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Broadening of Mildew Resistance in Wheat

Per-Olov Forsström

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Per-Olov Forsström
Department of Crop Science
Alnarp, Sweden

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Abstract

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Powdery mildew (*Blumeria graminis* f. sp. *tritici*) is a prevalent foliar disease in maritime and semicontinental climates and occurs wherever wheat is grown. The pathogen is an obligate fungus that seldom kills its host and winters at the asexual stage. Mildew-resistant lines was selected from a broad material derived from crosses between mildew-susceptible bread wheats and mildew-resistant: (1) triticale, (2) a double disomic wheat-rye substitution line, and (3) a wheat-*Leymus mollis* amphidiploid (containing 12 chromosomes from *L. mollis*). Mildew tests were conducted on later generations (BC_1F_4 or F_5) in order to increase the isolation of resistant lines under homozygous conditions. The choice of parents influenced the results and a large-sized population was a prerequisite in order to succeed in exploiting the full potential of these interspecific crosses.

A combined approach of a mildew test and cytogenetics was used to obtain lines with transferred rye chromatin from a double disomic wheat-rye substitution line. The material showed a fairly high rate of translocations derived from misdivision and centric fusions, *i.e.* lines that are suitable for plant breeding. The most prominent result was the isolation of a Robertsonian translocation, 2BS.2RL.

Molecular cytogenetics was used to characterise mildew-resistant lines that contained the rye chromosomes 1R + 2R, 1R + 4R, 1R + 6R, 1R and an inversion of 1R, all derived from triticale x wheat crosses. C-banding was used to identify the aberrant chromosome 1R and molecular cytogenetics was required to describe the chromosome as a pericentric inversion. The inverted 1R was characterised with five different repetitive probes using FISH and the breakpoints were localised between (1) the 5S rDNA and the NOR region on the satellite of the short arm, and (2) between two $ACC_{(5)}$ sites close to the centromere of the long arm.

We developed a strategy for converting a wheat RFLP-based assay into a PCR-based sequence-tagged site (STS). Two RFLP-loci located on 2L were chosen and STS markers were obtained for the three wheat genomes A, B, D and the rye genome R. The PCR markers can be used for marker-assisted selection (MAS) in the selection of mildew-resistant translocation 2BS.2RL.

Key words: wheat, rye, triticale, wheat-rye hybrids, wheat-rye introgressions, chromosomal substitution, pericentric inversion, *Leymus mollis* hybrids, powdery mildew (*Blumeria graminis* f. sp. *tritici*), resistance breeding, C-banding, molecular cytogenetics, physical chromosome mapping, GISH, FISH, molecular markers, RFLP, SSCP, STS, MAS

Author's address: Per-Olov Forsström, Department of Crop Science, Swedish University of Agricultural Sciences, SE-230 53 Alnarp, Sweden. E-mail: Per-Olov.Forsstrom@vv.slu.se

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Appendices

Papers I-IV

This doctoral thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Forsström PO and Merker A (2001) Sources of wheat powdery mildew resistance from wheat-rye and wheat-*Leymus* hybrids. *Hereditas* 134:115-119

II. Merker A and Forsström PO (2000) Isolation of mildew resistant wheat-rye translocation lines from a double substitution line. *Euphytica* 115:167-172

III. Forsström PO, Merker A and Schwarzacher T (2002) Characterisation of mildew resistant wheat-rye substitution lines and identification of an inverted chromosome by fluorescent *in situ* hybridisation. *Heredity* 88:349-355

IV. Forsström PO, Koebner R and Merker A (2002) The conversion of wheat RFLP probes into STS markers via the single-stranded conformation polymorphism technique. Submitted.

Abbreviations

AFLP	Amplified fragment length polymorphism
BSA	Bulk segregant analysis
cv(s)	Cultivar(s)
DNA	Deoxyribonucleic acid
FISH	Fluorescent *in situ* hybridisation
GISH	Genomic *in situ* hybridisation
GMO	Genetically modified organism
HR	Horizontal resistance
IF	Infection frequency
IT	Infection type
ISH	*In situ* hybridisation
ISSR	Inter-simple sequence repeat
LP	Latent period
MAS	Marker-assisted selection
NOR	Nucleolar organising region
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pm	Powdery mildew
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SCAR	Sequence characterised amplified region
SNP	Single-nucleotide polymorphism
SSCP	Single-stranded conformation polymorphism
SSR	Simple sequence repeat (microsatellites)
STS	Sequence-tagged site
SP	Spore production
VR	Vertical resistance

Introduction

The Evolutionary History of Wheat

A majority of all plant species in the world is cross-pollinated, while most cereals are self-pollinated. These are regarded as more suitable for domestication, because a natural barrier for pollination restricts the occurrence of introgressions. Selfing makes the crop more suitable for reproduction and it does not risk losing its identity. This in turn makes it easier to maintain the established character of the cultivar. For instance, an occasional mutant, such as the breakdown of the mechanism behind germination inhibition, was probably automatically selected and already more or less "fixed" in the same event. Cross-pollinated crops are less stable and require constant selection against out-crossing in order to maintain valuable characteristics. This is probably the main reason why cross-pollinators entered agriculture later and comprise a minority among traditional grain crops. The most natural candidates for successful domestication were therefore selfers that had overcome inbreeding depression. A general feature of these crops, including wheat and barley, is the occurrence of occasional cross-pollination as a source of genetic variability for adaptation and crop evolution. The development of self-pollinators hence opened up for improvements, since they could be selected for occasional out-crossing events that preserve valuable characteristics. Desirable adapted traits include the development of erect plant types, synchronised tillering and ripening, tougher rachis, the production of additional fertile florets and spikes and the reduction of glume thickness and awns (Harlan *et al.*, 1973).

The most significant development during domestication was probably the loss of the shattering mechanism. Non-shattering mutants are believed to have replaced the old brittle types over a few generations. This deficiency would have been lost in wild derivatives, but instead developed to a crop that was totally dependent on the interaction with man for its survival. The wild type has adapted to disseminate its seeds at maturity. This mechanism depends on a fragile rachis, which disarticulates at the internode and shatters into individual spikelets. The cultivated type has a tougher rachis which is thicker throughout the length of the ear and the kernels remain on the spike when mature. Cultivated wheat is classified into two groups according to threshing response. The primitive types have hulled grains with tight spikelet glumes and tough palea invested

around the kernels. The rachis breaks in fragments at the internodes, resulting in spikelets and not grains when threshed. The advanced form of cultivated wheat has thinner glumes and palea, which break and release naked kernels at threshing. The free-threshing feature of wheat has developed in two different directions. The polygenic system has evolved since the onset of cultivation and is scattered throughout most of the germplasm. It is most frequent in tetraploid species and reached completion in advanced emmer wheats (*Triticum turgidum* ssp. *carthlicum*). It is a compelling thought by Mac Key (1966) that the squarehead factor Q evolved at the hexaploid level as the addition of new genes for spelting and brittleness from the D genome challenged the evolution of a second completion. Although the Q factor can be found in emmer wheats, it is more common in the hexaploid subspecies, namely *Triticum aestivum* ssp. *vulgare*, ssp. *compactum* and ssp. *sphaerococcum* (Table 1). The shift from hulled to free-threshing hexaploid wheat is governed mainly by one mutation (the q to Q). This indicates that naked wheat probably evolved over a relatively short time span (Zohary and Hopf, 2000). The Q factor is dominant and located on chromosome 5A, which is a suppressor gene of the speltoid character. Carriers of the Q factor have a tougher rachis and the kernels become naked after threshing. The mutants *Triticum monococcum* ssp. *sinskajae* and *Triticum timopheevi* ssp. *militinae* probably also have the Q factor, due to their short history and morphology.

A shared feature among other members of the tribe *Triticeae* is the polyploidisation in wheat species. Polyploid species have an evolutionary advantage over diploid species. These species are less isolated and tolerate gene flow and recombination with other species through introgressions and hybridisations with retained fertility. According to the concept of pivotal genome (Zohary and Feldman, 1962), a shared genome buffers other genomes in a polyploid species during hybridisation. Owing to this constitution, other genetic events of gene shuffling – such as duplications and translocations – can occur to a greater extent and hence become less vulnerable to point mutations. All these factors contribute to the genetic flexibility of wheat and make it suitable for adaptation to different environments and climates.

In ancient times, before the involvement of man, wild tetraploid wheat and the goat grass *Aegilops squarrosa* were separated geographically since they had adapted to different climates. The tetra-

ploid wheat prefers the Mediterranean climate, while *Ae. squarrosa* thrives in areas with continental climate. The hybridisation between emmer and *Ae. Squarrosa* gave rise to hexaploid wheat. It probably has its origin in the south-western corner of the Caspian belt, which is located outside the "Fertile Crescent" of Mesopotamia and Syria. It is likely that this occurred after cultivated emmer had been domesticated and had expanded to Iran in the north and adjacent Transcaucasia. *Ae. squarrosa* has probably never been cultivated as a crop itself. As an opportunistic coloniser of secondary habitats, it invaded cereal fields as a weed when land was opened up by agriculture and cross-hybridised with cultivated emmer. This probably occurred in different habitats between several parent lines through recurrent hybridisation. After this event, it can be assumed that man made selections from alloploids, since there is no evidence of any hexaploid wheat in the wild. When wheat shifted from tetraploid to hexaploid form, its ability to adapt was greatly expanded. Tetraploid wheats adapted to thrive in the Mediterranean basin with mild winters and dry summers. The addition of the D genome made the hexaploid wheat better adapted to cooler continental winters and humid summers. This adaptability also explains the spread and distribution of tetraploid wheat centred around the Mediterranean basin and in the Near East, while hexaploid wheat spread over the continental plateau of Asia and the temperate areas of Europe.

Wheat has been a major staple crop in many civilisations and the history of wheat domestication covers almost 20,000 years. The earliest evidence that wheat is used comes from the Ohalo II site in Israel, where the wild tetraploid type *T. turgidum* ssp. *dicoccoides* has been found and dated back to 17,000 BC. In ancient times it was most likely a staple crop for hunters and collectors. Cultivated forms of wheat originate from between 8,000–7,000 BC. Both the diploid wheat *T. monococcum* ssp. *monococcum* and the tetraploid *T. turgidum* ssp. *dicoccum* came into cultivation simultaneously around 8,000–7,500 BC and the earliest findings of *T. aestivum* is at Can Hasan in Turkey, dated around 7,000 BC (Feldman *et al.*, 1995). Wheat has been able to hold such a superior position as a staple crop owing to characteristics that are feasible for cultivation and storage. These features are shared with other cereals. In addition, wheat has unique baking qualities and a comparatively high nutritive value. This makes it suitable as a staple food resource. The starch content is between 60 and 80 percent and today, starch constitutes over 20 per-

cent of all calories consumed by man. A normal cultivar has a protein content of at least 10 percent. The average yield world-wide is around 2,600 kg/ha, a relatively low productivity level. By comparison, most European countries are above this level owing to large amounts of fertilisers and pesticides, but also because of the predominance of winter wheat grown in some of these countries. In the current world production, wheats are one of the most important cultivated staple crops beside maize and rice, with an average annual production between 570–600 x 10^6 MT (metric tons) each (FAO Yearbook, 1998). Improvements of these crops are of paramount importance since food demands are increasing in the world. The growth curve for the world's population has historically been defeated by an increase in average yield. The most remarkable breakthrough was the introduction of semi-dwarf varieties by the employment of *Rth* genes and the "green revolution" during the 1960s (Borlaug, 1983; Feldman *et al.*, 1995). In the United States, there was an increase in productivity by approximately 40 percent for each decade during the 1950s and 1960s, to be compared with a stagnation of the total world-wide production of cereals during the 1990s. Wind and water erosion with soil loss, saline soils, limited water resources, reduced microbial activity in the soil and increased urban activity are the main reasons for the current decrease in agricultural productivity (Gardner, 1996). Seen from this perspective, it is rather obvious that agricultural development and productivity have to be taken seriously as major issues on our agenda for the future.

Taxonomy

The characterisation of species has predominantly been made according to morphological traits. This has created a tendency among taxonomists to exaggerate the classification of cultivated plants. There is an enormous variation within each species due to extensive breeding efforts, which have often been governed by single genes and do not justify a classification into species. Mac Key's (1966) evaluation of the genus *Triticum,* which was based on genetic concepts, has proved to be a valid classification corresponding to crucial differences between the species. He established that *Triticum* comprises the five species *T. monococcum*, *T. timopheevi*, *T. turgidum*, *Triticum zhukovskyi* and *T. aestivum*, based on the polyploidisation and the desynapsis system in *timopheevi* as the only evolutionary systems behind the processes of speciation (Table 1). Recently,

Triticum urartu, *T. monococcum* ssp. *sinskajae* and *T. timopheevi* ssp. *militinae* have been recognised and described. According to Miller (1987) and Feldman *et al.* (1995) they should be included in the genus *Triticum* (Table 1). If the same approach as practised by Mac Key (1966) is used, *T. urartu* can be classified as a species, since hybrids between *T. urartu* and *T. monococcum* are sterile although they both carry the same A genome. *T. urartu* is an endemic wild type with awns, two fertile florets per spikelet and fragile ears. An analysis of isoenzymes and storage proteins have also established differences between the species. *T. monococcum* ssp. *sinskajae* (AA) is a free-threshing mutant originating from *T. monococcum* ssp.

Table 1. Classification of the genus *Triticum* L. according to genetic conceptions (based on Mac Key, 1966; Feldman *et. al.*, 1995)

Species	**Genomes**	**Wild**	**Cultivated**	
		Hulled	**Hulled**	**Free-threshing**
Diploid (2*n* = 14)				
T. urartu	A	All		
T. monococcum	A	ssp. *boeticum*	ssp. *monococcum*	ssp. *sinskajae***
Tetraploid (2*n* = 28)				
T. timopheevi	AG	ssp. *araraticum*	ssp. *timopheevi*	ssp. *militinae***
T. turgidum	AB	ssp. *dicoccoides*	ssp. *dicoccum*	ssp. *turgidum*
			ssp. *paleocolchicum*	ssp. *durum*
				ssp. *turanicum*
				ssp. *polonicum*
				ssp. *carthlicum**
Hexaploid (2*n* = 42)				
T. zhukovskyi	AAG		All	
T. aestivum	ABD		ssp. *spelta*	ssp. *vulgare**
			ssp. *vavilovi*	ssp. *compactum**
			ssp. *macha*	ssp. *sphaerococcum**

*Carrier of Q factor; **Presumed carrier of Q factor

monococcum. The mutant resembles the progenitor, but has a shorter spike. *T. timopheevi* ssp. *militinae* (AAGG) is a free-threshing tetraploid, which is derived as a mutant from *T. timopheevi*. The spike is short, wide, dense and black, with an extra awn on the outer glume (Miller, 1987). Feldman *et al.* (1995), however, have excluded the species *T. zhukovskyi* and subspecies *T. turgidum* ssp. *paleocolchicum*. *T. zhukovskyi* is a hexaploid analogue to *T. timopheevi*, and *T. turgidum* ssp. *paleocolchicum* is a cultivated hulled form, similar

to *T. turgidum* ssp. *dicoccum* but with a broader compact ear. Zohary and Hopf (2000) have reduced the classification even further and exclude *T. urartu* as well. The complex of the genera *Triticum* and *Aegilops* has been combined into one genus based on their phylogenetic relationships (Bowden, 1959; Morris and Sears, 1967). Although the reclassification, where approximately 20 species from *Aegilops* were incorporated into the enlarged genus *Triticum*, was considered a valid treatment, it has so far not been accepted (Miller, 1987). Despite the fact that traditional classification holds *Aegilops* and *Triticum* apart, they are practically regarded as the "wheat group."

The bread wheat *Triticum aestivum* ssp. *vulgare* (AABBDD) is a segmental allohexaploid ($2n$=$6x$=42), which is a hybrid between einkorn wheat and two species of *Aegilops*. The basic chromosome set, x=7, is the same in the three homoeologous genomes. This is a feature shared with many other monocotyledon plant species and of significance in hybridisations with other grass species. Another characteristic of polyploid wheats is the diploidising mechanism that prevents the pairing of homoeologous chromosomes by a suppressor gene *Ph1*, located on the long arm of chromosome 5B. The development of this diploid-like behaviour ensures stable segregation, genetic stability and preserved fertility. The progenitor of the A genome is *T. monococcum*, while the origin of the B genome has evaded determination and is still unsettled. The ancestor may be extinct, the donor may still be undiscovered, or the genome may be derived and combined from more than one species. The third genome D, however, originates from *Ae. squarrosa*. The tetraploid emmer wheats consist of the two species *T. turgidum* (AABB) and *T. timopheevi* (AAGG). The A genomes are homologous, while the B and G genomes are closely related but non-homologous. The origin of the B and G genomes has been difficult to find and obtained results have given rise to several proposed hypotheses. *Aegilops speltoides* (SS) was earlier recognised as the progenitor of the B genome but later experimental evidence has suggested *Aegilops searsii* (S^sS^s), resembling *Aegilops longissima* (S^lS^l), as a candidate donor of the B genome (Feldman, 1978), while *Ae. speltoides* is a more likely candidate as donor of the G genome (Feldman *et al.*, 1995). However, both genomes have later been modified and this naturally makes it harder to identify their origin. In fact, both genomes may have been derived from the same S genome, which afterwards may have under-

gone modification into different genomes. An ancestral amphiploid with the genome formula AASS may have hybridised with similar polyploid species, which consisted of one common genome (here the A genome), and a second different genome, such as that of *Ae. longissima* (S^lS^l) or *Aegilops bicornis* (S^bS^b). The pivotal genome buffers the hybrids, while the second genome can exchange chromosome segments and develop modified, but convergent genomes. Hexaploid wheat (AABBDD) contains two genomes homologous with the A and B genomes in *T. turgidum*. This shows that dinkel wheat is a hybrid between emmer wheat (AABB) and *Ae. squarrosa* (DD).

The Gene Pool

The potential gene pool is an available source of recruitment of germplasm for crop improvement. The gene pool of bread wheat consists of the tribe *Triticeae*, which comprises around 350 species (Fig. 1) belonging to the family *Poaceae* (*Gramineae*). The tribe has evolved from a common ancestor since the early Tertiary or late Cretaceous (Löve, 1982). Cultivated cereals, such as wheat, barley and rye belong to the gene pool as do many of the most important forage and pasture grasses (Dewey, 1984; Bothmer *et al.*, 1992). The most important genomes with bearing on the wheats belong to the genera of *Triticum* and *Aegilops*. Traditionally, scientists and breeders have been of the opinion that the gene pool of each species consists of all other taxonomically related species, which can hybridise with the species itself. However, hybrids have variable abilities for chromosome pairing during meioses based on fertility, and some crosses can result in non-surviving hybrids. These circumstances have required additional differentiation of the gene pool concept. It is proposed in order to add a genetic perspective without formal expectations into the field, where practical considerations according to crossability between species have to be evaluated. The following definitions are according to Harlan and de Wet's (1971) original version "towards a rational classification of cultivated plants" and contains the primary (I), the secondary (II) and the tertiary gene pool (III) (Fig. 1).

The primary gene pool of bread wheat consists of subspecies to hexaploid wheat and hybrids are easily created. The polyploid level is the same and the chromosome pairing is homologous. Gene transfers and segregation is almost normal and the hybrids are fully

fertile. Most breeding in the field of germplasm enhancement focuses on crosses within the species, between different cultivars and lines in bread wheat.

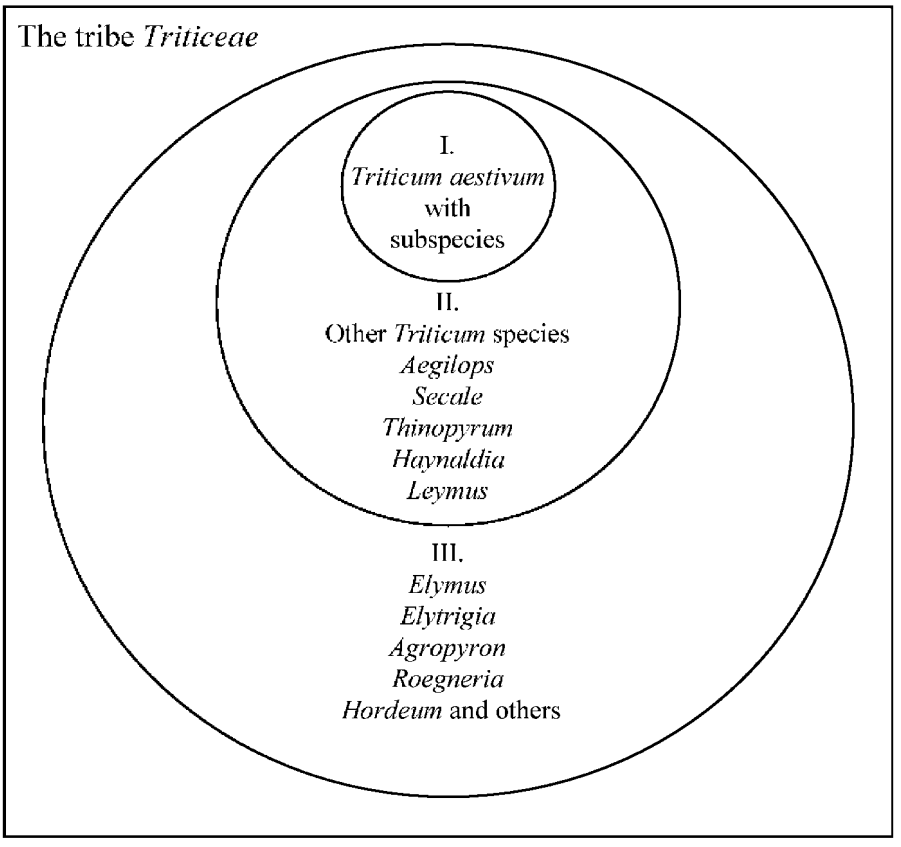

Fig. 1. The tribe *Triticeae* with the gene pools I, II and III for *Triticum aestivum* (based on Harlan and de Wet, 1971).

The secondary gene pool consists both of species at a lower ploidy level but with homologous genomes, and of other genera, such as *Aegilops, Secale*, *Thinopyrum, Haynaldia* and *Leymus*, with homoeologous genomes. Gene transfer is obtained and recombination occurs in the crosses, although hybrids have reduced ability for chromosome pairing and decreased fertility. Although the gene pool may be utilised, the gene flow is restricted and certain efforts are required to overcome incompatibility barriers between the species. Extensive backcrossing schemes are required to avoid deleterious traits that may accompany the desirable genes. However, as shown in a study by Knott (1989) low efficiency has to be expected, espe-

cially with respect to yield and less so for qualitative features (indicating a low level of recombination for polygenic traits).

The tertiary gene pool consists of the most distantly related species with non-homologous genomes. Crosses can be done but the hybrids may be anomalous or lethal and due to a lack of chromosome pairing, there is no spontaneous recombination or seed setting. Nevertheless, recombination can be attained by the use of X-ray in the induction of transfers. This is most conveniently carried out at the seed stage (Sears, 1993) or circumvented with chromosome doubling, bridging species and/or embryo culture. Notwithstanding the obstacles in the transfer of germplasm, these species mainly evolved from a common ancestor and carry desirable and functional traits, such as disease resistance and winter hardiness, which could be of interest in gene transfer and may be considered in the employment of gene technology.

Regarding the progress in GMO technology, the quarternary gene pool may include all other living organisms.

Powdery Mildew

Powdery mildew (*Blumeria graminis*) is one of the most common foliar diseases in cereals. The disease is most widespread and severe at temperate latitudes and in coastal areas with maritime climate, but occurs wherever host cereals are grown. Powdery mildew, classified as Higher Fungi of the subdivision *Ascomycotina* in the order of *Erysiphales*, is an obligate parasite, which depends on living tissue to survive and seldom kills its hosts (Agrios, 1997). The fungus is host-specific and wheat powdery mildew is classified into formae specialis *tritici*, *i.e.* f.sp. *tritici*.

The critical period for fungus survival can either be a hot summer or a cold winter. During a hot and dry summer in areas where host plants do not survive, the only way for the fungus to survive is probably the development of sexual ascospores (Koltin and Kenneth, 1970). The ascospores develop in groups of four or eight per ascus inside a closed ascocarp (cleistothecium), which is the fruiting body. The cleistothecia are closed spherical containers that appear as black pinheads interwoven with the secondary mycelium and develop at the end of the season after conidial production has declined (Agrios, 1997). Cleistothecia can winter, but play a minor role in spreading

the disease. Winter cereals are the most likely wintering hosts for the pathogen that survives vegetatively as dormant mycelium (Jenkyn and Bainbridge, 1978).

Any agricultural practises that reduce the primary inoculum are important to the delay of epidemic development, such as ploughing down crop debris and avoiding green bridges between winter and spring forms of the same crop. Early sown autumn crops and late sown spring crops are more frequently exposed to inoculum during the early growth stages. This can in turn have severe impact on yield loss. The crop is especially vulnerable to an infection of the disease during the seedling stage and results in fewer tillers and ears on the plant. A later attack affects the grain size and the thousand-kernel weight. Subsequently, the hormone balance of the plant is altered after an infection. The nutrient flow is allocated to the infection sites, creating general water stress. The physiology of the host is changed towards reduced photosynthesis, increased respiration and transpiration. The host responds with decreased growth, accelerated senescence and a lower leaf area index (Jenkyn and Bainbridge, 1978; Agrios, 1997).

The disease is first established in exposed field patches, where the crop is double-sown, or in areas with nitrogen surplus, because nitrogen fertilisers and a dense canopy increase the susceptibility. The optimal temperature for infection is around 15–20° C, but infection can take place between 5 and 30° C. Spores germinate better at high humidity, although this factor does not influence the mycelium development. The spores adhere with an appressorium, which is the absorbing organ of the nutrients, on the surface of the leaf and invade it by sending haustoria into the epidermal cells. The establishment of this organ is then essential for the mycelium on the leaf surface to continue to grow as the colony spreads. Additional epidermal cells are penetrated, while the mesophyll tissue below escapes intrusion (Jenkyn and Bainbridge, 1978). This primary mycelium produces conidiophores at the end of the hyphae, where small chains of rectangular or round conidia (the asexual form of the fungus) are developed and discharged passively by the wind (Agrios, 1997). Warm and sunny days with air currents followed by cold and bright nights with high humidity are typical weather conditions that accelerate the epidemic development of the disease. The life cycle of conidia is approximately one week during season and two generations can be sufficient for epidemic development (Benada, 1972). A high pro-

duction of conidia and a short generation time favours a high mutation frequency and the emergence of new virulent races. The mutation frequency has been estimated to be as high as 2,000 locus^{-1} ha^{-1} day^{-1} (Leijerstam, 1972).

Plant Defence

Different strategies of defence have evolved in the plant kingdom. They can be classified into three groups, namely avoidance, tolerance and resistance (Parlevliet, 1981). Avoidance and tolerance are both based on defence systems, while different forms of resistance have an active plant response mechanism in common, which is triggered after contact with a pathogen. Collinge *et al.* (2001) have estimated that the order of 1,000 genes are involved in response to pathogens. Avoidance is based on a strategy that evades the pathogen and can be regulated through time and space. Plant breeding for early cultivars of the crop can reduce the amount of inoculum during the critical growth stage, where the seedling stage is most vulnerable and can be passed during a lower level of primary inoculum. Spatial variation can also be of significant value, as shown by Gasowski (1990), where the deposition of leaf rust inoculum was reduced in plants with erect compared to horizontal leaf habit in cereals. The mechanisms of tolerance reduce the extent of damage caused by the pathogen, while the level of infection is not reduced, as in the case of avoidance and resistance. The plant has an ability to resist an infection through growth compensation. However, an infection always leads to a reduced yield due to increased respiration (Mac Key, 1980). This defence system therefore has limited value and active defence mechanisms are actually more useful in plant breeding.

Gene-for-Gene Interaction

Host resistance is the plant's ability to reduce growth and development in the pathogen after contact has been established (Niks *et al.*, 1993) The host-pathogen interaction was discovered by Flor (1955, 1956) in genetic studies of the flax and flax rust *Melampsora lini*. According to Flor, the host and the pathogen are interwoven with complementary genetic systems, where major resistance genes in the host interact specifically with so-called 'avirulence' genes in the pathogen. This is a paradigm that has influenced research in resis-

tance breeding ever since the 1950s. In general terms, an avirulence factor has to be recognised by a corresponding resistance factor in the host to trigger the defence mechanism. There is no reason to assume that an avirulence factor has to be a pathogenicity factor. It can be anything produced by a pathogen and detected by a plant recognition factor (Brown, 2001). Any mutation or the absence of an avirulence factor makes the pathogen undetectable to the host and the pathogen therefore stands a better chance of becoming virulent. Even if the gene-for-gene system has been proved for some diseases, there are several examples where no such relationship has been found (Johnson, 1992). However, advances in molecular biology have discovered a more complicated background of the gene-for-gene model.

A complete model of a typical resistance mechanism consists of a pathway including several stages such as the detection of the pathogen, a signal transduction pathway and the final disease resistance response. Song *et al.* (1995) revealed all these functions in one resistance gene *Xa21*, a cloned rice gene that gives resistance to a race of *Xanthomonas oryzae* pv. *oryzae*, while Salmeron *et al.* (1996) showed the complexity around the *Pto* gene which gives tomato plants resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* by contributing genes in the pathway of the defence mechanism.

Vertical versus Horizontal Resistance

van der Plank (1963, 1968) proposed two distinct resistance classes. Vertical resistance (VR) based on major genes, which was effective against certain races and follows a gene-for-gene relationship, while horizontal resistance (HR) was equally effective against all races and therefore beyond a gene-for-gene system. Parlevliet (1979) stated that van der Plank (1968) defined the classes in population kinetic terms, while the interpretation was epidemiological. The *a priori* definition of host resistance does not concern the spread of the disease and similarly, these classes are described in terms of the epidemiological parameters x_o and r. According to van der Plank, VR was of a race-specific nature and was supposed to decrease x_o, which is the amount of primary inoculum present at the start of an epidemic. HR was of a race-nonspecific nature and supposed to reduce r, which is the infection rate (van der Plank, 1968). Subsequently, in

terms of epidemiology VR and HR are defined as follows: VR reduces the amount of effective initial inoculum and HR is considered a rate-reducing resistance. This re-definition would have avoided all later confusion that arose regarding the original terminology (Nelson, 1978). Parlevliet (1979) showed that, from an epidemiological perspective, x_o and r were not as independent from each other as van der Plank presumed, since the *infection frequency* (IF), *i.e.* the proportion of spores that result in sporulating lesions, influenced them both. A popular assessment of so-called 'compound interest diseases', *i.e.* diseases which have many cycles of reproduction per year (van der Plank, 1963), a category that includes powdery mildews, has been to measure *infection types* (IT) that are crucial to the value x_o. Low IT is then often recorded as race-specific resistance with monogenic inheritance (Parlevliet, 1978), and r-reduced resistance is mainly recorded as polygenic, although not always (Nelson, 1978). Parlevliet (1979) also stated that both x_o-reducing and r-reducing resistances can be either race-specific or race-nonspecific. Others have argued that the distinction between VR and HR may be artificial and fabricated (Johnson, 1978). Nevertheless, none of the critics have abandoned van der Plank's general hypothesis, and several authors (Parlevliet and Zadoks, 1977; Nelson, 1978) have given him credit for the increased appreciation of epidemiology and the efforts to explain the dynamics of the host-pathogen relationship. In fact, there have been several attempts to refine the terminology as research has progressed (Shaner *et al.*, 1992; Bos and Parlevliet, 1995), although the controversies probably still have not been settled.

VR and HR have been presented as two distinct classes of resistance, but the crucial question is if there is any constitutional difference between them. Nelson *et al.* (1970) stated very early that there is no other difference between VR and HR than variable effects of their gene expressions in different genetic backgrounds. This tendency was observed with isolates of *Trichometasphaeria turcica* and inbreed lines of maize, where lines with a large number of resistance genes reacted more uniformly than inbreeds with few genes for resistance. This implicates that separate genes function vertically, while the same genes function uniformly when found together in one genotype, due to the cumulative effect. Parlevliet and Zadoks (1977) also concluded that there are no constitutional differences between VR and HR and that the major distinction between them is consid-

ered to be their oligogenic and polygenic nature. Here, they introduced the interaction model to include minor genes in the paradigm of the gene-for-gene relationship and found support in Nelson *et al.* (1970), although they admitted that minor interactions are difficult to distinguish from the error variance. The results are then measured in an additive way, in the same manner as the addition model by van der Plank (1975). The interaction model confirms the proposed idea that polygenic resistance represents archaic major genes. Although they have lost their crucial influence during co-evolution, together with others they contribute to substantial background resistance (Clifford, 1975; Nelson, 1978).

Plant breeding focused on VR does not exclude breeding efforts on HR, which can enhance the effect of VR. They can even be complementary to each other. However, a loss of HR may not be discovered in the presence of race-specific resistance. This is an unavoidable complication owing to the masking effect commonly known as the Vertifolia effect and caused by genetic erosion (van der Plank, 1968). An apparent procedure to obtain various goals in the breeding efforts would then be to introduce race-specific resistance at the final breeding stages. Since variation in resistance also can be observed between different plant stages, and even between different tissues of the plant (Bennett, 1981), further consideration and adjustment of the applied strategies may be needed.

Race-Specific Resistance

Race-specific resistance has been described in terms of vertical, specific, differential and qualitative resistance, as monogenic with major genes, complete, unstable and non-durable. Race-specific resistance is effective against certain isolates but ineffective against others. Its ability to resist and withstand an attack from the pathogen is decreased during high infection pressure from the pathogen and increased during lower selection pressure on the pathogen (obtained via a broader spectrum of resistance genes in the cv.). A common feature shared among diseases that have many cycles of reproduction per year is the development of new races that may already exist within the pathogen population or may be developed *de novo* by a high mutation frequency (Leijerstam, 1972; Parlevliet and Zadoks, 1977). A normal pattern shows either a complete resistance against all races of the pathogen in the area or a relatively quick turnover

with multiplication of one single race. Since the cultivars are extremely vulnerable if no other defence mechanisms can be used as plant protection, fungicide treatment may be used as a last resort.

Disease resistance is a character with high heritability and selection in an ongoing breeding programme can therefore be made already in F_3 (McIntosh, 1978). In general, single major genes control resistance to powdery mildew during the seedling stage. Until now, a total number of 25 *Pm*-loci with four cases of multiple allelism, recognised as race-specific resistance genes in wheat, have been identified (Keller *et al.*, 1999; Chen and Chelkowski, 1999). However, the introduction of novel mildew resistance genes remains to be extended as several reports have indicated (Bennett, 1984; Heun and Fischbeck, 1987a; Huang *et al.*, 1997a). The loci that are located in synteny with genomes of related species (Table 2) are of particular interest to the identification of novel mildew resistance genes.

A majority of identified race-specific resistance genes against powdery mildew originate from the 'wheat group', *i.e.* the genera *Triticum* and *Aegilops*. Other introductions from the secondary gene pool consist of four resistance genes from the genus *Secale* called *Pm7*, *Pm8*, *Pm17* and *Pm20*, and one gene from the genus *Haynaldia* called *Pm21* (Chen *et al.*, 1995). To a certain extent, synteny can be predicted in accordance with the major re-arrangements between rye and wheat genomes as studied by Devos *et al.* (1993). Co-linearity mainly exists between rye (1R) and the first chromosome group of wheat, the long arm of the second rye chromosome (2RL) and the wheat counterparts and the centromeric regions of other chromosomes. *Pm7* is located on a translocation between non-homoeologous chromosomes. The translocation 4B/2R was made with X-ray and introduced in the line Transec. The substituted segment of 4B has had little influence on the translocation (Driscoll and Anderson, 1967; McIntosh *et al.*, 1995a). *Pm8* is located either in a substitution or a translocation 1RS.1BL. The rye chromosome 1R has the ability to compensate the loss of the wheat chromosome 1B, but the translocation is superior in terms of agronomic qualities. Kavkaz and Aurora are two well-known 1RS-cultivars, mainly distributed in Eastern Europe (Zeller, 1973). The translocation 1RS.1AL is introduced to the line Amigo and contains *Pm17*, which is an allel to *Pm8* (Hsam and Zeller, 1997). The cultivar was created using X-ray treatment of F_1 seeds after a cross between a hexaploid

Table 2. Powdery mildew resistance loci and alleles in wheat (based on Chen and Chelkowski, 1999)

Chrom	Genomes						Derivation	Representative cultivar/line
	A	B	D	R	V	S^l		
1			*Pm10*				*T.aestivum*	Norin 4
			Pm22				*T.aestivum*	Virest
	Pm25						*T.boeticum*	NC96BGTA5
1S	*Pm3a*						*T.aestivum*	Asosan x Cc^8
	Pm3b						*T.aestivum*	Chul x Cc^8
	Pm3c						*T.aestivum*	Sonora x Cc^8
	Pm3d						*T.aestivum*	Kolibri
	Pm3e						*T.aestivum*	W150
	Pm3f						*T.aestivum*	Michigan Amber x Cc^8
				Pm8			*S.cereale*	Disponent
				Pm17			*S.cereale*	Amigo
2L		*Pm6*					*T.timopheevii*	TP114 x $Starke^2$
	Pm4a						*T.dicoccum*	Khapli x Cc^8
	Pm4b						*T.carthlicum*	Armada
				Pm7			*S.cereale*	Transec
	Pm23						*T.aestivum*	81-7241
3S						*Pm13*	*Ae.longissima*	R1A, R1D
4	*Pm16*						*T.dicoccoides*	BRG3N
5S			*Pm2*				*Ae.squarrosa*	Ulka x Cc^8
6		*Pm14*					*T.aestivum*	Norin 10
			Pm24				*T.aestivum*	Chiyacao
6S		*Pm11*					*T.aestivum*	Salmon
		Pm12					*Ae.speltoides*	Wembley line #31
					Pm21		*Haynaldia villosa*	C215
6L				*Pm20*			*S.cereale*	Prolific (rye cv.)
7			*SuPm8*				*T.aestivum*	Caribo
	Pm18						*T.aestivum*	Weihenstephan stamm MIN
			Pm19				*Ae.squarrosa*	XX 186
7S			*Pm15*				*T.aestivum*	Norin 26
7L	*Pm1a*						*T.aestivum*	Axminster x Cc^8
	Pm1b						*T.aestivum*	MocZlatka
	Pm1c						*T.aestivum*	Weihenstephan stamm MIN
	Pm1d						*T.spelta*	*duhamelianum*
		pm5a					*T.dicoccum*	Hope
		Pm5b					*T.aestivum*	Xiaobaidong
	Pm9						*T.aestivum*	Normandie

Cc^8 – seven times backcrossed with cv. 'Chancellor'
$Starke^2$ = once backcrossed with cv. 'Starke'

wheat line and the octaploid triticale cultivar Gaucho (Heun *et al.*, 1990). *Pm20* is located on the long arm of rye chromosome 6R and translocated as a 6RL.6BS from the rye cultivar Prolific. The transfer was carried out as a homologous recombination between a mono-

somic substitution line 6RL(6D) and an existing susceptible translocation (Heun and Friebe, 1990; Friebe *et al.*, 1994).

Race-Nonspecific Resistance

Race-nonspecific resistance has such epithets as horizontal, general and uniform and has been described as polygenic with minor genes, quantitative, incomplete, stable and durable, as partial resistance and field resistance. Durable resistance is mostly partial or incomplete and these terms have therefore been tightly connected with each other. However, partial resistance itself can not be taken as a criterion for the selection of durable resistance, as *e.g.* partial resistance to yellow rust in wheat has sometimes turned out to be non-durable (Johnson, 1978). In addition, Johnson (1984) stated that there are several examples of durable resistance due to single genes.

Race-nonspecific resistance is, however, regarded as reliable because of the nature of the resistance, which is quantitative and complex, *i.e.* functional in an additive way with separate sets of genes and contributions from several so-called 'minor genes'. Selective progress is measured as continuous variation in the level of resistance and results are compared as relative values of different lines and cultivars. In this way, breeders have developed a useful and standardised strategy to deal with polygenes, which may be scattered all over the genome but sometimes also gathered in groups on particular chromosomes (Thoday, 1961; Johnson, 1978), although Bennett (1984) concluded that the German cv. Diplomat so far is the only known example in wheat where quantitative resistance to mildew has been shown to be of polygenic character. In addition, two examples of durable resistance are known, *i.e.* the cv. Maris Huntsman and cv. Knox from the United States.

A significant feature of race-nonspecific resistance is that the disease develops slower than with race-specific resistance. Parlevliet (1979) concluded that reduced infection frequency (IF), prolonged latent period (LP) and decreased spore production (SP) are the key components in the resistance that influence the pathogen's reproduction rate (r). The impact of race-nonspecific resistance is more significant when the selection pressure is extended over a longer period in the season and/or a wider geographical region. The displacement of a wind-dispersed pathogen population such as powdery mildew is

on average 110 km per year in the predominant wind direction. Basically, successful varieties should therefore be introduced in the opposite wind direction, *i.e.* from east to west in Europe (Limpert, 1987).

General Perspectives of Resistance

In the process from natural populations of self-pollinated cereals to the cultivation of crops, there has been a genetic shift from highly diversified populations towards genetically uniform varieties grown over large areas. Naturally, the co-evolutionary change in pathogen populations has not been less dramatic during this man-made evolution. The selection pressure favours multiplication of pre-existing virulent races in the pathogen populations, something that makes cultivated crops far more vulnerable than a natural host population at genetic equilibrium. All resistance genes in the natural host population, more or less, are presumed to have virulent counterparts in the pathogen population, at genetic equilibrium, but there are no fully developed genotypes that can resist all pathotypes and no virulent races that are virulent on all host genotypes. Thus, genetic equilibrium is based on different sets of genes operating both on the host and the pathogen populations and the outcome is the combined effect of these systems.

Resistance durability is not solely a question of its sovereignty, but also a reflection of genetic homeostasis acting in the pathogen population. Genetic homeostasis restricts genetic changes due to the already attained maximum fitness regarding the differentiation of the genotypes and their respective frequencies in the population. The number of loci involved and the recombination frequency govern the efficiency of genetic homeostasis (Lerner, 1954). For practical reasons, it is therefore beneficial to explore genetic homeostasis in pathogen populations. It has been suggested that genetic equilibrium similar to that which prevailed between natural populations of hosts and pathogens also can be obtained with race-nonspecific resistance in modern crops (Nelson, 1978). Alternatively, it is possible to use an approach where a differential set of race-specific resistance genes are deployed in the concept of gene pyramiding (Hittalmani, 2000). In addition to the polygenic and the pyramiding approach, the same result can be obtained with a multiline approach (Wolfe and Barrett, 1980).

Wide Hybridisation

The introduction of alien germplasm into wheat by means of wide hybridisation has mainly been attributed to three different methods reviewed by Sharma and Gill (1984): (i) The use of ionising radiations (recommended for use on seeds by Sears, 1993), which causes breakage and a subsequent fusion of chromosomal segments. Sears (1956) successfully applied this method as early as the 1950s with irradiation applied to pollen mother cells. (ii) The induction of homoeologous chromosome pairing according to one of the following two methods: (1) using a *Ph1* mutant (*ph1b*) (Sears, 1977) that normally suppresses the mechanism, or, (2) the method used by Riley *et al.* (1968), where the same effect was obtained through a cross with *Triticum speltoides* (Tausch), which contains genetic factors that inactivate the suppressor gene *Ph1*. Friebe *et al.* (1996) summarised that 10 of 57 spontaneous and induced wheat-alien translocations had their breakage-fusion in the centromeric region. A majority of the transfers (45) were terminal translocations, with an alien segment translocated to the wheat chromosome arm, and two were intercalary transfers. Translocations caused by induced homoeologous pairing generally have the ability to compensate for the lost (substituted) segment, whereas radiation treatments cause random chromosome breakage, which means that transfers normally occur as non-compensating translocations with the implement of genetic imbalance. The latter method is seldom used, although it has been employed successfully by several researchers (Sears, 1983). However, it is not recommended except when the target gene is located in the centromeric region beyond the range of recombination (Jiang *et al.*, 1994). (iii) Exploiting the tendency of univalents to misdivide and reunite. For example in F_1 hybrids between hexaploid triticale and hexaploid wheat, there is one set of the R and the D genome forming two sets of univalents. These univalents are under pressure from the centric breakage-fusion mechanism during meiosis and can be selected as homozygous translocations and substitutions after some generations (Sears, 1952; Merker, 1984). Studies on the recovery rate of rye chromosomes at BC_1F_3 and F_4 generation have shown that the chromosome 1R was recovered most frequently, 2R and 3R to some extent, while 4R and 7R were most likely transmitted together due to chromosomal rearrangements in the rye genome compared to the wheat homoeologous (Merker, 1984; Gustafson *et al.*, 1985; Devos *et al.*, 1993). Several agronomic traits have been successfully

transferred to wheat from alien germplasm, such as disease resistance, winter hardiness, stress and salt tolerance from *Aegilops* species, *T. timopheevi*, *Haynaldia villosa*, *Thinopyrum* species and *Secale cereale* (for review see Friebe *et al.*, 1996; Jiang *et al.*, 1994).

Aims and Design of this Study

The overall aim of this study was to broaden mildew resistance in bread wheat. Novel sources from alien germplasm, *i.e.* triticale and *Leymus mollis*, were used for the introgression and stabilised wheat lines were obtained for investigation and are now available for breeding programmes. The specific aims were:

- To select mildew-resistant wheat lines from advanced generations of crosses between sources of novel resistance and susceptible wheat cvs. (*Paper I*).
- To characterise obtained mildew-resistant lines with different cytogenetic methods (*Papers II and III*).
- To develop chromosome-specific PCR markers located on chromosome 2L, including 2RL as a carrier of mildew resistance (*Paper IV*).

Our study was based on an extensive amount of pre-breeding material, including interspecific crosses between mildew-susceptible wheat cvs. and mildew-resistant triticales, a wheat-rye substitution line and a wheat-*Leymus* mollis hybrid (Forsström and Merker, 2001; Merker and Rogalska, 1984; Anamthawat-Jónsson, 1999). These were assumed to contain novel sources of mildew resistance. It was assumed that chromosome substitution and meiotic misdivision and reunion would occur when the material was developed. This is especially feasible when specific alien traits can be selected and traditional breeding methods are used without any need for complicated laboratory techniques. Introgressions in plant material from crosses were hence achieved without any particular technique such as induced homoeologous pairing or irradiation treatment. The material was propagated through self-pollination and investigated in advanced generations.

(1) *Isolation of alien germplasm.* In the first paper of the study, we made a selection of powdery mildew-resistant genotypes. Heun and Fischbeck (1987a) were first to use a differential set of isolates of the pathogen to differentiate between commercial cvs. and afterwards it has been widely used (Heun and Fischbeck, 1987b; Hovmøller, 1989; Leath and Heun, 1990; Lutz *et al.*, 1992; Huang *et al.*, 1997a; Huang *et al.*, 1997b). A small set of particularly virulent isolates was used in our study in order to reduce our material into a sensible number of mildew-resistant genotypes. We used the same isolates

that are applied by commercial breeders in Sweden and the results of using these isolates are compiled in *Paper I.*

(2) *Characterisation of alien germplasm.* In the second paper of the study, we characterised resistant genotypes derived from a double wheat-rye substitution line. Available methods for characterisation can be biochemical, molecular or cytogenetic technologies, recently reviewed by Berzonsky and Francki (1999). Wang *et al.* (1998) showed a combination of the used methods Southern hybridisation, PAGE of storage proteins, C-banding and *in situ* hybridisation (ISH). A standard karyotype for wheat (Endo and Gill, 1984; Gill *et al.*, 1991) and rye (Gill and Kimber, 1974; Sybenga, 1983) enables the use of the Giemsa C-banding method to identify transferred chromosomes in wheat-rye hybrids. The rye chromosomes are predominantly recognised by their large heterochromatic regions of the telomeres. Our results are assembled in *Paper II.*

(3) *Chromosome painting of alien germplasm.* In the third paper of the study, we characterised resistant genotypes derived from interspecific crosses between resistant triticale and susceptible bread wheats. According to Islam-Faridi and Mujeeb-Kazi (1995) and Lavania (1998), molecular cytogenetics is the most appropriate method for characterisation of alien chromosome introgressions. The advantage of genomic *in situ* hybridisation (GISH) compared to other methods is the "painting" of all alien germplasm located in the nucleus. The method has been widely used to investigate the origin of genomes, chromosomes and parental genomes in hybrids (Schwarzacher *et al.*, 1989) and to analyse derived introgressed lines from interspecific crosses (Murata *et al.*, 1992; Schwarzacher *et al.*, 1992; Taketa *et al.*, 1997). Fluorescent *in situ* hybridisation (FISH) combined with GISH enables the detection of translocations and other aberrations, including particular breakpoints, by the use of specific repetitive DNA probes, as in our study *Paper III.*

(4) *Development of PCR markers.* In the fourth paper of our study, we developed sequence-tagged sites (STS) markers. The aim was to develop chromosome-specific PCR markers located on the long arm of chromosome group 2, designed for the translocation T2BS.2RL, which is a carrier of mildew resistance (see *Paper II*). Here a novel method is applied for the development of PCR-based markers that takes advantage of exploring already mapped and sequenced RFLPs. Our study revealed that internal sequences of the RFLP probes offer

a sufficient degree of polymorphism that even allele-specific STS markers could be obtained. The results are presented in *Paper IV*.

Results and Discussion

Isolation of Alien Germplasm (Paper I)

Since the early 1980s it has been known that interspecific crosses between triticale and bread wheat can conduct transfers of rye chromatin. Lukaszewski and Apolinarska (1981) have shown that winter triticale has the benefit of being able to maintain the complete R genome, while spring triticale can be inclined to lose rye chromosomes and replace them by their D-homoeologous chromosomes in secondary triticales (crosses between triticale and wheat). Merker (1984) suggested that triticale used as a female in the cross with wheat produces viable seeds, while in the reciprocal cross, the endosperm development is disturbed and the embryos have to be rescued by an embryo culture. Lukaszewski (1982) also showed that it would be preferable to use wheat pollen in crosses with F_1 hybrids in order to facilitate the transmission of rye chromosomes in the egg cells. This was the method used to develop the material used in our study. The crosses and hybrids in our study have been designed in accordance with these findings. Furthermore, a delay in the isolation of resistant lines to later generations (BC_1F_4 or F_5) increased the possibility of isolating genetic transfers in a homozygous state.

The overall aim was to isolate a feasible number of mildew-resistant genotypes using a robust and reliable test method. According to Hovmøller (1989) there were no detected differences between the use of intact seedlings or detached leaf segments when the scale 0 to 4 was used in the mildew test. However, intermediate infection types had a tendency to be exaggerated when leaf segments were used instead of intact seedlings. Intermediate infection types were also more sensitive to temperature. We had no statistical aims and hence a 0 to 9 scale was unnecessary. The scale 0 to 4 was chosen and the experiment was conducted on intact seedlings. We used three of the most virulent isolates found in Sweden, currently used by commercial plant breeders. Previously, another mildew isolate (less virulent) was used for pre-selection. Moreover, the technique used to infect intact seedlings in the greenhouse is less vulnerable to fluctuations in drought and temperature. We reduced an extensive amount of genotypes (approximately 13,000 lines) to a handful valuable resistant wheat lines. By comparison, the line Riebesel 47-51, which is the main source for the currently most used wheat cvs. that

carry the translocation 1BL/1RS, was based on the selection of more than 10,000 spikes (Rabinovich, 1998).

It has been proven that wheat-rye translocations can increase the yield, especially in lower-yielding environments and under dry conditions (Moreno-Sevilla *et al.*, 1995a; Villareal *et al.*, 1998). In addition to disease resistance (Zeller and Hsam, 1983; Rajaram *et al.*, 1983) and other traits such as winter hardiness (Moreno-Sevilla *et al.*, 1995b), earliness, tolerance to low pH and drought tolerance (Merker, 1984; Plaha and Sethi, 1993; Schlegel *et al.*, 1998), they also contain genes for adaptability (Plaha and Sethi, 1993). They are therefore considered the most important alien sources of enhancing wheat germplasm (Bartoš, 1993; Jiang *et al.*, 1994; Friebe *et al.*, 1996).

Lines with a mildew reaction similar to the formerly known *Pm8* and *Pm17* represent the highest scores of resistant genotypes from the interspecific crosses between mildew-resistant triticale and susceptible bread wheat. It indicates the presence of 1RS in these lines, *i.e.* RSS, as shown in Table 3 and Tables 1 and 2 of *Paper I.*

Table 3. Highest score(s) of resistant genotypes in the different interspecific crosses between mildew-resistant triticale and susceptible bread wheat

Triticale x wheat*	Highest score(s) **
TC1 x H	SRS
(TC1 x H) x K	RSS
(TC2 x H) x K	RSS and RRS
(TC3 x H) x K	RSS
(TC2 x H) x G	RSS

* TC1-3 = Triticale 1-3; H = cv. Holme; K = cv. Kraka (contains recessive resistant gene pm5; G = wheat line Goerzen

** Mildew reaction: R = Resistant; S = Susceptible; Order of isolates: M_{Da}, Egt1 and M_{Ha}; See Table 1 in *Paper I* for a characterisation of these isolates.

Lines resistant to the isolate Egt1 in the cross TC1 x H and (TC2 x H) x K presumably contains an additional source of resistance. This is in accordance with the hypothesis we used in *Paper I* and we could analyse these categories to examine if they really contain alien germplasm from the rye genome. However, at this stage we are also concerned about the hidden formulas behind some of the other categories and this requires further examination.

Why does the population TC1 x H contain a negligible number of lines resistant to the isolate M_{Da}? Is it because of a suppression of 1RS or because of the absence of 1RS and 1R? A reasonable explanation could be that the population is still segregating. Other categories from this cross must therefore be examined before a final conclusion can be drawn. Whatever the reason, we can expect the same pattern in the cross (TC1 x H) x K if it is due to the origin of the TC parent. Two dominant categories have been scored in the cross (TC2 x H) x K, while the other crosses lack a second category. There is no association with either TC or wheat backgrounds and this distribution of lines can therefore not solely be explained by either the origin of triticale or the used bread wheats in the crosses. It would therefore, for instance, be necessary to bulk different categories of genotypes in the above populations and score the differences in a bulk segregant analysis (BSA) (Michelmore *et al.*, 1991). BSA would be suitable for use in conjunction with the AFLP marker system (Vos *et al.*, 1995) to reveal the complexity of interactions between different used combinations of bread wheats and triticale. Since we, obviously, did not isolate any novel resistance located at the *Pm8/Pm17* locus (Hsam and Zeller, 1997) there is no need to exploit mildew resistance in these categories. However, it has been shown before that the yield and end use quality of 1RS cvs. may depend on the interaction between the rye genes and the wheat backgrounds (Schlegel and Meinel, 1994; Moreno-Sevilla *et al.*, 1995a; Moreno-Sevilla *et al.*, 1995b; Lee *et al.*, 1995; Villareal *et al.*, 1998). This emphasises the importance of a broader genetic diversity of 1RS sources in different wheat backgrounds. Further examinations of the parameters yield and baking properties could therefore be made in order to investigate the value of these categories and perhaps affirm a combined BSA/AFLP analysis. The broader resistance shown in the eight RRR-resistant genotypes in the triticale x wheat material are likely to be far more important than the genotypes above and the use of these lines is discussed extensively in *Paper III.*

The absence of category RSS in the cross SUB x G (Table 2 in *Paper I*) can be explained by the presence of a suppressor gene in the line Goerzen similar to the suppressor gene located on 7D and described by Hanušová *et al.* (1996). It was probably selected against already in the test with the isolate M_0. This hypothesis could be examined by a cross with Goerzen and representatives from RSS in the crosses SUB x H and SUB x K and a study of the mode of inheri-

tance. It would be of minor importance except that these results show the beneficial value of making crosses with wheats of different backgrounds. Similarly, the RRR category is probably influenced by the wheat background, both in the crosses TC x wheat, (TC x wheat) x wheat and (AD99 x wheat) x wheat, whereas SUB x wheat seems more independent of such interactions.

Characterisation of Alien Germplasm (Paper II)

The aim was to evaluate the efficiency of methods used to make a selection based on mildew resistance and fertility in combination with the C-banding method used to isolate translocations from a mildew-resistant double disomic (1R+2R) wheat-rye substitution line, crossed with susceptible bread wheats. The most prominent result of this investigation was the isolation of the translocation 2BS.2RL, which showed a broader resistance than *Pm7*, *i.e.* the previous rye-derived resistance gene from this particular chromosome arm, which is transferred to the line Transec. In *Paper IV* we report the development of PCR markers for T2BS.2RL that can distinguish between 2RL and 2BL.

When alien introgression is used, there is always concern about detrimental effects. It is well-known that the 1RS cultivars provide increased yield and decreased bread-making qualities, *i.e.* dough weakness and stickiness, poor extensibility and sensitivity to over-mixing (Martin and Stewart, 1986; Koebner and Shephard, 1988; Fenn *et al.*, 1994). The Hamlet translocation T2BS.2RL was developed and derived from a cross with "Chaupon" rye at Kansas State University (Lapitan *et al.*, 1984; Friebe *et al.*, 1990). It is a carrier of Hessian fly [*Mayetiola destructor* (Say)] resistance. In a study intended to clarify the quality of the Hamlet translocation T2BS.2RL, different parameters such as test weight, flour yield, kernel hardness, mixograph mixing time and bake-mixing time were used. It was established that differences in milling and baking properties were so minor that they could be overcome by breeding efforts and the line is currently used in wheat breeding programmes in Kansas (Knackstedt *et al.*, 1994). There are no major storage proteins located on 2BL and this is probably why the baking properties in the T2BS.2RL translocation could be retained. The rye chromatin of 2RL shows homoeology with 2BL and therefore compensates the substituted segment, as

there are no chromosome rearrangements in 2RL compared to chromosome group 2L in wheat (Devos *et al.*, 1993).

Wernersson (2001) has analysed several agronomic characters, such as resistance to lodging, straw length, heading date, fertility, hectolitre weight, 1000-kernel weight, yield and amylase activity on our translocation T2BS.2RL. Two days later heading date of translocation carriers was the only significant difference compared to non-carriers. The later heading is similar to the 1RS-cvs. Another character of 1RS-cvs. is a higher kernel weight, but our 2BS.2RL carriers were closer to the non-carriers. Results showed that the yield was higher although it should not be considered as reliable since the experiment was conducted in small "hills." Rye has in general a lower hectolitre weight and a weaker straw compared to wheat and results indicate that these characters have other chromosome locations than 2RL. Rye also has a taller straw than wheat, and here in the study it was slightly taller but still within the limits of selection. Both categories showed the same fertility, which indicates that the translocation is able to compensate for the lost segment 2BL. Sprouting in the ear can decrease the quality, especially during periods of humidity. Results showed that this character was not affected and may even enhance the product, because a formerly known alpha-amylase inhibitor is located on 2RL (Sadowski *et al.*, 1988). However, further examinations must be done regarding quality and yield, especially as it can be used directly in wheat breeding and contains other important traits (Schlegel *et al.*, 1998). For instance, leaf rust resistance could be scored since 2RL is a formerly known carrier of leaf rust resistance (McIntosh *et al.*, 1995b).

Chromosome Painting of Alien Germplasm (Paper III)

Molecular cytogenetics can be used to determine the physical location of specific DNA sequences on the chromosomes. A simultaneous multicolour approach first applied by Leitch *et al.* (1991), and Mukai (1996) succeeded in using seven different probes in one experiment by combining three fluorochromes. The advantage of simultaneous FISH is that specific DNA sequences can be placed in order. The physical distance in the hybridisation of different probes can have major impact on the results. Conventional FISH techniques have a lower limit for resolution (approximately 1–10 kb) (Mukai and Yamamoto, 1998; Schubert *et al.*, 1998) and two hybridised se-

quences must be separated by a distance of at least 1Mbp. A shorter distance between the hybridisation sites results in a single intermixed signal. The mapping resolution on metaphase chromosomes is usually restricted to 2 Mbp in plants and the relatively low resolution compared to human chromosomes is partly due to the plant cell construction and particularly to a higher degree of condensation of the chromosomes at this stage. However, the digestion by enzymes can break down the cell wall barrier and cell debris can be wiped away with a pectinase treatment. An examination of interphase chromosomes may otherwise be an alternative due to the decondensed nature of the chromosomes at this stage (Jiang and Gill, 1994; Lavania, 1998).

Physical mapping of repetitive DNA sequences by FISH can be obtained using cloned probes from both wheat and rye. The chromosomes in those genomes can hence be identified (Cuadrado *et al.*, 1995). The *Aegilops squarrosa* clone pAs1 and the homologous clone dpTa1 can both be used to identify the D-genome chromosomes (Rayburn and Gill, 1987; Pedersen and Langridge, 1997; Vershinin *et al.*, 1994). The rye clone pSc119.2 can be used to identify the R-genome and B-genome chromosomes (McIntyre *et al.*, 1990; Mukai *et al.*, 1993; Cuadrado *et al.*, 1995). Simple sequence repeats (SSRs), in particular the motifs GACA, AAC and AAG, can identify R-genome and B-genome chromosomes (Pedersen and Langridge, 1997; Cuadrado and Schwarzacher, 1998; Cuadrado *et al.*, 2000). The clone pTa71 can identify major NORs in 1R, 1B and 6B and minor NORs in 1A and 5D (Gerlach and Bedbrock, 1979; Mukai *et al.*, 1991). The clone pTa794 can be used to identify the 5S rDNA (Castilho and Heslop-Harrison, 1995) (see Table 1 in *Paper III*).

We were able to determine the breakpoints of an inverted 1R chromosome (rye genome) at metaphase I by using five of those different repetitive DNA probes combined with GISH. We showed that the breakpoints were between (1) the 5S rDNA site and the NOR region on the satellite of the short arm, and (2) between two $AAC_{(5)}$ sites close to the centromere on the long arm, *i.e.* a pericentric inversion. A measurement of the physical length of the chromosome and GISH revealed no indication of any other rearrangement of the chromosome. Lines with an inverted 1R are difficult to maintain in crosses with other lines carrying 1R. The aberrant structure of 1R in these lines could instead be explored in the development of recombinant wheat-rye lines, in order to minimise alien chromatin or

to create a dosage effect through deletions or duplications of the rye chromatin.

Five other mildew-resistant lines were also analysed at metaphase I by C-banding, GISH and FISH. They were found to be either addition or substitution lines and rye chromosome 1R was present in all lines. This indicates that the mildew resistance is located on 1R. Rye chromosome 1R and pre-dominantly *Pm8* has been regarded as the main source of mildew resistance derived from rye (Zeller, 1973). However, the breakdown of *Pm8* in Europe and in China may have changed the genetic equilibrium between the host and the pathogen. This implicates that breeding must consider resistance from other sources, a fact that may still be used or investigated. For instance, *Pm17* still resists the pressure in Europe due to the fact that it is currently mainly used in the United States. It has already been concluded by Heun and Fischbeck (1987a and 1987b) that extended efforts are needed to broaden the genetic basis for wheat. The limited number of currently used major resistance genes/alleles in wheat cvs. in China (6) and Europe (9), reminds us that the level of protection from resistant sources must be enhanced (Huang *et al.*, 1997a). Rabinovich (1998) summarised that 330 cultivars and wheat lines bred in 35 different countries on five continents contained either the substitution 1R or the translocation 1RS, while this rye germplasm originated from four different rye sources. Evidently, the main reason why 1RS cvs. are consistently so popular can be attributed to its higher yield. Our triticale-derived lines that contain 1R may, if they hold further examination, be a welcome contribution to the genetic diversity in the stream of released 1RS cvs. This refers both to mildew resistance and the constitution of the derived rye chromosome, and its interactions with different wheat backgrounds. As far as we can see, it can be assumed that 1R is the most likely carrier of mildew resistance derived from the interspecific crosses with triticale.

Formerly derived mildew resistance genes from rye have been transferred from four chromosomes of the genome, *i.e.* 1R, 2R, 4R and 6R (Zeller, 1973; Heun *et al.*, 1990; Driscoll and Jensen, 1965; Lind, 1982; Friebe *et al.*, 1994). Their presence in our material is probably the result of a selection for broad mildew resistance during the development of these lines. Subsequent crosses with the addition/substitution lines (1R + 2R; 1R + 4R; 1R + 6R) and susceptible bread wheats could reveal if the rye chromosomes 2R, 4R and 6R are involved in the defence mechanisms of these lines, and to what ex-

tent. It may not be a coincidence that these chromosomes are formerly known carriers of mildew resistance. In order to elucidate the precise effects on resistance in the different rye chromosomes, co-segregation between resistance and rye chromosomes could be studied in populations from crosses between the different resistant lines and susceptible bread wheat cultivars.

Development of PCR Markers (Paper IV)

DNA markers have several advantages over biochemical and phenotypic markers, *e.g.* a higher degree of polymorphism and independence of gene expression. They are not environmentally influenced and can basically be extracted from all living tissues at all plant stages (Gale and Sharp, 1988). However, a suitable DNA marker system must be used according to the aim and specific objectives of each study. RFLPs has been the most extensively used marker system in comparative mapping and the study of synteny, since it can be utilised to detect homoeologous loci. PCR marker systems are more feasible in studies of genetic diversity and practical breeding. The current map of SSRs in wheat has approximately 1,000 mapped loci with a high degree of polymorphism. It is hence the most common method for marker-assisted selection (MAS) in practical wheat breeding. Multiplexed methods such as AFLPs and ISSRs are more suitable for studies of genetic diversity (Gupta *et al.*, 1999). SNPs and other non-gel-based technologies like microarrays and pyrosequencing are attractive alternatives to the above methods, although it has currently not been established that they could replace the current technologies for financial reasons (Qi *et al.*, 2001; Koebner *et al.*, 2001).

Molecular marker technology has generally been restricted to use with specific traits that would otherwise be difficult to tag, such as particular pathogen diseases and quality characters (Mohan *et al.*, 1997). Another approach to tagging has been to conduct selection in early generations (Ribaut and Hoisington, 1998). Evidently, selection could be made more efficient if additional efforts were made to develop tightly linked DNA markers to several traits for the subsequent development of elite lines from large populations. In the case of disease resistance genes, selection can be made without subjecting plants to the pathogen and several desirable traits could be controlled simultaneously. In a polyploid species such as wheat it is possible

that effective genes in other genomes epistatically mask the effect of recessive genes. Masking effects may therefore be held in order by means of markers and gene pyramiding of such genes has been a successful way to increase the durability of resistance against severe diseases in the crop (Yoshimura *et al.*, 1995; Ogbannaya *et al.*, 1998; Hittalmani *et al.*, 2000).

The conversion of RFLP probes into STS markers in a diploid species such as barley is a straightforward process, involving two major steps: the sequencing of the RFLP probe and the design of convenient primer pairs for the PCR reaction (Blake *et al.*, 1996). In wheat, however, we have to consider the hexaploid nature of this species and conduct the same process simultaneously for the homoeoloci in the wheat A, B and D genomes. The aim of our study was to perform STS assays for the translocation T2BS.2RL. In order to do this, we had to consider the rye genome, something which could of course not be accomplished without discriminating between the products derived from these homoeoalleles. We applied a method based on single-stranded conformation polymorphism (SSCP), a sensitive technique that exploits the conformational mobility of single-stranded DNA separated on polyacrylamide gels under non-denaturing conditions and allows for the detection of single-nucleotide polymorphism (SNP). This is a high-resolution and user-friendly technique, which only requires basic equipment available in small laboratories. SSCP is known to be most effective for DNA with high GC contents and smaller DNA fragments give higher sensitivity. In our design of primer pairs, derived from the sequence of the RFLP probe, we used different combinations in the range between 200–300 bp and obtained stringent results. These primers amplified all the homoealleles at the specific RFLP locus that were present in the templates we used. The gel matrix differentiates the templates depending on the secondary structure of the DNA. Selected ditelosomic and nullisomic lines were applied in the separate lanes and thus used to reveal the origin of the homoealleles. Specific bands were excised directly from the gel and used for sequencing. We could hence identify sequences that were used for the design of chromosome-specific primer pairs. The results were validated with a set of F_3 families of mildew-resistant and susceptible genotypes (derived from a cross between T2BS.2RL and a susceptible bread wheat), which were correlated with RFLP profiles and mildew resistance and showed the presence and absence of 2RL and 2BL, re-

spectively. The mildew resistance was confirmed to correlate with 2RL. Further examinations were made using an array of substitution lines and one 2RL allele-specific primer combination could be identified. In the future, it would be fruitful to examine whether RFLP-derived STS markers can be more polymorphic than the RFLP probes themselves. A broader screen of a set of rye and wheat cvs. could in fact reveal whether our allele-specific STS marker is an indication or an exception of polymorphism between different cvs.

Chromosome-specific markers developed in this study are based on the amplification of one single fragment, something which makes them suitable for stained PCR assays where the DNA double helix is visualised directly in the plate by ethidium bromide (Gu *et al.*, 1995). The development of STS markers designed to select the powdery mildew-resistant wheat-rye translocation 2RL.2BS could therefore be even more accelerated if this method was adopted in MAS. The requirements for MAS in breeding programmes are as follows: (1) a co-segregation between the marker and the trait, (2) efficient technology for high-throughput adapted to screening of large populations of breeding material, (3) marker validation to confirm the overall usefulness in crosses with different genotypes, and (4) a reliable and robust screening technique with reproducibility across different laboratories. In a review by Gupta *et al.* (1999), a total number of 36 agronomic traits in wheat have so far been recorded and tagged with different molecular marker systems, such as RFLP, RAPD, SCAR, AFLP, SSR and STS. RFLP belongs to the first generation of DNA markers, which is based on a hybridisation between a RFLP probe and digested templates of DNA, while the others are PCR-based. The most eminent application of RFLP has been the construction of linkage maps. The RFLP mapping in wheat has identified more than 1,000 markers. It is thus the most explored and used DNA marker system in this crop. However, RFLP analysis has not been integrated into the application of breeding programmes to any great extent, since it is time-consuming, labour-intensive and has a low level of detected polymorphism between adapted wheat varieties. Bryan *et al.* (1999) estimated the variation at DNA level to be approximately 1:1000 basepairs among adapted genotypes of wheat. It is hence ineffective when used for intervarietal detection of polymorphism. On the other hand, it has a co-dominant character and can be used to detect homoeologous loci suitable for comparative mapping and to study synteny between closely related species as

in the case of the grass family. Gale and Devos (1998) stated, in fact, that if PCR had been discovered five years earlier, we might have been unaware of the conserved gene order among plant species. However, introgressions of alien germplasm into wheat have in general been restricted to the tribe *Triticeae*, something which is comprehensible in the context of synteny with substituting ability from adaptive linkage blocks (Sharma and Gill 1983; Jiang *et al.*, 1994). Due to homoeology and suppressed chromosome pairing, these introgressions are inherited as completely linked blocks. This means that a DNA marker located on the introgressed segment always is linked not only to the trait but also to the entire segment. It can therefore be useful for MAS aimed at transferring the alien segment in breeding programmes (Cenci *et al.*, 1999).

Genetic factors required to express the trait in gene transfers are more likely to be linked in a Robertsonian translocation. By contrast, a GMO approach would have severe problems to overcome, even if progress is foreseen in monocots as well, where biolistics seems to be replaced by the *Agrobacterium* approach as the method of choice. The T-cassette is normally equipped with a single gene (without genetic factors) in addition to other components necessary for its transfer. Apparently, GMO technology does not have the advantage of linked beneficial gene complexes that can be transferred in translocations. Although the transformation efficiency in cereals has been improved during the past decade, other problems have to be resolved in the development of GMO, such as integration control and expression failures (Cheng *et al.*, 1997; Rasco-Gaunt *et al.*, 2000). In the currently used technology, there is no control of the integration event and the transgene insert is integrated randomly over the genome. At transgene inserts, transgene expression is hampered by structural defects in the T-cassettes and has to be monitored and designed in a more robust fashion. Methylation is another problem shown as an effect in progressive silencing over the generations. It indicates that the entire transgene insert has been "marked" in the act of host surveillance systems.

Our choice of RFLP-loci was governed by the decision that it should be within the linkage block, *i.e.* the chromosome arm 2RL, where no recombination could occur due to the chromosome pairing suppressor gene *Ph1* located on 5B. Normally, the introgressions from other species are seldom accepted and introduced into plant breeding programmes, but no negative effects are expected from the

compensating translocation T2RL (Knackstedt *et al.*, 1994). In addition, the introduction of 2RL cultivars offers an opportunity to build up a new gene complex of valuable traits. Desirable and adaptive traits are to be expected in the linkage block (Jiang *et al.*, 1994) and it can be assumed that the translocation 2BS.2RL contains other beneficial traits besides mildew resistance and hence is an attractive approach in the gene transfer of mildew resistance. The effect of a linkage drag can either be beneficial or deleterious to the line, and these effects should not be under-estimated. In the scenario of negative linkages in the 2BS.2RL line, it should be possible to choose a linked RFLP-locus for conversion into STS markers in accordance with our developed method, that is co-segregating with the character in question. Further crosses can then be conducted in order to minimise the size of the introgression and thus avoid unwanted deleterious traits in the linkage block.

Conclusions

Wide hybridisation between triticale and bread wheat has been known since the early 1980s. Earlier studies have shown that winter triticale is preferable to spring triticale. Higher transmission rates of rye chromosomes are expected if bread wheat is used as a pollinator. These procedures were used to develop the pre-breeding material that was screened for mildew resistance in this study. According to our findings, wide hybridisation using methods that promote transfer of alien germplasm into the wheat genome gives a sufficient number of lines with alien introgressions in advanced generations of pre-breeding material.

The mildew isolates used in the study were convenient for selection of breeding material in the northern part of Europe and Sweden in particular. However, it would be interesting to investigate if the material contains a broader resistance towards continental mildew races in Europe, as mildew has been adapted to hotter and drier climate with the spread of semi-dwarf cultivars and increased use of nitrogen fertilisers. Further screening of the material with a broader set of mildew isolates has therefore been discussed with Dr Hsam and colleagues in Germany. More complex traits such as yield and drought tolerance may require the identification of QTLs and advanced crossing procedures using the induction of chromosome pairing and marker technology. In our study, it was sufficient to select lines for mildew resistance as presumed carriers of rye chromatin. Our study has shown the preferential selection of lines carrying the rye chromosomes 1R, 2R, 4R and 6R.

Alien introgressions in a plant material must be developed and selected, but also characterised in an appropriate way. Characterisation of introgressions has been made with different methods, such as C-banding, FISH, GISH, RFLP and PCR markers, where the purpose of the investigations has determined the choice of method. Cytogenetics and preferably C-banding is a well-documented method used to identify chromosomes by their karyotype. The C-banding method combined with a selection of mildew resistance has proven an efficient approach to obtain and characterise wheat lines with incorporated genetic transfers in the form of substitutions and translocations from the rye germplasm.

Molecular cytogenetics can be used to detect and describe alien introgressions exclusively and is therefore useful in the characterisation of plant material in plant breeding. However, the method is most useful if the alien species does not belong to the primary gene pool of the species, because of cross-hybridisation of total genomic probes between closely related species. GISH was an efficient and accurate technique to estimate the amount of rye chromatin in a set of triticale-derived wheat-rye substitution/ addition lines. ISH applications showed a sufficient degree of resolution to characterise and localise the breakpoints of a pericentric inversion (1R) contained in two similar wheat-rye substitution lines.

RFLP technology has been successful in characterising alien introgressions in wheat due to the synteny among other species in the grass family, although RFLP is not feasible for the purpose of practical breeding. Considerable efforts have instead been made to develop PCR markers, while non-gel-based approaches are still too expensive to pay dividends for the application breeding programmes. In this perspective it is an attractive approach to convert RFLPs into PCR markers. The conversion of wheat RFLP probes into STS markers has proven a robust and financially feasible method to develop PCR assays and it makes use of already mapped low copy RFLP probes. STS markers could be an alternative to the currently used microsatellites for minor crops such as rye and millet and may also be used to characterise sources of wild relatives stored in gene banks all over the world.

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