8	2-3/7-8	–	–	–	AABBDD	BC_2F_3
325	29-N3-5 × SLU820	2-3	2-3/4-5	7-8	2-3	–	–	–	AABBDD	BC_2F_3
354	29-N3-5 × SLU820	2-3/4-5	2-3/4-5	2-3/7-8	2-3	+	+	+	Not tested	BC_2F_3
354 (IT 7-8)	29-N3-5 × SLU820	–	–	7-8	–	–	–	–	AABBDD	BC_2F_3
356	29-N3-5 × SLU820	8-9	7-8	2-3/4-5/7-8	2-3/4-5	+	–	–	Not tested	BC_2F_3
356 (IT 7-8)	29-N3-5 × SLU820	8-9	7-8	–	–	–	–	–	Not tested	BC_2F_3

[a] Infection types (ITs) were observed based on a 0-to-9 scale (McNeal et al. 1971). Plants with ITs of 1-2 to 2-3 were considered resistant, plants with ITs of 4-5 were considered moderately susceptible, and plants with ITs of 7-8 to 8-8 were considered susceptible to highly susceptible. The "/" symbol divides the resistant and susceptible plants, thus indicating the segregation in the family.

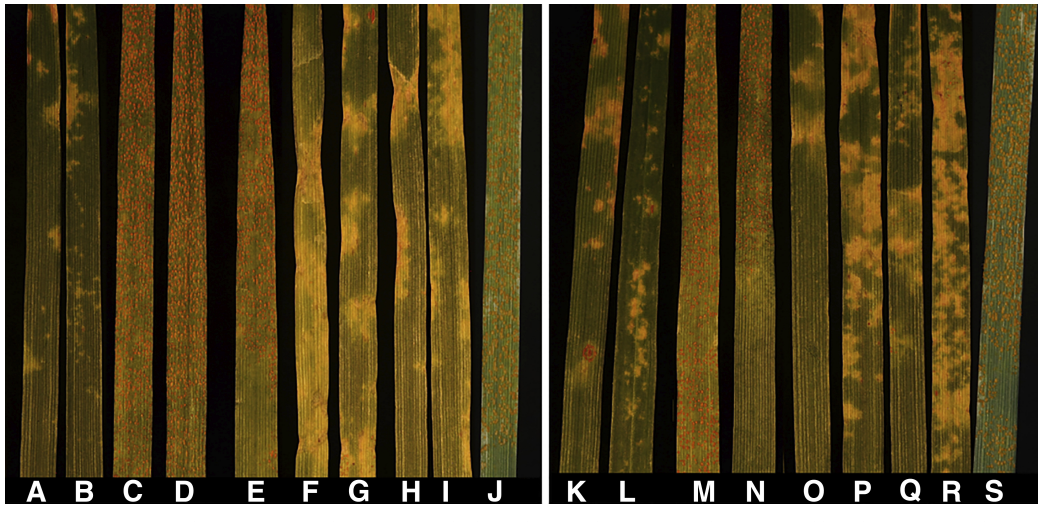

Fig. 1. Reaction to *Puccinia striiformis* f. sp. *tritici* race PSTv-14 + PSTv-37 of **A,** SLU126 first leaf; **B,** SLU126 second leaf; **C,** CS ph1b; **D,** CSA; **E,** SLU820; **F,** line 119; **G,** line 383; **H,** line 386; **I,** line 484; and **J,** line 484 (infection type [IT] 7-8) in BC_1F_4 populations; and race PSTv-218 of **K,** SLU126 first leaf; **L,** SLU126 second leaf; **M,** CSA; **N,** SLU820; **O,** line 129; **P,** line 136; **Q,** line 354; **R,** line 356; and **S,** line 356 (IT 7-8) in BC_2F_3 populations.

with increased total protein and arabinoxylan contents, as well as with additional agronomic and physiological features (Schneider et al. 2016). Several resistance genes have also been identified on 6RL (e.g., cereal cyst nematode resistance gene *Cre10*, Hessian fly resistance gene *H25*, and powdery mildew resistance gene *Pm20*) (Friebe et al. 1996; J. Li et al. 2020). Our study reports the transfer of *S. cereale* 6RL cryptic chromatin to wheat that confers stripe rust resistance.

Table 6. Backcrossing of 29-N3-5 family to CSA and SLU820

Cross	Generation	*P. striiformis* f. sp. *tritici* races PSTv14+37 Resistant	Susceptible	χ^2	*P* value
BC_1F_1 29-N3-5 × CSA	BC_2F_1	32	78	19.23	1.15
	BC_2F_2	170	216	–	–
BC_1F_1 29-N3-5 × SLU820	BC_2F_1	36	94	25.87	3.63
	BC_2F_2	224	302	–	–

The resistance segregation pattern for the lines in the present study in response to five *P. striiformis* f. sp. *tritici* races used (PSTv-14, PSTv-37, PSTv-40, PSTv-218, and PSTv-221) indicates the presence of an additional gene or genes. We observed segregation distortion of stripe rust resistance in the BC_1F_1 (CSph1b × SLU126 × CSph1b) and BC_2F_1 (29-N3-5 × CSA and 29-N3-5 × SLU820) populations. The segregation distortion is a common feature when wild relatives are used for crossing with wheat (Marais et al. 2010). In this study, the CS *ph1b* mutant recessive condition possibly influenced the segregation patterns. Rahmatov et al. (2016a) developed a new 2DS·2RL Robertsonian translocation using CS *ph1b* mutant lines and segregation distortion also was observed in that case. In total, 16 polymorphic SSR markers in parental lines could not

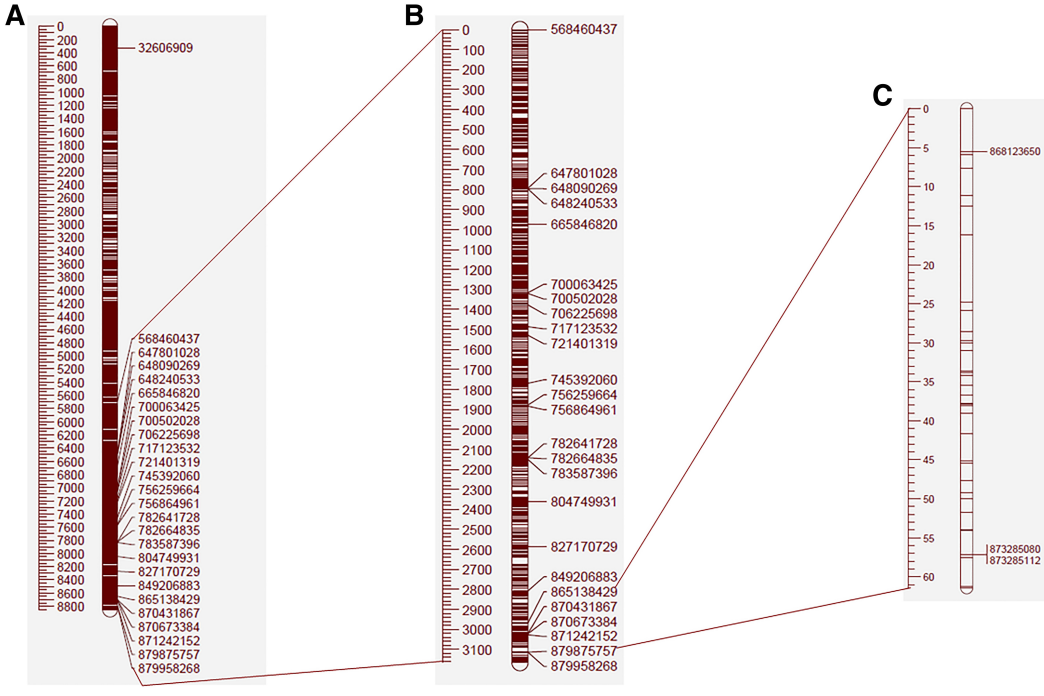

Fig. 2. A, Physical map of 26 nucleotide-binding leucine-rich repeat (NLR) gene positions of 6R in SLU126; **B,** physical map of 25 NLR gene positions of 6RL in SLU126; and **C,** physical region of kompetitive allele-specific PCR markers in SLU126.

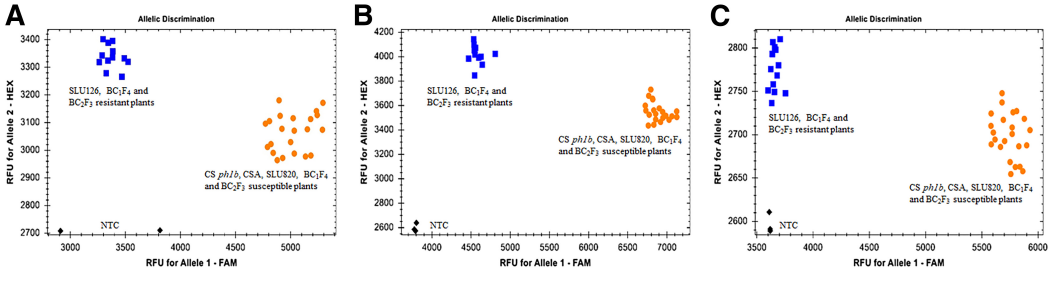

Fig. 3. Allele discrimination plots of the kompetitive allele-specific PCR markers used for **A,** 375C1 (868,123,650 bp); **B,** 387C2 (873,285,080 bp); and **C,** 392C1 (873,285,112 bp).

differentiate the resistant and susceptible plants in the BC_1F_2 and BC_2F_1 populations in that case. Therefore, Rahmatov et al. (2016a) developed three dominant rye 2RL-specific KASP markers suitable for detecting small introgressions whereas no polymorphisms were detected for the SSR or PLUG markers. The GBS in the present study determined a huge polymorphism between parental lines (CS *ph1b*, CSA, Holme, SV876012, SLU820, and SLU126), resulting in a high-density physical map in the parental lines. The genome-wide NLR complement of rye was efficiently identified using the NLR-Annotator tool from the rye reference genome, in which 792 NLRs were fully annotated (Rabanus-Wallace et al. 2021). In this study, BLASTN search of 184 full-length annotated rye NLR genes to the GBS reads identified a physical position of 26 NLR in 6R, of which 25 resided in the 6RL segment. Additionally, 272 full-length rye NLR genes for 4R and 83 for 5R were used as queries for BLASTN search against the GBS reads. The numbers of NLR genes in chromosomes 4R (13 NLRs) and 5R (9 NLRs) were fewer than for chromosome 6R. There are relatively few stripe rust resistance genes on rye chromosomes 4R and 5R (An et al. 2019; Li et al. 2016) compared with chromosome 6R. This was the reasons why we first focused on 6R in the present article, in the search for novel stripe rust resistance genes, although 4R is also a potential source that needs further evaluation. The results here indicated that the stripe rust resistance genes from SLU126 were residing in the 6RL segment; thus, harboring SNPs around NLR gene positions were used to develop KASP markers. By evaluating the 38 KASP markers in the 6RL chromosomal segment, we identified 9 (158C2, 180C2, 344C1, 375C1, 387C2, 390C1, 392C1, 393C1, and 398C1) that were able to clearly distinguish CS *ph1b*, CSA, and SLU820 from SLU126 (Table 4). Of these nine KASP markers, three (375C1, 387C2, and 392C1) were amplified in the BC_1F_1 family 29-N3-5 (Tables 4 and 5). The NLR protein family contains the majority of disease resistance proteins, which results in opportunities to recognize specific microbial effectors conferring resistance (Kourelis and van der Hoorn 2018). Many functional resistance genes are NLRs mediated by defense signaling mechanisms, leading to effector-triggered immunity, which often involves a hypersensitive response resulting in programmed cell death (Jones and Dangl 2006). In the present study, putative GBS-SNPs accurately enabled the detection of rye introgressions in the wheat genome. Tiwari et al. (2014) developed high-throughput SNP markers using GBS technology that allowed mapping and cloning of novel genes from the wild relatives of wheat.

The characterization of the small rye translocation, which contains stripe rust resistance genes, provides an additional genetic resource for developing resistant cultivars. We developed KASP markers for the detection of the small rye translocation in the wheat genome. The small size of the translocation reduces linkage drag when the desired region is transferred to an elite background.

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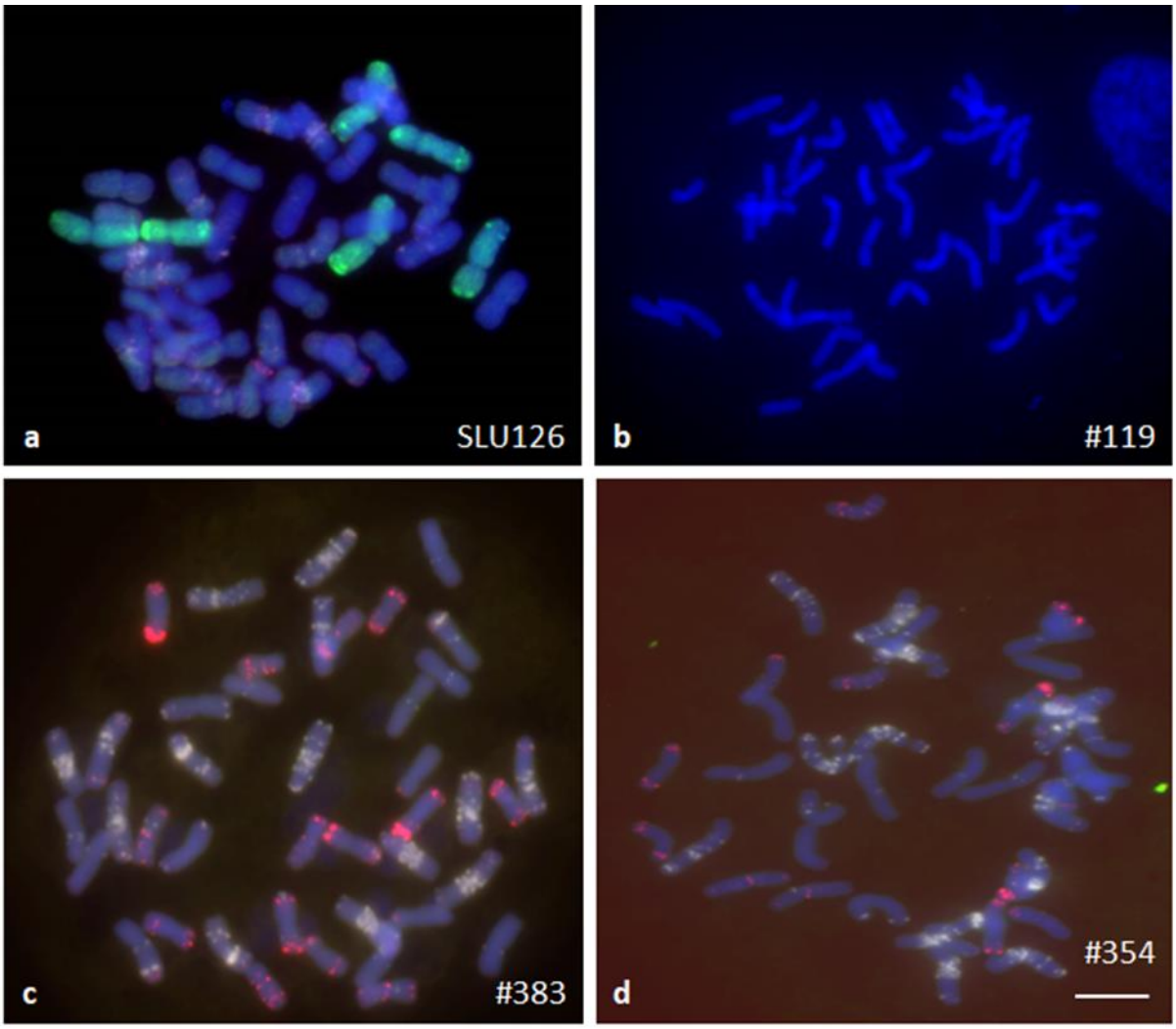

Supplementary Figure S1. a, GISH visualization of SLU126, multiple wheat-rye disomic substitutions line 4R (4D), 5R (5D), and 6R (7D), 2n = 42; **b**, GISH visualization of resistant BC_1F_4 progeny line #119, 2n = 42; **c**, FISH visualization of resistant BC_1F_4 progeny line #383, 2n = 42; **d**, FISH visualization of resistant BC_2F_3 progeny line #354, 2n = 42. Bar corresponds to 10 μm

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This thesis identified wheat-rye introgression lines with novel stripe rust resistance genes. Molecular markers associated with stripe rust resistance genes (*YrSLU*), were developed and confirmed a cryptic translocation. Marker-assisted gene pyramiding successfully combined stripe and stem rust resistance genes, enriching the wheat disease resistance gene pool. This precision breeding approach exemplifies the potential to introduce and combine multiple disease resistance genes within a single wheat genotype. Phenotypic and genotypic analysis confirm presence of stripe rust resistance genes which are retained over several generations.

Rimsha Ashraf has attained a Master of Philosophy (M.Phil.) in Plant Genetics and Genomics from Quaid-i-Azam University, Islamabad, Pakistan, complemented by a Master of Science in Botany, from Quaid-i-Azam University, Islamabad, Pakistan. She also received her Bachelor of Science degree with a specialization in Botany, Zoology and Chemistry from University of the Punjab, Pakistan.

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