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Development of Molecular Markers for the Adult Plant Leaf Rust Resistance Genes *Lr13* and *Lr35* in Wheat (*Triticum aestivum* L.)

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1. Summary

Susceptible wheat cultivars which are not protected by chemical fungicides suffer severe losses when attacked by the fungal pathogen *Puccinia recondita* Rob. Ex Desm. f.sp. *tritici*, the causal agent of leaf rust. As chemical fungicides can have a negative impact on the ecosystem, breeding for resistance is one of the most economical and ecological ways of plant protection.

The aim of this investigation was to develop molecular markers which can be used in marker-assisted selection (MAS) to support the introgression of the two leaf rust resistance genes *Lr13* and *Lr35*. Both genes are acting at the adult stage in wheat. The adult plant growth stage is very vulnerable to leaf rust attack resulting in reduced floret set and yield loss. During the adult period of the growth the inoculum density is very high due to the optimal growing and developing conditions for the fungus. The adult leaf rust resistance gene *Lr13* is considered to be one of the most widely distributed leaf rust resistance genes of the world. In combination with other resistance genes it increases resistance. *Lr13* is located on the short arm of chromosome 2B of wheat and originated from common wheat. We developed two F_2 populations derived from the crosses between the resistant line Th*Lr13* and the susceptible lines Frisal and Thatcher, respectively. We established an infection test in the growth chamber to detect the adult resistance at the seedling stage. Eighty-two RFLP probes and 14 microsatellites that were previously located on chromosome 2B were tested for polymorphism between the parents. Three RFLP probes and four microsatellites were mapped in the segregating population derived from the cross Th*Lr13* x Frisal. The closest marker (GWM630) was located at 10.7 cM from *Lr13*. In the second population (Th*Lr13* x Thatcher) one RFLP probe and one microsatellite were linked with the resistance gene. GWM630 again was the closest linked marker (10.3 cM). Although many polymorphisms were detected, no closer marker could be found by using the AFLP technique.

The origin of the resistance gene *Lr35* gene is *Triticum speltoides*, a wild relative of wheat. As this gene is not yet exploited in wheat breeding it is still effective against leaf rust worldwide. The resistance gene *Lr35* is also located

on wheat chromosome 2B. An F_2 population from the cross Th*Lr35* x Frisal was developed. We established an infection test on adult plants in the growth chamber to detect resistance or susceptibility. Ninety-six F_2 plants of the segregating population were tested at the adult stage. Fifty-one of the tested 80 RFLP probes which were located on chromosome 2B were polymorphic between the parents. Fourteen of these probes were mapped. We developed a dominant STS (sequence-tagged-site) marker from the completely linked RFLP probe BCD260. This STS marker can be used efficiently in resistance breeding programs to introgress *Lr35* into elite breeding material.

2. Zusammenfassung

Weizenbraunrost, hervorgerufen durch das Pathogen *Puccinia recondita* Rob. Ex Desm. f.sp. *tritici,* ist eine der weltweit wichtigsten Pilzkrankheiten im Weizen. Im Falle einer Infektion reagieren anfällige Sorten mit empfindlichen Ertragseinbussen. Der Einsatz chemischer Fungizide zum Schutze der Pflanzen kann einen nachteiligen Effekt auf die Umwelt ausüben. Züchtung auf Resistenz ist somit eine ökonomisch und ökologisch bevorzugte Strategie des Pflanzenschutzes.
Ziel dieser Arbeit war es, molekulare Marker für die Braunrostresistenzgene *Lr13* und *Lr35* zu entwickeln. Beide Resistenzgene sind nur in adulten Pflanzen effektiv. Eine Infektion während diesem Entwicklungsstadium ruft einen verminderten Blütenansatz und somit einen deutlich verringerten Ertrag hervor. Molekulare Marker würden es erlauben, diese beiden Resistenzgene durch markergestützte Selektion gezielt in der Züchtung einzusetzen.

Das Resistenzgen *Lr13* ist eines der weltweit am meisten verbreiteten Braunrostresistenzgene. In Kombination mit weiteren Braunrostresistenzgenen erhöht es die Resistenz. *Lr13* stammt aus dem hexaploiden Weizengenpool. Es ist auf dem kurzen Arm von Chromosom 2B lokalisiert. In dieser Arbeit wurden zwei segregierende F_2 Populationen entwickelt: Dafür wurden für *Lr13* die resistente Linie *ThLr13* und die anfällige Sorte Frisal bzw. *ThLr13* und die anfällige Linie Thatcher gekreuzt. Um die adulte Resistenz im Keimlingsstadium erkennen zu können, wurde in der Klimakammer ein spezifischer Infektionstest für *Lr13* durchgeführt. Zwischen den Kreuzungseltern wurden 82 RFLP Sonden und 14 Mikrosatelliten für Polymorphismen getestet. Diese Sonden und Mikrosatelliten waren bereits früher auf Chromosom 2B kartiert worden. Drei RFLP Sonden und vier Mikrosatelliten konnten in der Population *ThLr13* x Frisal kartiert werden. Der am nähesten lokalisierte Marker (GWM630) zeigte einen Abstand von 10,7 cM zum Resistenzgen. In der zweiten Population (Th*Lr13* x Thatcher) waren eine RFLP Sonde und ein Mikrosatellit mit dem Gen gekoppelt. GWM630 war wiederum der am engsten gekoppelte Marker (10,7

cM). Mit der AFLP Technik konnten viele Polymorphismen entdeckt werden. Allerdings konnte kein gekoppelter Marker mit dieser Technik entwickelt werden.

Triticum speltoides, ein diploider Verwandter des Weizens, ist der Donor des *Lr35* Resistenzgenes. Dieses Gen ist weltweit noch wirksam, da es noch nicht kommerziell ausgenutzt worden ist. Auch dieses Gen ist auf dem kurzen Arm von Chromosom 2B lokalisiert. Aus den zwei Kreuzungseltern Th*Lr35* und Frisal wurde eine spaltende F_2 Population entwickelt. 96 F_2 Pflanzen wurden auf Resistenz im adulten Stadium in der Klimakammer getestet. 51 der 80 getesteten RFLP Sonden zeigten Polymorphismen zwischen den elterlichen Linien. 14 dieser Sonden konnten kartiert werden. Die mit *Lr35* vollständig gekoppelte RFLP Sonde BCD260 wurde in einen dominanten STS (sequence-tagged-site) Marker umgewandelt. Dieser auf PCR basierende Marker erlaubt die effiziente Einführung des *Lr35* Resistenzgenes in fortgeschrittenes Zuchtmaterial.

3. General Introduction

3.1 Wheat and leaf rust

Wheat is the most widely grown and consumed food crop in the world. It is the staple food of nearly 35% of the world population (Braun et al. 1998). Today wheat occupies approximately 20% of the world's cultivated land and is the most important agricultural commodity in international trade (Wiese, 1991). The term wheat is normally used to refer to the cultivated species of the genus *Triticum* (Knott, 1989). The genus *Triticum* is very diverse and it includes diploid, tetraploid and hexaploid wheat. Today the cultivation is restricted to the tetraploid durum wheat (*Triticum durum* L.) and hexaploid common wheat (*Triticum aestivum* L.).

Leaf or brown rust caused by *Puccinia recondita* Rob. Ex Desm. f.sp. *tritici* is considered to be one of the most important fungal diseases of wheat worldwide. It usually does not cause spectacular yield losses, but on a world wide basis it probably causes more damage than wheat stem rust or wheat yellow rust (Samborski, 1985). Under favorable conditions for the fungus, losses can be severe and reach up to 30% (Roelfs et al. 1992).

Puccinia recondita belongs to the order uredinales of the class of basidiomycetes. It can be subdivided into *formae speciales*, which are specific for wheat (f.sp. *tritici*) and rye (f.sp. *secalis*). Other agronomically important rust fungi which are belonging to the basidiomycetes are *Puccinia graminis*, (stem or black rust of wheat, barley and triticale), *Puccinia stiiformis* (stripe or yellow rust of wheat, barley, rye and triticale), *Puccinia hordei* (leaf rust of barley) and *Puccinia coronata* (crown rust of oats). As *Puccinia recondita* is macrocyclic, the sexual life cycle of the fungus includes two different hosts: major primary hosts for wheat leaf rust are hexaploid wheat (*Triticum aestivum* L.em. Thell) and spelt (*Triticum spelta* L.), tetraploid wheat (*Triticum durum*) and triticale (X *Triticosecale* Wittmack), whereas *T. monococcum* L., *T. dicoccum* L. and *T. speltoides* are of minor importance (Roelfs et al. 1992). The asexual life cycle

can be completed in the cereals. On these hosts, the fungus is in the dicaryotic phase. On the alternate hosts, such as *Thalictrum, Anchusa, Clematis* and *Isopyrum fumarioides* the fungus is monocaryotic and undergoes a sexual cycle. Therefore, the alternate hosts may play an important role for genetic exchange between races of the fungi (Roelfs et al. 1992). The importance of the alternate host in generating changes in the pathogen population for virulence combinations is unknown (Roelfs et al. 1992). The aeciospores which are again dicaryotic are released from the alternate host to infect the primary host. The importance and frequency of some events like sexual cycle or wheat cropping seasons may vary among areas and regions in the world (Roelfs et al. 1992). The regions where alternate hosts occur and the pathogen is able to develop do not have more severe epidemics than areas where the alternate host is not growing (Saari and Prescott, 1985).

As the pathogen is windborn, urediniospores are spread in the field and over large distances. For example, spores are transported annually from the southern autumn-sown wheat area of North America to the northern spring-sown wheat area (Roelfs et al. 1992). After the dispersal of the pathogen over large distances, the spores remain viable. This is one of the reasons of the economic importance of leaf rust (Saari and Prescott, 1985).

3.2 Adult plant resistance

Seedling, adult plant and overall resistance describe the plant stage at which the resistance is fully expressed. Seedling resistance means resistance expressed at the seedling stage and it is nearly always associated with resistance in the other plant stages. It is therefore almost identical to overall resistance, which is expressed in all plant stages (Parlevliet, 1997). Resistance against a pathogen which is only effective in the advanced plant growth stage of a plant but not at the seedling stage was defined by Zadoks (1961) as adult plant resistance (APR). The onset of APR can vary. In the case of leaf rust resistance the resistance is active when infection is most likely to occur

because of high inoculum density and suitable environmental conditions for infection (Roelfs et al. 1992). An attack of *Puccinia recondita* during heading can cause severe grain yield losses due to reduced floret set, reduced rate of photosynthesis, increased rate of respiration and decreased translocation of photosynthates from infected tissue (Roelfs et al. 1992; Agrios 1997). Therefore, resistance at the adult stage is very important in wheat breeding. Until now more than 45 race specific leaf rust resistance genes have been described (McIntosh et al. 1995) as well as quantitative trait loci (QTL) for leaf rust (Nelson et al. 1997; Messmer et al. 2000). The major resistance genes are inherited in a simple Mendelian fashion. Almost all of them are effective at the seedling stage or throughout the whole life cycle of the plant. A few of these major genes, such as *Lr12*, *Lr13*, *Lr22a*, *Lr22b*, *Lr34*, *Lr35* and *Lr37*, however, are only effective at the adult stage (McIntosh et al. 1995). APR can act as a hypersensitive resistance but also in a quantitative way. Seedlings show a susceptible infection type whereas adult plants respond to the same race of the pathogen with cell death (hypersensitive response, HR) or with increased latency period, decreased infection frequency and reduced sporulation rate. Although APR has been found against viruses, bacteria and fungi, little is known about the mechanism of APR (Boyle and Aust, 1997). No concerted attempt has been made to determine the factors which affect its expression (Qayoum and Line, 1985).

3.3 Molecular markers and marker-assisted selection

Until recently, morphological markers were used in plant breeding to define linkage to desired traits and to select indirectly for these traits in crop plants (Weber and Wricke, 1994). Classical phenotypic markers were plentiful in only a few well characterized species such as maize (Rafalski et al. 1996). It took a long time and much patience, however, to obtain several markers for one species. Often genes with negative effects such as dwarfism, albinism or other subvital mutations had to be used (Weber and Wricke, 1994). Moreover,

morphological markers often can only be detected in adult plants. For these reasons, the use of morphological markers in plant breeding has been limited.

Biochemical markers (protein markers) are based on differences in charged amino acids of enzymes with little or no effect on the enzymatic activity. In this case, the variation between different varieties and lines is high, which means that polymorphisms on the protein level are frequent. With the present techniques, however, only few isoenzymes are easily distinguishable. For example, in maize less than 40 proteins are verifiable and this number is to small to find close linkage to agronomically important genes (Becker, 1993). In wheat, the important leaf rust resistance gene *Lr19* can be detected with the biochemical marker *Ep-D1c* developed by Winzeler et al. (1995).

Molecular (DNA) markers based on polymorphisms in the nucleic acid sequence are theoretically almost unlimited in number and are overcoming the disadvantage of morphological and biochemical markers. DNA markers are being applied to a wide variety of problems central to plant genome analysis (Rafalski et al. 1996) such as:

- creation of genetic maps
- mapping of simple traits
- mapping of quantitative trait loci (QTL)
- mapping of mutations
- identification or transgenics
- genetic diagnostics for plant breeding
- population genetics
- molecular taxonomy and evolution
- identification of individuals
- germplasm characterization
- identification of proprietary germplasm
- estimation of genome size

According to Winter and Kahl (1995), in plant breeding the use of molecular markers focuses on three major issues: (I) the acceleration of the introgression of single disease resistance genes from wild species or cultivated donor lines into superior cultivars, (II) the accumulation (pyramiding) of major and/or minor resistance genes into cultivars to generate multiple and more durable resistance and (III) the improvement of the agronomic value of crops by breeding for quantitatively inherited traits such as yield, protein content or drought and cold tolerance.

There is now a large number of research projects that address questions and prospects of marker-assisted selection (MAS) in theoretical or applied form. A considerable number of molecular markers have been developed for qualitative and quantitative characters. Particularly for disease and pest resistance much effort was and is made to develop different types of molecular makers for the main staple crops such as wheat, maize, rice, barley, potato and sugarbeet (reviewed by Mohan et al. 1997). Numerous papers on the theoretical application of MAS were published in the last years. For example, Hospital et al. (1997), Xie and Xu (1998), Knapp (1998), Ribaut and Hoisington (1998) and Frisch et al. (1999) addressed questions such as where to integrate molecular markers in the breeding program, the number of markers which should be used, the number of progeny which should be tested and whether molecular markers could actually have an impact in applied plant breeding. There are, however, few reports on MAS experiments. Successful work has been done in rice (Yoshimura et al. 1995; Reddy et al. 1997), barley (Han et al. 1997) and maize (Ribaut et al. 1997; Stuber, 1997). Yoshimura et al. (1995) successfully developed rice lines with major resistance gene pairs against bacterial blight. One major resistance gene against bacterial blight in rice was introgressed into a popular rice variety of eastern India by Reddy et al. (1997). Different breeding strategies to increase malting quality traits in barley by MAS were compared by Han et al. (1997). These authors conclude that the combination of phenotypic and genotypic selection is more effective than phenotypic selection alone. In maize, Stuber (1997) found that identifying and mapping of QTLs is effective.

Furthermore, the transfer of two to four QTLs from one line to another by MAS was optimal. By increasing the number of introgressed QTLs, the recipient genome was replaced by donor chromosomal segments with deleterious effects. Due to the application of the fast and reliable MAS Ribaut et al. (1997) were able to increase the selection pressure in backcrosses in their maize breeding program.

The essential requirements for MAS in plant breeding programs are:

- marker(s) which co-segregate or are closely linked (1 cM or less is probably sufficient for MAS) with the desired trait
- an efficient way for screening large populations with the molecular markers
- the marker technique should be highly reproducible across laboratories, economical and breeder-friendly (Mohan et al. 1997)

At present, the two last points can be achieved relatively well with the use of PCR-based markers such as sequence-tagged sites (STS) and microsatellites. These marker types allow:

- an earlier plant sampling because of the small amount of tissue required
- a faster DNA preparation because of the small amount of template DNA required
- a more efficient handling of large sample size, because of the efficiency of PCR technology
- the possibility of automation as the extraction of genomic DNA and amplification reactions can be performed in the 96 or 384 microtiter plate-format

3.4 Breeding for resistance and sources of resistance genes

Breeding for resistance requires a constant inflow of new resistance genes. The occurrence of new virulent pathogen races makes it necessary to permanently breed for new resistant elite lines. Over the years, effective resistance genes

are often overcome by the pathogens. This is due to several reasons: sexual recombination is known to occur on the intermediate host. Asexual recombination can serve as well as a source for new virulent races. The fusion of germ tubes and hyphae which is called anastomosis, occurs readily in rust fungi and it is reasonable to expect mitotic recombination to occur. In fact, new strains have been isolated from susceptible wheat plants inoculated with mixtures of urediospores of two races of various rusts (Samborski, 1985). Mutations provide the basic variation that occurs in leaf rust. Parlevliet and Zadoks (1977) estimated that 1 ha of susceptible wheat with 1% infection of leaf rust could produce 10^{11} uredospores per day. With a mutation frequency of 1×10^{-5} to 1×10^{-6} and assuming heterozygosity of the dikaryotic pathogen, there is a potential for production of many mutant spores each day. Depending on the area where the variety with the resistance gene is grown, the number of years and the climatic conditions, single resistance genes can become ineffective after a few growing seasons. The average longevity of conventional, monogenic inherited resistance to wheat rusts throughout the world was less than 10 years (Kilpatrick, 1975). To prevent the pathogen to become virulent on resistant lines and reduce yield, several breeding and agricultural strategies are existing. Agricultural measures to reduce the development of the pathogen could be realized on the farm level and on a region level. On the farm level, agricultural methods can be an early seeding time to escape the epidemic occurrence of the pathogen, an adequate application of nitrogen, the use of multilines which is the combination of several resistance genes in one variety, or the use of seed mixtures with lines containing different resistance genes. On a regional level, the use of different combinations of resistance genes in different areas of an epidemiological zone, called gene-deployment, is a promising strategy (Knott, 1989). In practice, however, this is difficult unless all breeders, farmers and government agencies in a given zone agree to cooperate. In addition, seed distribution must be controlled and adequate and good resistances must be available (Roelfs et al. 1992).

Breeding for durable resistance is the most ambitious project to protect plants from the attack of a pathogen. Several ways are existing to achieve this goal. The use of polygenic inherited resistance in breeding is challenging, due to the fact that these resistances are genetically complex. Because several genes are involved in the expression of polygenic traits, they generally have smaller individual effects on the plant phenotype. This implies that several regions (QTLs) must be combined at the same time in order to have a significant impact and that the effect of individual regions is not easily identified. This kind of resistance does not confer complete resistance to the pathogen but is supposed to be durable and to reduce the fungal attack considerably.

Another promising approach to breed for durable resistance is the combination, also called pyramidisation, of different single resistance genes and/or QTLs against the same pathotype. When a cultivar has several genes for resistance to the same disease, it is generally assumed that the genes act independently. A cultivar with two genes, each determining a different level of resistance, usually exhibits the rust reaction phenotype of the more effective gene; the gene conferring the least resistance is masked, i. e. the more effective gene is epistatic to the one that confers lower resistance. Furthermore, a cultivar with two or more genes will not only be resistant to the rust races against which the individual genes are effective but in some cases also against races which have overcome the individual resistance genes. Genes for disease resistance, however, do not invariably act independently. Thus, several resistance genes may interact to give higher levels of resistance. A single gene by itself might have only a small effect, but in combination with other genes the effects can be greater (Knott and Yadav, 1993). Extreme forms of complementary resistance require the presence of two or more genes for the resistance to be expressed. There are numerous examples of genes for disease resistance that interact to give an enhanced level or resistance (Samborski and Dyck, 1982; Ezzahiri and Roelfs, 1989). This complementary interaction, which may be additive, results in a higher level of resistance than that conferred by the single genes. The most durable resistance to leaf rust is associated with a few gene combinations (Roelfs, 1988). *Lr13* and perhaps *Lr12*, both adult plant resistance genes, in

combination with *Lr34* are the basis of most of this resistance. The combination of *Lr13* (adult resistance) and *Lr16* (seedling resistance) also results in enhanced resistance: rust races virulent to *Lr16* did not result in a compatible interaction but produced an incompatible infection type (Samborski and Dyck, 1982).

In order to stay ahead of constantly changing rust pathogens it has been necessary for wheat breeders to maintain genetic diversity by seeking resistance genes from sources other than common wheat. The different gene pools of wheat are the sources of not yet exploited resistance genes. According to their crossability with hexaploid wheat the species are divided into three gene pools.

The primary gene pool

Genes of species belonging to the primary gene pool of common wheat can be transferred to wheat with almost no problems (Dyck and Kerber, 1985). This group includes modern hexaploid wheat (*T. aestivum*) with the AABBDD genome, old land races and as well hexaploid spelt (*T. spelta*). Wheat species with lower ploidy levels such as tetraploid durum wheat (*T. durum*) with the AABB genome and wild relatives of durum wheat (*T. dicoccoides*) are also belonging to this group. The diploid Einkorn (*T. monococcum*), related to the A genome donor *T. urartu* and the diploid D genome donor, *T. tauschii*, also belong to the primary gene pool. Within this group sexual crosses are possible to transfer desired traits from one variety to another, from one ploidy level to another. Because normal chromosome pairing and genetic recombination occurs in hybrids produced from crosses between hexaploid wheat and the tetraploid (AABB) and diploid (AA and DD) progenitors of wheat, the transfer of resistance genes is possible without problems of F_1 sterility (Dyck and Kerber, 1985). The direct cross between some genotypes of hexaploid and diploid wheat, however, is sometimes difficult and the hybrid is sterile. Bridge crosses are the solution in such cases. Genes can be transferred first from diploid to tetraploid level and then to hexaploid wheat. Another bridging method

applicable to the transfer of genetic material to both tetraploid and hexaploid wheat is with natural amphiploids that have the A genome in common with these two wheats or the D genome in common with hexaploid wheat. The amphiploid selected for resistance is crossed to wheat, and the partially fertile hybrid is then backcrossed several times to the wheat cultivar to obtain fertile plants. Although 25,000 cultivars of wheat are available, there is ongoing need to search for new resistance genes in the other sources. The known genes for leaf rust resistance in most of the cereal crops are now being utilized on an international scale. Consequently, few new genes for resistance can be expected from advanced breeding stocks (Dyck and Kerber, 1985).

The secondary gene pool

Out of the 49 numbered and 11 unnumbered leaf rust resistance genes (McIntosh et al. 1995; Kolmer, 1996; Dubcovsky et al. 1998), 31 originated from hexaploid wheat and one from hexaploid spelt (*Triticum spelta*). Ten leaf rust resistance genes were introgressed into breeding material from the secondary gene pool. This gene pool includes the polyploid *Triticum* species that have at least one genome in common with *T. aestivum*. Gene transfer from these species by recombination is possible. For example, *T. ventricosum* with the DDMM genome was the donor of the *Lr37* resistance gene. The diploid S genome species, *T. speltoides*, which is supposed to be the progenitor of the B genome of common wheat (Sarkar and Stebbins, 1956), also belongs to the secondary gene pool. This species has contributed several resistance genes that are used in cultivar improvement, for example: *Lr28*, *Lr35*, *Lr36*, Neepawa*6/*T. speltoides* F-7, Manitou*6/*T. speltoides* E-11, Neepawa*6/*T. speltoides* H-9 and Neepawa*5/*T. speltoides* 0-1 (McIntosh et al. 1995). As the S genome is related to the B genome, recombination between the two homoeologous chromosomes can occur but the frequency is very low (see chapter about the resistance gene *Lr35*). Often the embryos of such crosses appear to develop normally for a number of days and then show signs of degeneration. This probably results from a breakdown of the endosperm and its inability to nourish the embryo. If the embryos have developed for at least 8-10

days, they can often be removed, cultured on special medium and induced to produce plants (Knott, 1989). Embryo rescue is often the only choice to obtain fertile hybrids.

The tertiary gene pool

Species belonging to the tertiary gene pool are more distantly related. Resistance genes from *T. umbellulatum* (*Lr9*), *Thinopyron ponticum* (*Lr19*, *Lr24*, *Lr29*), *Thinopyron intermedium* (*Lr38*) and *Secale cereale* (*Lr25*, *Lr26*, *Lr45*) were transferred to hexaploid wheat (McIntosh et al. 1995, Knott, 1996). Their chromosomes are homoeologous to those of wheat, but during meiosis there is no recombination between the homoeologous chromosome pairs. Several methods are available for inducing recombination. Radiation can cause random breaks in chromosomes which will reunite at random and result in translocated chromosomes. Numerous successful transfers have been induced by this method. An alternative method for inducing homoeologous pairing and recombination is to use the X-ray induced *ph1b*/*ph1b* mutant line developed by Sears (1977), which is a deletion line for the *ph1* locus, located on the long arm of chromosome 5B. The gene product PH1 ensures that only homologous chromosomes can pair and recombine.

According to Knott (1989) there is little evidence that resistance in the wild relatives of wheat is genetically or physiologically different from that in cultivated wheat. Consequently, there is no reason to assume that it will be more durable: in fact, in a number of cases resistance from an alien species has been overcome by a new, virulent race of a rust pathogen.

Alien gene integration into wheat is a long and laborious process so that screening should be done thoroughly to ensure maximum results from the integration process.

3.5 The objectives of the study

In this work, we wanted to develop molecular markers for the monogenically inherited adult plant leaf rust resistance genes *Lr13* and *Lr35* in wheat. These markers would allow to integrate the resistance genes efficiently into advanced breeding material via MAS. Furthermore, MAS would allow to combine several resistance genes against the same pathogen in one line to achieve a more durable resistance.

4. Molecular mapping of the adult leaf rust resistance gene *Lr13* in wheat

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Abstract

Resistance of wheat to leaf rust can be expressed either at the seedling stage or only at later stages of plant development (adult-plant resistance). Among more than 45 leaf rust resistance (*Lr*) genes, seven are adult-plant resistance genes. *Lr13* is effective only at the adult stage and is considered to be one of the most important leaf rust resistance genes. The objective of this work was to develop a linkage map around *Lr13* and a molecular marker for this resistance gene. We developed two segregating populations derived from the crosses between the wheat lines Th*Lr13* and Frisal and Th*Lr13* and Thatcher, respectively. Eighty-two RFLP probes, 14 microsatellites and 226 AFLP primer combinations were tested for linkage. In the first population (Th*Lr13* x Frisal) three RFLP probes and four microsatellites were linked with *Lr13*, whereas in the second population (Th*Lr13* x Thatcher) one RFLP probe and one microsatellite were linked. No AFLP amplification product was linked with the gene. In both populations the wheat microsatellite GWM630 was the closest marker at 10 cM from *Lr13*.

Introduction

Wheat leaf rust caused by *Puccinia recondita* Rob. ex Desm. f.sp. *tritici* Eriks. & Henn. is one of the most devastating fungal diseases in wheat worldwide. The use of resistant varieties is an economical and environmental-friendly way for minimizing the losses caused by leaf rust. Resistant varieties, however,

particularly those carrying only one resistance gene are frequently not durably resistant. The ability of the pathogen to evolve new pathotypes which overcome the host resistance requires constant efforts in resistance breeding.

Among the more than 45 leaf rust (*Lr*) resistance genes identified in wheat, *Lr13* is probably the most widely distributed gene worldwide (McIntosh et al. 1995). For example, most of the wheat varieties of the CIMMYT (Centro International de Mejoramiento de Maíz y Trigo, Mexico) are expressing the *Lr13* resistance gene (Rajaram et al. 1988). Under normal conditions, this resistance gene only acts at the adult plant stage which is characteristic for the so called adult-plant resistance (APR). As a single gene *Lr13* is no longer effective in most wheat-growing areas (McIntosh et al, 1995). However, in combination with *Lr34* it appears that *Lr13* is the basis of the most durable resistance against leaf rust (Ezzahiri and Roelfs, 1989). The combination of *Lr13* and *Lr16* also showed an increased resistance (Samborski and Dyck, 1982). These examples illustrate the observation that pyramiding resistance genes against the same pathogen can confer broad-spectrum and durable resistance presumably due to quantitative complementation (Yoshimura et al. 1995). However, the combination of two or more resistance genes in one line using traditional host-parasite interactions is time-consuming and sometimes impossible due to the lack of isolates with specific virulence genes. The hybrid necrosis gene $Ne2^m$ has been previously defined as a morphological marker for *Lr13* (McIntosh et al. 1995). However, $Ne2^m$ can not be used for accurate detection of the resistance gene *Lr13* (Anand et al. 1991) as it maps at 33 cross over units from the resistance gene. In addition, hybrid necrosis is an agronomically negative trait as the yield is drastically reduced. The introduction of molecular markers in the breeding process to select lines which contain the desired composition of resistance genes is therefore very valuable.

Here, we have developed two linkage maps of the region of chromosome 2B where the resistance gene *Lr13* is located. Furthermore, we have found a microsatellite marker, GWM630, which segregates at 10 cM distance from the *Lr13* resistance gene.

Material and methods

Plant material

Studies were performed on the susceptible spring wheat variety Frisal (FAL, Zürich-Reckenholz), the susceptible spring wheat line Thatcher and on the resistant near isogenic line (NIL) Thatcher*7/Frontana, R.L.4031 (referred to as Th*Lr13*) which was developed by Dyck et al. (1966).

Two segregating populations were developed for linkage analysis. The first population consisted of 156 F_2 individuals derived from the cross between the NIL Th*Lr13* and Frisal. The second population comprised 151 F_2 individuals derived from the NIL Th*Lr13* crossed with the recurrent parental line Thatcher.

Although the resistance gene *Lr13* is effective at the adult stage, detection of the gene was done at the seedling stage according to the method developed by Pretorius et al. (1984). Infection was performed with the leaf rust isolate 96505 (FAL, Zürich-Reckenholz) avirulent for *Lr13* under controlled conditions in a growth chamber. Plants of the resulting F_2 progeny of the first population (Th*Lr13* x Frisal) were tested at the seedling stage for resistance. To confirm the infection type 50 F_2 plants were re-tested as F_3 families at the seedling stage. Each F_3 family consisted of 20 individuals. The second F_2 population originating from the cross Th*Lr13* x Thatcher was also infected and scored for resistance at the seedling stage.

Scoring of the artificially infected plants was done 10 days after spraying urediospores as a suspension with mineral oil "Soltrol" (Philips, Paris). A scale ranging from 0 to 4 was used to determine infection types (Roelfs et al. 1992).

Parental screening for polymorphism with molecular markers

Isolation of genomic DNA from leaf tissue of 5-week-old wheat seedlings was performed as described by Graner et al. (1990). Seven restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I) were used for genomic DNA digests. RFLP probes were labeled with [^{32}P]dCTP using a random primer labeling kit (Amersham, Switzerland) according to the manufacturer's

instructions. Eighty-two RFLP probes mapping to chromosome 2B of wheat were used to screen the resistant parent Th*Lr13* and the susceptible parents Thatcher and Frisal for polymorphisms. Out of these probes, 48 probes had been mapped on chromosome 2B (Devos et al. 1993; Laurie et al. 1993; http://wheat.pw.usda.gov), whereas the position of the other 34 probes on the chromosome was not known. The cDNA and genomic DNA probes used were derived from wheat (PSR and GLK), barley (BCD) and oat (CDO). The probes BCD1709 and CDO388 are described in the Graingene database (http://wheat.pw.usda.gov) and were kindly provided by Dr. M.E. Sorrells (Cornell University, USA). The probe PSR912 was mapped by Devos et al. (1993). The probe GLK456 was developed by Liu and Tsunewaki (1991).
The 14 wheat microsatellites used in this study were derived from the wheat variety Chinese Spring and were kindly provided by Dr. M. Röder (IPK, Gatersleben, Germany). The microsatellites GWM 120, 319, 388 and 630 were described by Plaschke et al. (1995) and Röder et al. (1998). PCR-amplification of microsatellites was performed as described by Röder et al. (1998). Fragment analysis was carried out on automated sequencers ALFexpress (Pharmacia, Switzerland) and LiCor 4200 (MWG, Germany) with fluorescence labeled primers.

AFLPs were performed as described by Zabeau and Vos (1993) and Vos et al. (1995) with minor modifications on the F_2 plant material of the cross Th*Lr13* x Frisal. Digestion of genomic DNA, ligation of adapters and PCR amplification were performed according to Hartl et al. (1999). 226 *Sse*8387I/*Mse*I primer combinations with two selective nucleotides extending into genomic sequences were tested. The labeling reaction of the *Sse*8387I primers was made in a volume of 25 µl containing 10 µM *Sse*8387I primer, 2 MBq [γ-^{33}P] ATP, 1 X kinase buffer (10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate) and 1U T4 polynucleotide kinase. The incubation time was 1 hour at 37°C, followed by 10 min at 65°C for enzyme inactivation. Screening for polymorphisms was done by bulked segregant analysis (BSA) (Michelmore et al. 1991). Phenotypic bulks were composed by sampling an equal amount of

DNA (250 ng) from 10 F_2 individuals showing a susceptible infection type and 10 F_2 individuals showing a resistant infection type after inoculation with leaf rust. Polymorphisms found between the parental lines and the pools were tested on 30 F_2 individuals different from those used for the BSA. The amplification products were separated on a 5% denaturing polyacrylamide gel. Electrophoresis was done for 105 min at constant power (60 W). The dried acrylamide gel was exposed for 2-3 days to Biomax MR films (Kodak).

Linkage analysis

Linkage estimation was based on the maximum likelihood method using MAPMAKER (Lander et al. 1987). The recombination fraction was transformed to centiMorgan (cM) according to Kosambi (1944).

Results

Segregation analysis of the F_2 populations

Two different F_2 populations were developed for mapping of the *Lr13* resistance gene. The first population was derived from the cross Th*Lr13* x Frisal and consisted of 156 F_2 individuals. Ten days after infection of the seedlings, 39 F_2 individuals showed a resistant phenotype, 83 an intermediate phenotype and 34 plants a susceptible phenotype. This indicated that the gene followed a 1:2:1 segregation (X^2 = 1.05) and is inherited as an intermediate gene in this population. Genetic mapping was carried out on a subset of 50 F_2 plants which were retested as F_3 families at the seedling stage.

The second mapping population was derived from the cross between the NILs Th*Lr13* and Thatcher. Out of 151 F_2 plants, 50 showed a resistant reaction, 67 showed an intermediate phenotype and 34 a susceptible reaction. The 1:2:1 segregation (X^2 = 5.22) for a monogenically inherited resistance gene confirmed the intermediate action of *Lr13*. A subpopulation of 89 F_2 individuals was used for mapping the resistance gene *Lr13*.

Genetic mapping of the *Lr13* gene

Linkage studies were performed on the two different segregating F_2 populations. RFLP probes and microsatellites used in this work had previously been shown to be located on chromosome 2B of wheat. Out of the 82 RFLP probes tested for polymorphism between the parental lines, five were polymorphic between Th*Lr13* and Frisal (Tab. 4/1). Only the probes BCD1709, CDO388 and GLK456 were linked with the resistance gene. The closest RFLP probe, BCD1709, showed a genetic distance of 16.9 cM to the *Lr13* gene (Fig. 4/1a). Two polymorphic probes mapped by Laurie et al. (1993) in the distal region of the short arm of chromosome 2B were not linked with the resistance gene.

The microsatellites were more polymorphic than the RFLP probes between the parents Th*Lr13* and Frisal. Indeed, five of the 14 microsatellites tested were polymorphic between the two parents Th*Lr13* and Frisal (Tab. 4/1). Four of them (GWM120, GWM319, GWM388 and GWM630) were linked with the *Lr13* resistance gene (Fig. 4/1a). The closest microsatellite, GWM630, was located at 10.7 cM from *Lr13*. and thus is the marker which showed the closest linkage with the *Lr13* resistance gene in this mapping population. All probes mapped proximal to *Lr13* on chromosome 2B. No molecular marker was found to be linked distal to the resistance gene 2B (Fig. 4/1a).

We used the AFLP technique in the F_2 population generated by the cross Th*Lr13* x Frisal to find new markers at the *Lr13* resistance gene locus. In total, 226 primer combinations were used. Each primer combination generated between 70 and 100 amplification products per genotype. In total 65 primer combinations revealed three to six polymorphic fragments differing between the parental lines and the bulks (data not shown). Although the degree of polymorphism was high, no linkage was found with the resistance gene *Lr13* when the polymorphic fragments were tested on the F_2 population.

The second mapping population was derived from the cross between Th*Lr13* and the recurrent parent Thatcher. Marker analysis showed that the frequency of polymorphisms was lower than in the first population Th*Lr13* x Frisal. Only one RFLP probe (PSR912) and one microsatellite (GWM630) were found to be

polymorphic between the NILs Th*Lr13* and Thatcher. Both markers were linked with the resistance gene *Lr13*. The RFLP probe PSR912 was dominantly inherited whereas the microsatellite GWM630 was codominantly inherited. Therefore, the mapping was performed with combined data. Microsatellite GWM630 showed a linkage of 10.3 cM with the resistance gene while PSR912 mapped at 22 cM (Fig. 4/1b). As in the first population, the mapped probes were located proximal to the resistance gene.

Table 4/1: Number of tested markers, polymorphic markers and linked markers found with different marker techniques in two different crosses, Th*Lr13* x Frisal and Th*Lr13* x Thatcher.

Cross	**Th*Lr13* X Frisal**	**Th*Lr13* x Thatcher**
Tested RFLP probes	82	82
Polymorphic RFLP probes	5	1
Linked RFLP probes	3	1
Tested microsatellites	14	14
Polymorphic microsatellites	5	1
Linked microsatellites	4	1
Tested AFLP combinations	226	-
Polymorphic combinations	65	-
Linked AFLP	0	-

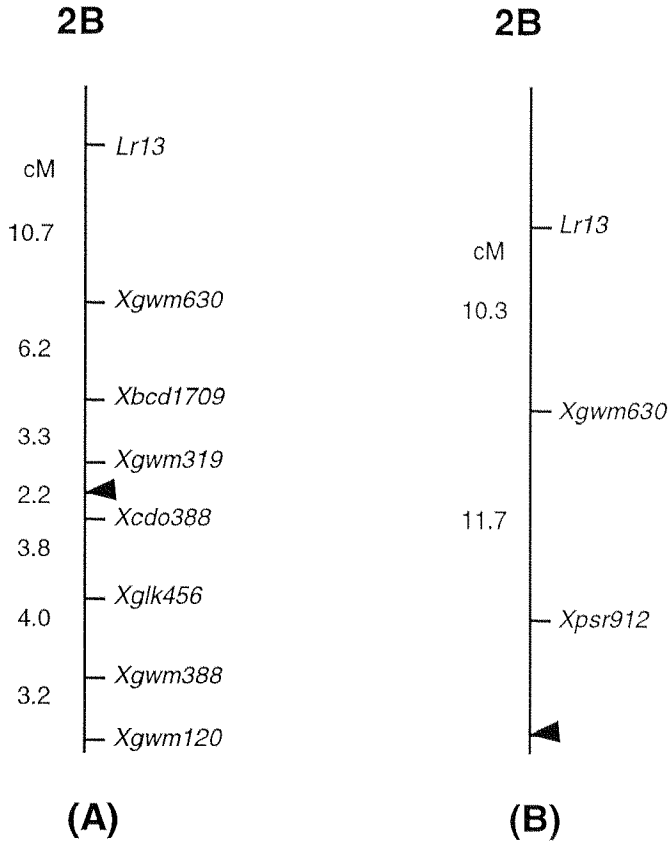

Figure 1: Linkage map of RFLP probes and microsatellites on chromosome 2B of wheat. The approximate position of the centromere is indicated by an arrowhead. (A) Map of chromosome 2B in the cross Th*Lr13*x Frisal. (B) Map of chromosome 2B in the cross Th*Lr13*xThatcher. RFLP probes: BCD, CDO, GLK and PSR; microsatellites: GWM.

Discussion

In this study, two different F_2 populations were developed for mapping the *Lr13* resistance gene. The level of polymorphism detected by RFLP was low in the population originating from the parents Th*Lr13* and Frisal. Only 6% of the RFLP probes tested were polymorphic between these two parental lines. In hexaploid wheat, the lack of polymorphism between lines revealed by RFLP is well known (Liu and Tsunewaki, 1991). The microsatellites tested in the first segregating population showed a higher rate of polymorphism (35%), which is in agreement with Röder et al. (1998) who found that microsatellites had a much higher level of polymorphism and information content in hexaploid wheat than any other marker system. Only one RFLP probe and one microsatellite were polymorphic in the second population which was derived from the cross between Th*Lr13* and Thatcher. This is possibly due to the close relationship of the crossing partners which are NILs and therefore theoretically share 99% of the genome. Despite the low level of polymorphism, NILs represent some of the best material for finding molecular markers linked to desired traits because any polymorphism is likely to be linked to the gene of interest. NILs have been successfully used to develop molecular markers for disease resistance genes in wheat, e.g. for the *Lr9* and *Lr24* leaf rust resistance genes (Schachermayr et al. 1994, 1995) or for the *Pm1*, *Pm2* and *Pm3* powdery mildew resistance genes (Hartl et al. 1993, 1995).

In both populations all molecular markers were mapped proximal to the resistance gene and the microsatellite GWM630 was the closest molecular marker at 10 cM from *Lr13*. All the tested RFLP probes and microsatellites which were located distal to the *Lr13* gene according to previous maps (Devos et al. 1993, Laurie et al. 1993; Röder et al. 1998; http://wheat.pw.usda.gov) were monomorphic between the parental lines. Two RFLP probes located in the telomeric region of chromosome 2B were polymorphic but not linked. Thus, the region around the centromere showed a normal level of polymorphism whereas we observed a complete absence of polymorphism in the region distal to the resistance gene. The mapping of the RFLP probes and microsatellites in this

region has been performed so far in wide crosses (wheat x spelt or wheat x synthetic wheat) (Liu and Tsunewaki, 1991; Devos et al. 1993, Laurie et al. 1993; Röder et al. 1998). Therefore, the lack of polymorphism that we have found here could be due to the use of two hexaploid wheat parents as crossing partners. New segregating F_2 populations with different parents would possibly increase the chance to find polymorphism in this region and therefore more precisely determine the location of the resistance gene. So far we used all available RFLP probes and microsatellites in the region of *Lr13*. Therefore, new probes and microsatellites are needed in the future to find a closer marker for the resistance gene *Lr13*. Other molecular marker techniques could also be used.

We have used the AFLP technique on the Th*Lr13* x Frisal mapping population. Unfortunately, none of the polymorphic fragments which was amplified by using 226 different primer combinations was linked to the resistance gene. Although it was shown that with a low number of primer combinations (96) completely linked markers could be developed (Hartl et al. 1999), in other cases more than 1900 different primer combinations had to be tested to find a completely linked marker (Büschges et al. 1997). In our population the testing of more primer combinations could also increase the chance to obtain a closely linked marker.

Morphological markers can also serve as tools for the indirect selection of the desired traits. Only a very limited number of morphological markers have been found which were linked to disease resistance genes and were useful for practical breeding. Singh (1992, 1993) demonstrated a genetic linkage between leaf tip necrosis and a number of resistance genes against leaf rust (*Lr34*), yellow rust (*Yr18*) and barley yellow dwarf virus (*Bdv1*) on chromosome 7DS. In the case of *Lr13*, the resistance gene was shown to be linked to $Ne2^m$, a member of the complementary gene pair (*Ne1*, *Ne2*) responsible for hybrid necrosis (McIntosh, 1995). Because of the frequent recombination between $Ne2^m$ and *Lr13*, however, this morphological marker can not be used for accurate detection of the resistance gene (Anand et al. 1991). The microsatellite GWM630 which is a PCR-based marker and shows a closer

linkage represents a potentially better marker for the resistance gene *Lr13*. Nevertheless, the genetic distance is still quite big and integration of GWM630 into MAS seems not feasible yet. According to Mohan et al. (1997) molecular markers should co-segregate or be linked with less than 1 cM with the resistance gene for a successful application in MAS.

The genetic analysis of *Lr13* presented here provides the basis for future high-density mapping of this resistance locus. The development of additional microsatellite markers, the derivation of new probes from coding sequences in large scale EST sequencing and mapping projects as well as other new sources of cloned sequences such as BAC clones will provide the basis for the future development such closely linked markers for *Lr13*.

5. Development of a molecular marker for the adult-plant leaf rust resistance gene *Lr35* in wheat

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Abstract

The objective of this work was to develop a marker for the adult plant leaf rust resistance gene *Lr35*. The *Lr35* gene was originally introgressed to chromosome 2B from *T. speltoides*, a diploid relative of wheat. A segregating population of 96 F_2 plants derived from a cross between the resistant line Thatcher*Lr35* and the susceptible variety Frisal was analyzed. Out of 80 RFLP probes previously mapped on wheat chromosome 2B, 51 detected a polymorphism between the parents of the cross. Three of them were completely linked with the resistance gene *Lr35*. The cosegregating probe BCD260 was converted into a PCR-based sequence-tagged-site (STS) marker. A set of 48 different breeding lines derived from several European breeding programs was tested with the STS marker. None of these lines has a donor for *Lr35* in its pedigree and all of them reacted negatively with the STS marker. As no leaf rust races virulent on *Lr35* have been found in different areas of the world, the STS marker for the *Lr35* resistance gene is of great value to support the introgression of this gene in combination with other leaf rust (*Lr*) genes into breeding material by marker-assisted selection.

Introduction

Leaf rust caused by *Puccinia recondita* f. sp. *tritici* is considered to be one of the most important fungal diseases of wheat. To date, more than 40 leaf rust resistance genes have been characterized (Knott 1989; McIntosh et al. 1995).

Most of them are effective from the seedling stage through the whole life of the plant, whereas a few of them are only effective at the adult stage. Resistance during this period is called adult plant resistance and was defined by Zadoks (1961) as a resistance that is only effective in the advanced plant growth stage but not at the seedling stage. An attack of the pathogen during heading (the adult stage) can cause severe grain yield losses due to reduced floret set (Roelfs et al. 1992). Therefore, resistance at the adult stage is of very important economical significance in wheat breeding. Until now, seven genes which are only effective in the adult stage have been described (McIntosh et al. 1995). Although they are inherited in a monogenic fashion, the type of resistance differs between the adult resistance genes. The resistance gene *Lr34* expresses resistance in a quantitative way (Drijepondt and Pretorius 1989; German and Kolmer 1992; Singh 1992). It causes an increased latency period and decreased infection frequency and uredium size (Drijepondt and Pretorius 1989). In contrast, the resistance gene *Lr13* induces a hypersensitive reaction upon infection with an avirulent leaf rust race (McIntosh et al. 1995). So far, *Lr34* was used in many breeding programs in the world, as it confers durable resistance in combination with other genes (German and Kolmer 1992). *Lr13* is probably one of the most widely distributed resistance genes worldwide (McIntosh et al. 1995) but it shows enhanced effectiveness only in combination with other resistance genes (Kolmer 1992). The combination of the adult resistance genes *Lr13* and *Lr34* appears to be the basis of most of the durable leaf rust resistance (Roelfs 1988). This example shows that combination of APR genes is an important factor for successful resistance breeding. *Lr35* also confers hypersensitive reaction upon infection by an avirulent race (Kolmer 1997). In contrast to the resistance genes *Lr13* and *Lr34* which were derived from common wheat, the resistance gene *Lr35* was transferred by Kerber and Dyck (1990) from chromosome 2S of the diploid wild relative *Triticum speltoides* to chromosome 2B of hexaploid wheat. To our knowledge no virulent leaf rust races for *Lr35* have been found until know (Kerber and Dyck 1990; Kloppers and Pretorius 1995; Kolmer 1997, RF Park, personal communication, own data). The *Lr35* gene has not yet been used in modern varieties (McIntosh et

al. 1995).

To maintain a wide spectrum of resistance against pathogens in cultivated wheat, the introgression of resistance genes derived from hexaploid wheat and wild relatives is very valuable. The efficiency of introgression of alien genes to hexaploid wheat can be improved by the use of molecular markers (Asiedu et al. 1989). Molecular markers for translocated resistance genes have already been developed (Schachermayr et al. 1994, 1995; Autrique et al. 1995; Procunier et al. 1995; Dedryver et al. 1996; Naik et al. 1998). Morphological markers for *Lr13* and *Lr34* have been described. A gene for hybrid necrosis (*Ne2*^{m}) was found to be linked to *Lr13* (Singh and Gupta 1991). However, *Ne2*^{m} can not be used for accurate detection of *Lr13* (Anand et al. 1991) in wheat breeding programs. A strong genetic association or pleiotropism with leaf tip necrosis was described as a morphological marker for *Lr34* (Singh 1992). To our knowledge no practically useful molecular markers for adult plant leaf rust resistance genes have been reported so far.

In this study, we developed molecular markers based on RFLP and PCR technologies for the adult plant leaf rust resistance gene *Lr35*. These markers are completely linked with the *Lr35* resistance gene and will be a valuable tool to combine *Lr35* with other effective resistance genes and thus improve the durability of leaf rust resistance.

Material and Methods

Plant material

The resistant line R.L.6082 (Tc*6/R.L.5711, a near isogenic line [NIL] of the spring wheat line Thatcher with the *Lr35* gene, developed by Dr P. Dyck, Winnipeg, Canada) and the susceptible spring wheat variety Frisal (FAL-Reckenholz, Zürich, Switzerland) were crossed. Hundred and thirty-seven plants of the resulting F_2 progeny were tested for resistance under controlled conditions in a growth chamber. The linkage analysis was performed with a subset of 96 F_3 families also evaluated for resistance in the field. Forty-eight

European wheat and spelt breeding lines (described by Siedler et al. 1994) that do not contain the *Lr35* resistance gene were used to validate the specificity of the molecular marker for *Lr35*.

Artificial infection of adult plants under controlled conditions

Seeds were germinated on wet Whatman paper 3MM to ensure regular germination. Rooting seedlings were transferred into plastic tubes filled with sand. The plants were grown in a growth chamber under strict hygienic regime to avoid contamination with other wheat pathogens. In the first week of growth, the light intensity was set to 150 µmol/m^2·s. Light intensity was increased continuously in the following two weeks up to 450 µmol/m^2·s. The photoperiod was 16h, with a day/night temperature of 19°C/15°C. Humidity was set permanently to 50%. 80 ml of a nutrition solution according to Hoagland (Jones, 1982) was given twice daily.

F_2 individuals were artificially infected at growth stage DC 51-55 (Zadoks et al. 1974) with leaf rust isolate 95502 avirulent for *Lr35* (kindly provided by Dr R. Park, University of Sydney, Australia). Urediospores were sprayed as a suspension with mineral oil «Soltrol 170» (Philips Petroleum, Paris) on the entire plant. After inoculation, plants were kept at 16°C and 100% humidity for 24 h in the dark. Ten days after inoculation (growth conditions: photoperiod 16 h, 19°C/15°C day/night temperature, 90 % relative humidity) plants were scored for the infection type on the flag leaves. A scale ranging from 0 to 4 (Roelfs 1984) was used to describe infection type (IT): ITs 0 (immune), ; (fleck), 1 (small uredinia with necrosis), and 2 (small uredinia with chlorosis) were considered as resistant, and ITs 3 (medium-size uredinia with or without chlorosis) and 4 (large uredinia without chlorosis) were considered as susceptible.

Scoring for leaf rust resistance in a field trial

A field trial was carried out in a field rust nursery (Haag, Switzerland) in 1998. Eleven seeds of each F_3 family chosen for linkage analysis were planted in 1.5 m rows. The field rust nursery was artificially inoculated with a mixture of 16

Puccinia recondita f.sp. *tritici* races prevalent in Switzerland. The parents Th*Lr35* and Frisal were also evaluated for resistance in the rust nursery. Host response data were recorded three times on the flag leaf. The F_3 families were classified as homozygous resistant, homozygous susceptible or segregating (resistant and susceptible individuals in a family) relative to Th*Lr35* and Frisal.

DNA isolation and RFLP analysis

For DNA isolation, plant material from F_3 families consisting of 11 plants was harvested and pooled. DNA isolation and Southern blotting were performed as described by Graner et al. (1990). Genomic DNA of Frisal, Th*Lr35* and Thatcher was digested with five restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I).

Southern hybridization was performed with 80 RFLP probes previously mapped on chromosome 2B. The cDNA and genomic DNA probes used were derived from wheat (FBA, FBB, PSR, TAM and WG), *Triticum tauschii* (KSU), barley (BCD) and oat (CDO). The probes CDO370 and WG996 were described by Heun et al. (1991). The probes BCD260, BCD1119, CDO405 are described in the Graingene data base (http://wheat.pw.usda.gov). The probe MWG950 was described by Graner et al. (1991). The probe PSR540 was mapped by Devos et al. (1993). Dr P. Leroy (INRA, Clermont-Ferrand and GIS Genoble Club, France) kindly provided the probes FBA199, FBA374, FBB4, FBB47 and FBB75. Probe KSUF11 and TAM18 were kindly provided by Dr B.S. Gill (Kansas State University, USA) and Dr G.E. Hart (Texas A&M University, USA), respectively.

Probe labeling with ^{32}P was performed with a labeling kit (Amersham, Switzerland) according to the manufacturer's instructions.

PCR amplification of wheat genomic DNA with specific primers

Polymerase chain reaction (PCR) was performed in a 25µl reaction volume. It contained 0.625 units of Taq DNA polymerase (Sigma, Switzerland), 1 x PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl 1.5mM $MgCl_2$, and 0.001% gelatin), 1mM of each dNTP, 10µM primers and 50ng of genomic DNA template.

Amplification was performed in a PTC-200 thermocycler (MJ-Research, Bioconcept, Switzerland) as follows: after one cycle at 94°C for 3 min, the reaction was subjected to 30 cycles at 94°C for 45 sec, 59°C for 45 sec and 72°C for 1 min. The extension of the amplified product was achieved at 72°C for 5 min.

Conversion of an RFLP probe to a STS marker

A *Bam*HI / *Hin*dIII fragment of 0.9kb of the probe BCD260 was subcloned. The resulting clone BCD260/0.9 was used as a RFLP probe in mapping analysis. This fragment was sequenced and the primers BCD260F1 (5' GAA GTT AAA GAG GTC TTG AC 3') and BCD260R2 (5' GAA GTA GTC CGC TAC CAC AG 3') were designed at each end of the cloned fragment (Microsynth, Switzerland). They were used for PCR amplification of genomic DNA isolated from Th*Lr35*, Frisal and the F_2 population. A CAPS (Cleaved Amplified Polymorphic Sequence) marker was developed by digesting the amplified fragments with the restriction enzyme *Dde*I (4bp recognition site, CTNAG). Ten units of restriction enzyme were added directly to the reaction after amplification. After 2h of digestion at 37°C, the fragments were separated on a 2% agarose gel.

The PCR fragments amplified between BCD260F1 and BCD260R2 (1.5 kb) were subcloned into the «pGEM-T Easy» vector (Promega, Switzerland) and sequenced. The specific STS primer 35R2 (5' TTT TGA GAA TCA GTC ATC AC 3') was designed in the insertion found in the Th*Lr35* allele. Visualization of the PCR product amplified by the primer combination BCD260F1 and 35R2 was done on a 1% agarose gel stained with ethidium bromide.

Linkage analysis

Linkage estimation was based on the recombination frequency r (defined as the summed frequency of recombination types among the total progeny). The recombination frequency was transformed to map units (centiMorgan) without using the Kosambi function. The recombination frequency was so low that we

estimated the probability of double crossing overs as zero and the recombination frequencies as additive.

Results

Segregation analysis of the F_2 population derived from the cross Th*Lr35* x Frisal

To map the *Lr35* resistance gene, 137 F_2 individuals derived from a cross between the line Th*Lr35* and the variety Frisal were scored for resistance. Evaluation of resistance was done after artificial infection at the adult stage under controlled conditions in a growth chamber. The infection type of the F_2 plants was either 0 - 1 (immune - small uredinia) like the resistant parent Th*Lr35* or 4 (large uredinia without chlorosis) like the susceptible parent Frisal (Fig. 5/1). Intermediate reaction types of heterozygous plants could not be distinguished (data not shown). To confirm the results obtained with the F_2 plants, additional resistance tests were performed in F_3 and F_4 generations. Out of 137 F_2 individuals, 103 F_2 individuals showed a resistant reaction whereas 34 F_2 individuals were susceptible. The 3:1 segregation (X^2: 0.0024) for a monogenically inherited gene confirmed the dominant action of the *Lr35* resistance gene.

A subset of 96 F_3 families (74 resistant plants, 22 susceptible plants) was randomly chosen and scored for susceptibility or resistance in a field trial. The progeny of the susceptible F_2 plants were homozygous susceptible in the field, whereas the progeny of the resistant F_2 plants were either homozygous resistant or segregating. Ninety F_3 families showed the same infection type as the F_2 individuals in the growth chamber. For six F_3 families no phenotypic data were obtained in the field. These results demonstrated that the phenotypic data generated in the growth chamber are identical to those obtained under field conditions. Genetic mapping was performed in this subpopulation as heterozygous F_2 individuals could be distinguished from the homozygous resistant plants by segregation in the F_3 families.

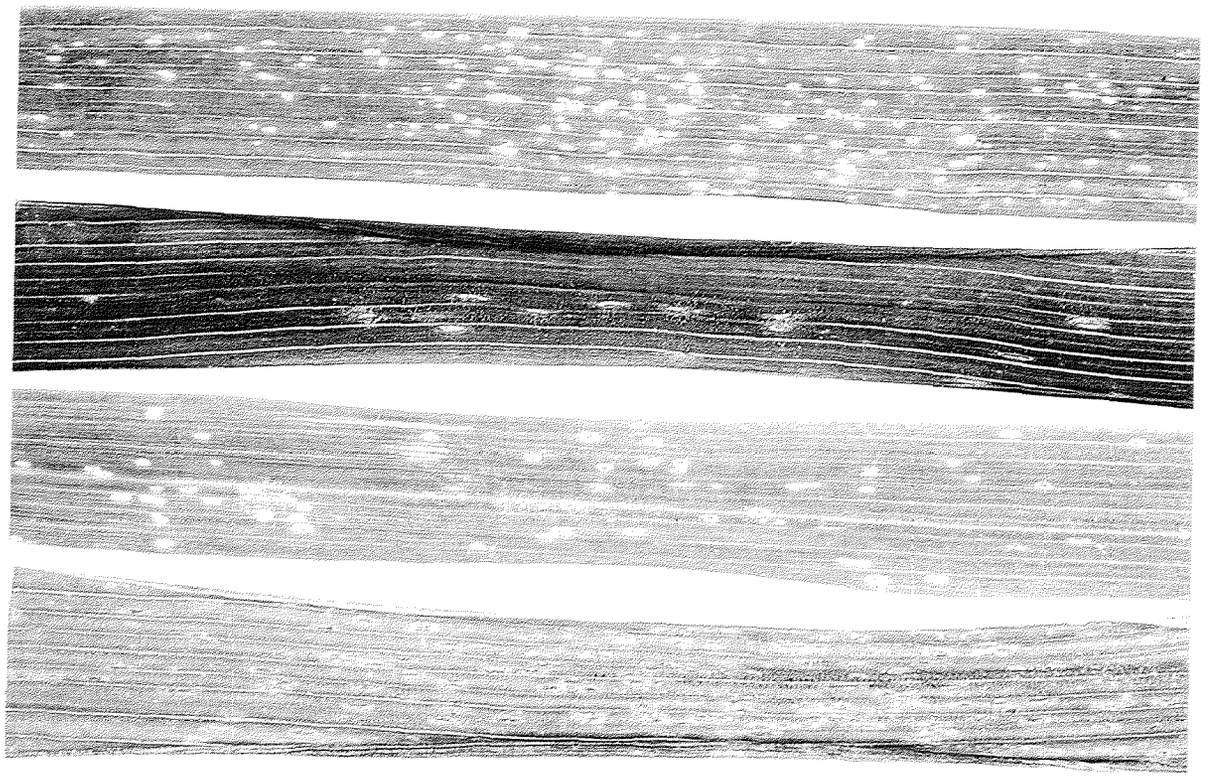

Figure 4.2/1: Flag leaves of wheat grown in the growth chamber 10 days after artificial infection with the leaf rust isolate 95502. Resistant parent Th*Lr35* (A), susceptible parent Frisal (B), resistant F_2 individual (C) and susceptible F_2 individual (D).

Genetic mapping of the *Lr35* gene

Eighty RFLP probes which were previously shown to be located on chromosome 2B were chosen for hybridization. Out of this set, 51 probes showed polymorphism between the two parents Th*Lr35* and Frisal. Fourteen probes which gave a clear hybridization pattern and were inherited as codominant markers, were mapped in a segregating population consisting of 90 F_3 families grown in the field. The 14 codominant RFLP probes formed one linkage group. Three probes BCD260/0.9, WG996 and PSR540 showed complete linkage to the *Lr35* resistance gene in the mapping population (Fig. 5/2). The probes FBB4 and MWG950 were located at 0.5 cM from the gene *Lr35*, whereas the probes FBA374 and TAM18 mapped at 0.5 cM on the opposite side of the resistance gene. The probes BCD1119, CDO370, CDO405 and FBA199 cosegregated at 1.1 cM from the resistance gene while the probe FBB47 was located on the other site of the *Lr35* gene at 1.1 cM. Thirteen of the probes used have already been mapped in the cross Synthetic x Opata (http://wheat.pw.usda.gov/ggpages/linemaps/Wheat/SxO_2.html) and covered a distance of approximately 39 cM (Fig. 5/2). In the cross Th*Lr35* x Frisal, the genetic region which was covered on chromosome 2B by these markers spanned only 3.3 cM, suggesting that recombination frequency was reduced.

Conversion of the RFLP marker BCD260 into a sequence-tagged-site (STS)

Hybridization with the probe BCD 260/0.9 resulted in a simple hybridization pattern with only three fragments in each parental line (Fig. 5/3). The polymorphic fragment of 4.3 kb and 4.5 kb in Th*Lr35* and Frisal, respectively, showed a complete linkage with the *Lr35* resistance gene.

The probe BCD 260/0.9 was sequenced. A primer was designed at each end of the fragment (BCD260F1/BCD260R2). PCR amplification with these primers on genomic DNA of both parents Th*Lr35* and Frisal resulted in non polymorphic amplification products of 1.5 kb. The larger size of the fragment amplified from wheat genomic DNA is due to the presence of an intron of 900 bp compared to the barley cDNA BCD 260/0.9 (data not shown). Digestion of the amplification

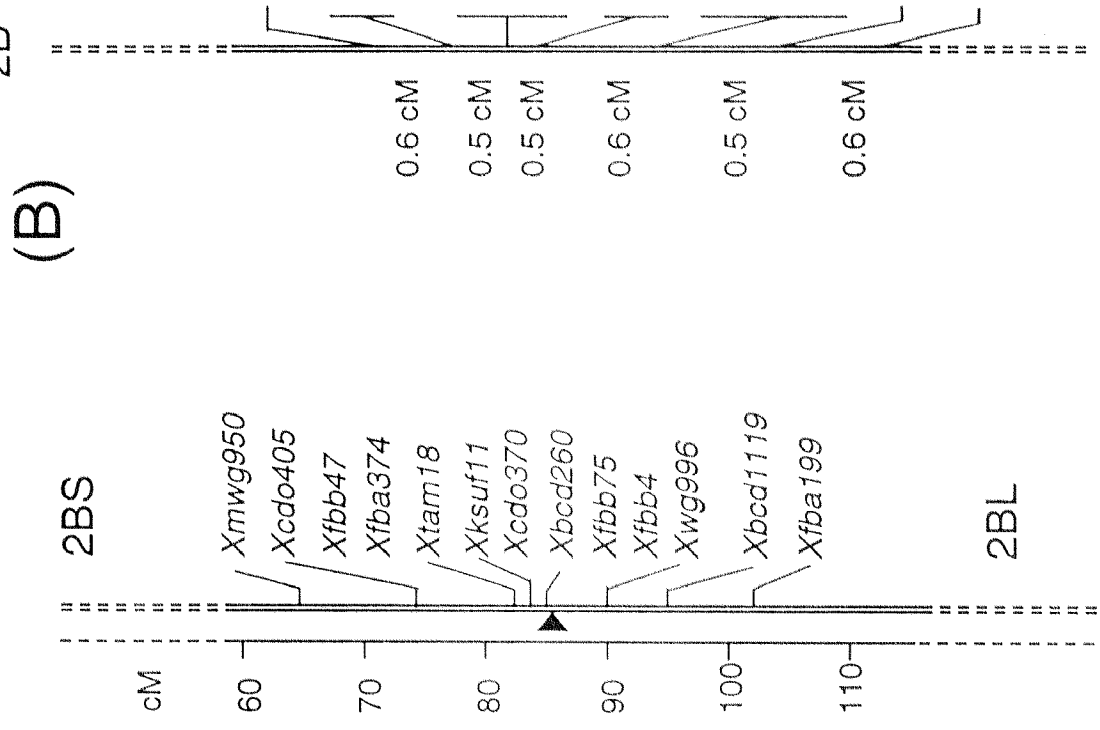

Figure 4.2/2: Genetic map of chromosome 2 B in a cross between Synthetic x Opata (A) and a cross between Th*Lr35* x Frisal (B). The arrowhead indicates the centromere of the chromosome 2B of Synthetic x Opata. The orientation of chromosome 2B in the cross Th*Lr35* x Frisal is not known.

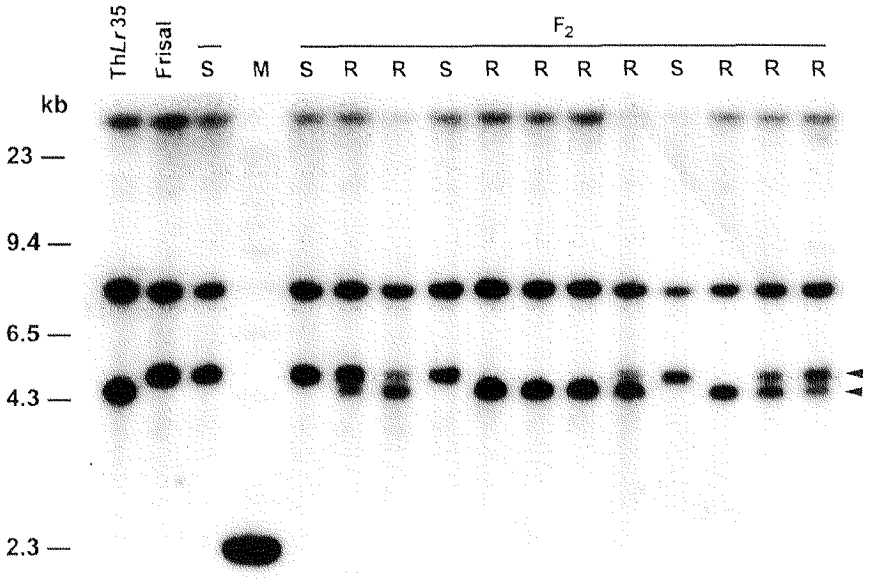

Figure 4.2/3: Southern hybridization pattern of *Xba*I digested genomic DNA with the probe BCD260/0.9. DNA was extracted from the Th*Lr35*, Frisal and the pooled F_3 progeny of 13 F_2 individuals from the segregating mapping population (R resistant phenotype, S susceptible phenotype). The molecular weight marker M is λ DNA digested with *Hin*dIII. The arrowheads indicate the polymorphic bands of 4.3 kb and 4.5 kb from the resistant and the susceptible alleles, respectively.

products with the restriction enzyme *Dde*I resulted in one additional fragment of 450 bp in Th*Lr35* (Fig. 5/4). This CAPS marker showed dominant inheritance in the mapping population and a complete linkage to the *Lr35* resistance gene.

The 1.5 kb fragments amplified with BCD260F1 and BCD260R2 from the parents Th*Lr35* and Frisal were subcloned and sequenced (Fig. 5/5). Five independent clones from the resistant line Th*Lr35* as well as from Frisal were analyzed. Two different types of sequences were obtained for Th*Lr35* which likely correspond to two homoeologous chromosomal locations of BCD260/0.9. One type of sequence derived from Th*Lr35* had an additional insertion of 29 bp at position 906 compared to the second type (Fig. 5/5). This insertion includes the *Dde*I restriction site which allowed the development of the CAPS marker. Thus we conclude that the sequence with the insertion corresponds to the resistance locus on chromosome 2B. Two different classes of sequences were also generated from genomic DNA of Frisal but none showed the 29 bp insertion (Fig. 5/5).

A specific primer (35R2) was designed from the 29bp insertion in Th*Lr35* (Fig. 5/5). In combination with primer BCD260F1 it led to the amplification of a single fragment of 0.9 kb in the resistant line Th*Lr35* whereas no product was amplified in Frisal. The 0.9 kb fragment was amplified from all the phenotypically resistant F_3 families whereas no amplification product could be obtained from the susceptible F_3 families (Fig. 5/6). Thus, this STS marker showed no recombination in the F_2 progeny and was completely linked with the resistance gene *Lr35*.

Validation of the CAPS and STS markers in wheat breeding material

A set of 48 European breeding lines derived from different breeding programs (Siedler et al. 1994) was used to test the specificity of the markers developed for the resistance gene *Lr35*. These breeding lines are known not to contain *Lr35*, as this resistance gene has not yet been exploited until now in wheat breeding programs. With the CAPS marker all the breeding lines showed the characteristic pattern of the susceptible variety Frisal (data not shown). The

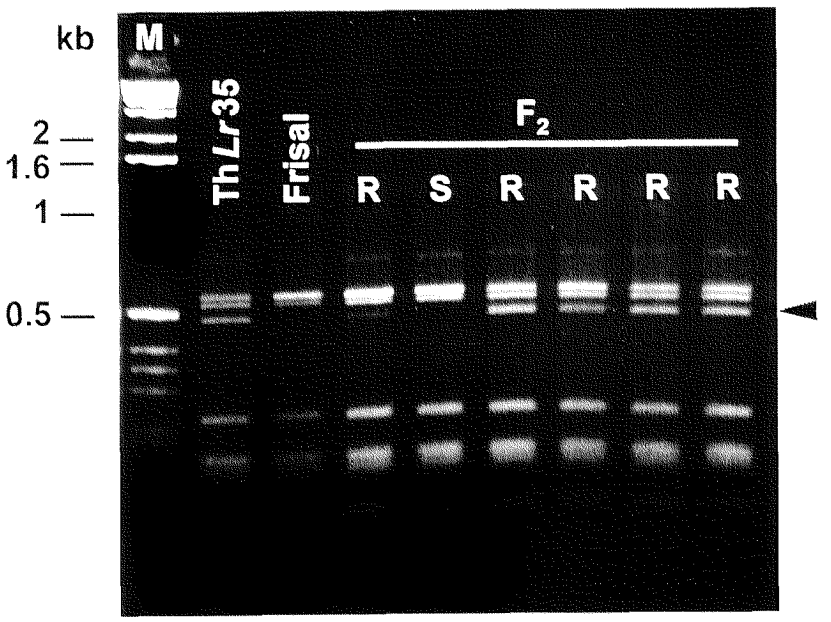

Figure 4.2/4: A CAPS marker for the *Lr35* resistance gene. PCR was performed on genomic DNA of Th*Lr35*, Frisal, five resistant (R) and one susceptible (S) F_2 individuals with the primers BCD260F1 and BCD260R2 followed by digestion with *Dde*I. The arrowhead shows the additional fragment of 450bp found in the resistant parent and the resistant F_2 individuals. The fragments were separated on an agarose gel.

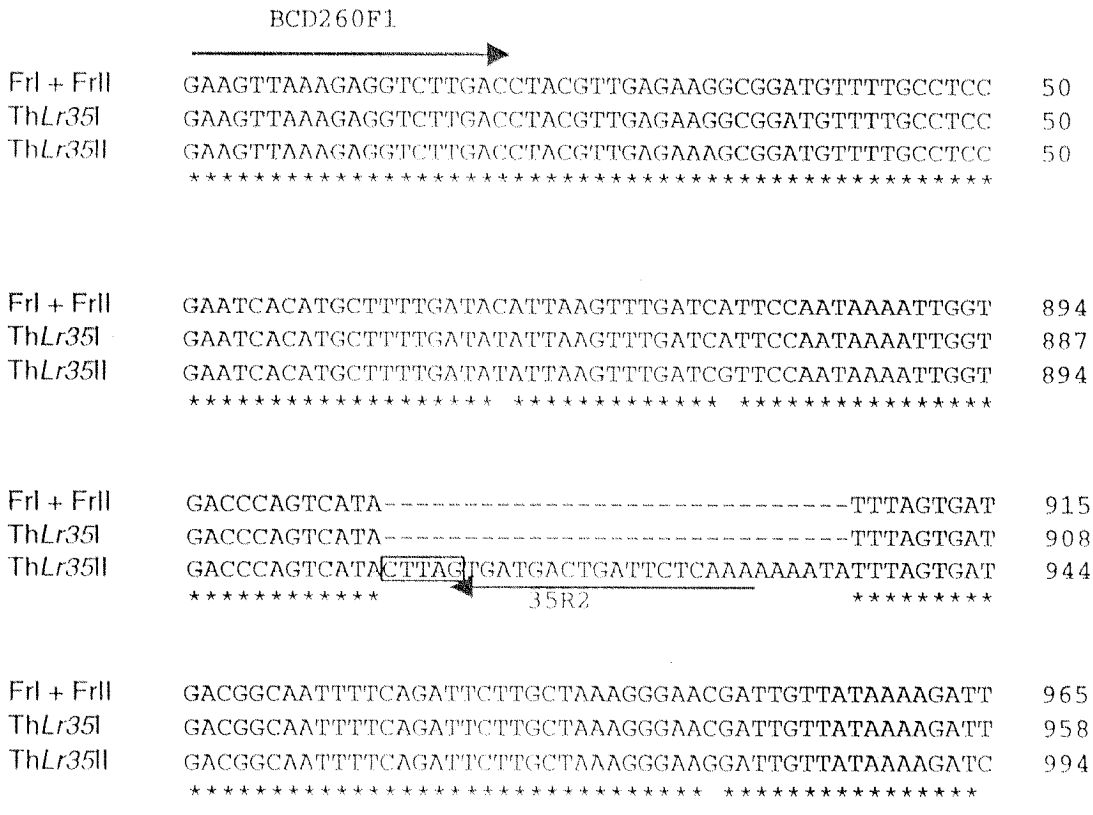

Figure 4.2/5: Nucleotide sequence comparison of the products amplified from genomic DNA of Frisal (Fr I and Fr II) and Th*Lr35* (Th*Lr35* I and Th*Lr35* II). Sequences were amplified with the primers BCD260F1 and BCD260R2. An additional 29bp was found in the Th*Lr35* II allele. The arrowheads indicate the primer sequences used to develop the dominant STS marker. The boxed nucleotides indicate the additional *Dde*I restriction site (CTNAG) in the allele on chromosome 2B of Th*Lr35*. Differences between the two classes of Frisal sequences are outside the DNA region shown in the Figure.

additional 450 bp fragment obtained with Th*Lr35* was not detected in any of these lines. Moreover, the use of the STS marker in the breeding lines did not result in any amplification product (Fig. 5/6, data not shown). We conclude that the two primers BCD260F1 and 35R2 are amplifying a specific sequence only in lines containing the *Lr35* resistance gene.

Discussion

Development of molecular markers for the detection of *Lr35*

In this work, the goal was to develop a molecular marker for the adult plant leaf rust resistance gene *Lr35* which is derived from a wild relative of wheat, *T. speltoides*. Linkage analysis showed that three codominant RFLP probes (BCD260, PSR540 and WG996) were completely linked with the resistance gene *Lr35*. A tight linkage of probes was expected due to the low level or complete lack of pairing and the reduced rate or absence of recombination between wheat and alien chromatin (Zeller and Hsam 1983; Dyck and Kerber 1985). Complete linkage between molecular markers and introgressed leaf rust resistance genes from alien species into wheat was already found for *Lr9* (Schachermayr et al. 1994; Autrique et al. 1995), *Lr19* (Autrique et al. 1995), *Lr24* (Autrique et al. 1995; Schachermayr et al. 1995; Dedryver et al. 1996), *Lr25* and *Lr29* (Procunier et al. 1995) and *Lr28* (Naik et al. 1998). In the case of the *Lr32* gene which was transferred from *T. tauschii*, the D Genome donor of wheat, recombination between the resistance gene and two RFLP markers was observed (Autrique et al. 1995). For *Lr35*, eleven RFLP markers showed a close linkage to the resistance gene. Between the most distant probes KSUF11 and FBB47 a genetic distance of 3.3 cM was found. This indicated that there was recombination between these loci as well as between these loci and the resistance gene. Interestingly, the probes which span 3.3 cM in the Th*Lr35* x Frisal cross are covering approximately 39 cM in the cross Synthetic x Opata. This shows that recombination is about 10 fold lower than in a cross between hexaploid wheat. These results suggest that pairing and crossovers between

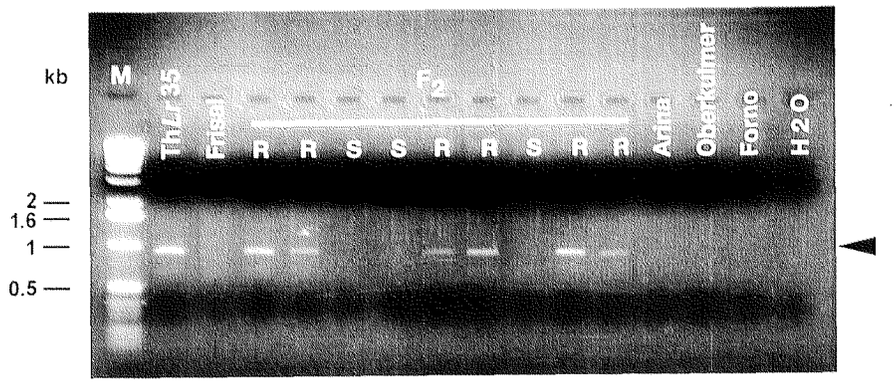

Figure 4.2/6: STS marker for the *Lr35* resistance gene. The amplification products from genomic DNA with the primer combination BCD260F1/35R2 are shown. The DNA was isolated from the resistant parent Th*Lr35* (R.L.6082), the susceptible parent Frisal, nine F_2 individuals (R resistant individual, S susceptible individual), two winter wheat varieties Arina and Forno and the spelt variety Oberkulmer. The arrowhead indicates the dominantly inherited DNA fragment of 931 bp, which showed a complete linkage with the resistance gene *Lr35*. The size marker (M) is the 1kb ladder.

chromosome 2B and fragment of chromosome 2S introgressed in Th*Lr35* are possible but at a reduced rate. This is in agreement with the hypothesis that the S genome of *T. speltoides* is the B genome donor of hexaploid wheat as previously suggested by Daud and Gustafson (1996) and Maestra and Naranjo (1998). With a *T. speltoides* specific probe, Daud and Gustafson (1996) detected a signal in the genome of tetraploid and hexaploid wheat. In contrast, signals in the genome of the *sitopsis* species, which have been proposed previously as B genome donor of wheat, were barely detected. Maestra and Naranjo (1998) showed that homoeologous pairing between chromosomes of *Triticum aestivum* and *T. speltoides* occurred. A pattern of preferential pairing of two types, A-D and B-S, confirmed that the S genome is very closely related to the B genome of wheat. When characterizing wheat-alien translocations by C-banding analysis, Friebe et al. (1996) could not determine the translocation breakpoint of chromosome 2B in the *Lr35* donor line R.L. 5711. The authors suggested that the translocation chromosome 2B was composed of several fragments derived from chromosome 2S of *T. speltoides* and that recombination could occur between the translocated parts. We found that the relative position of the 13 probes used in our analysis was not identical between the cross Synthetic x Opata and the cross Th*Lr35* x Frisal. A decreased recombination frequency and different arrangements of the fragments transferred from *T. speltoides* could explain the different order of the markers in the two maps.

Marker assisted selection for *Lr35*

RFLP markers are very reliable markers for plant breeding but as well labour intensive and expensive (Mohan et al. 1997). The high number of plants that has to be analyzed in plant breeding programs requires a rapid diagnostic assay. PCR provides a simple and fast screening method adapted to MAS (marker-assisted-selection). PCR-based STS markers, which identify a short and unique sequence at a known genetic locus are reliable and efficient diagnostic tools. STS markers have been successfully used to detect polymorphisms in cereals (D'Ovidio et al. 1990; Weining and Langridge 1991;

Williams et al. 1991; Tragoonrung et al. 1992; Talbert et al. 1994; Schachermayr 1994, 1995).

One of the probes (BCD260/0.9) which showed complete linkage with the *Lr35* resistance gene was converted into two different PCR-based markers: CAPS and STS markers. Although the CAPS marker is a PCR-based marker, an additional step for restriction enzyme digestion makes it less practical than the STS marker which only needs a PCR reaction. The insertion of 29 bp in Th*Lr35* with the additional *Dde*I restriction site allowed to convert the RFLP marker into a STS marker. Thus, large populations in breeding programs can now be screened for presence or absence of *Lr35* in a fast and easy way. Since no STS could be amplified from all the European breeding lines we have tested here, we conclude that this STS marker is highly specific for the *Lr35* resistance gene.

The pyramiding of different resistance genes can be supported by the use of molecular markers in classical breeding programs. In the case of the resistance gene *Lr35,* a combination with other genes should be achieved. No virulence has been reported in Canada, South Africa, Australia and Switzerland (Kerber and Dyck 1990; Kloppers and Pretorius 1995; Kolmer 1997; RF Park, personal communication, own data) until now. The use of this gene as a single resistance source would certainly lead to the emergence of virulent races which would overcome the resistance in a short time. With the help of the STS marker, the combination of *Lr35* with other leaf rust resistance genes which are active at the seedling and/or the adult stage should allow a more efficient breeding for durable resistance against this disease.

6. General Discussion

6.1 Marker types and marker-assisted selection

A first map of the human genome based on molecular markers (Botstein et al. 1980) was the catalysator for the development of genomic maps in other organisms. Polymorphisms that were detected in genomic DNA of different plant varieties paved the way for the development and use of molecular markers in plant breeding. Unlike isoenzymes, their number is nearly unlimited, their expression is not necessary for their detection and all markers can be detected with a single technique (Melchinger 1990). The increasing number of marker techniques has resulted in a large number of markers for agronomically important traits in nearly all crops. Molecular markers have been used to tag useful traits for breeding and also for map based cloning strategies. Restriction Fragment Length Polymorphism (RFLP), microsatellites, Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) were used to generate molecular markers.

PCR-based markers like STS markers or microsatellites are the most convenient marker types to be used in plant breeding due to their requirement of low amounts of DNA, their fast application and reliability as a selection tool. In maize the usefulness of microsatellites has already been shown (Ribaut et al. 1997). Röder et al. (1998) and Korzun et al. (1999) claimed that microsatellites are an excellent source of molecular markers for the mapping of agronomically important genes, due to their high rate of polymorphism, the even distribution over the genome and the possibility to automate the analysis of large plant numbers. The wheat microsatellites used in our study showed a high level of polymorphism between the distant parents Th*Lr13* and Frisal. GWM630 turned out to be the closest linked marker for the resistance gene *Lr13*. Another type of PCR-based marker (STS) has been shown to be a reliable and efficient marker type to detect wheat leaf rust resistance genes (Schachermayr et al. 1994, 1995). The STS marker we have developed for the resistance gene *Lr35*

in wheat is ready to be introduced into breeding schemes. As it is a dominant marker, direct staining and visualization of the PCR product through ethidium bromide can eliminate the need for electrophoresis.

A new promising technique to generate more molecular markers was developed independently by Michales and Amasino (1998) and Neff et al. (1998). This technique represents a modification of the CAPS (Cleaved Amplified Polymorphic Sequence) analysis and is called dCAPS (derived Cleaved Amplified Polymorphic Sequence). A point mutation has to be found between two parents which does not generate a novel restriction site. The different sequences are amplified by using primers which incorporate a mutation into the amplification products which together with the single nucleotide polymorphism creates a new restriction site in one of the alleles. The products are afterwards digested with the appropriate restriction enzyme and separated by gel electrophoresis. It was already shown that this new technique works in *Arabidopsis thaliana* (Michales and Amasino, 1998; Neff et al. 1998) but so far there are no such reports in agronomically important crops. The disadvantages of CAPS and dCAPS methodology is the requirement of an additional restriction enzyme digest of the PCR product.

Conventional plant breeders adopt breeding methods which increase their breeding efficiency but are conservative when making methodological changes. In a small survey of wheat programs having unrestricted access to new biotechnological methods, few research programs and no main wheat breeding program routinely used MAS or quantitative trait loci (QTL) (Braun et al. 1998). An exception is the wheat breeding program of the SARDI (South Australian Research and Development Institute, Australia), where 7000 RFLP marker assays are made per year (Dr. K. Williams, ITMI meeting 1999, personal communication). Depending on the kind of molecular markers used in MAS, differences between the type of application, the speed of detection and the costs exist. Each marker type has its advantages and drawbacks which make it more or less applicable in practical plant breeding where large numbers of plants have to be tested.

The main question which has to be solved for MAS is where in the classical breeding it should be integrated. Classical plant breeding is divided into four different categories, depending on the kind of reproduction. There are clonal plants which are vegetatively propagated, lines which are self fertile, populations which are open pollinating and hybrids which are selectively cross-pollinated. All categories have a common breeding scheme: each breeding method is consisting of three phases. (I) The first and most important step is to provide the basic variation. (II) The second part consists of the selection of the potential parents of the variety and (III) the testing of the selected lines. In each phase the application of molecular markers is possible and useful. Here, the application of MAS for self pollinating species such as wheat or barley is considered. First of all, the identification of elite lines with the best allelic complementarity has to be done before crossing. Molecular characterization could simplify the decision which lines should be crossed. Molecular markers can also be used as a predictive tool to reduce the number of necessary crosses. In the second phase, markers can be applied in one key selection step to maximize their impact. An early stage of recombination which could be the segregating F_2 generation would be such a critical step. The application of some markers in the first segregating population would fix already in this stage the desired traits whereas the rest of the genetic variance is not reduced. The number of progenies from a cross can already be reduced in this way, which is crucial as space and time for field trials is often limited. In the third phase of plant breeding, molecular markers could be used as diagnostic tools, when the allelic value has already been identified. The application of molecular markers in this step would ensure that the wanted genetic composition of the new line is correct. Also other traits could be detected with different molecular markers to predict the genetic value which was not selected for and therefore characterize the line precisely as a potential new crossing partner.

6.2 Allelism of resistance genes on chromosome 2BS and cloning of these genes

The three leaf rust resistance genes *Lr13*, *Lr23* and *Lr35* are located close to the centromere of chromosome 2B (McIntosh et al. 1995, Seyfarth et al. 1999a,b). *Lr13* and *Lr23* are very closely linked (McIntosh et al. 1995). The relationship between *Lr13* and *Lr35* as well as between *Lr23* and *Lr35* is not known. The resistance gene *Lr35* was derived from *Triticum speltoides* and could be allelic to *Lr13* or *Lr23* which were derived from the primary wheat gene pool. It was shown in the case of the resistance gene *Lr22* that two alleles came from distinct sources. One originated from hexaploid wheat (*Lr22b*) and the other one from the diploid *Triticum tauschii* (*Lr22a*) (McIntosh et al. 1995). With our current knowledge, we can not exclude that *Lr13*, *Lr23* and *Lr35* are alleles of the same gene.

The cloning of the two genes *Lr13* and *Lr35* is not feasible at the moment. In the case of *Lr13* closely linked and flanking markers, the prerequisite of map-based cloning, are still missing. *Lr35* does not derive from hexaploid wheat but is located on a translocated chromosome fragment derived from *T. speltoides* which shows a highly reduced recombination rate. Thus, the close linkage between the linked marker and the gene does not necessarily reflect the physical distance between them. Moreover, the large insert libraries that have recently been constructed are for the A genome (*T. monococcum*, Lijavetzky et al. 1999) and the D genome (*T. tauschii*, Moullet et al. 1999). So far, no library is existing for the B genome, a related species or hexaploid wheat.

6.3 Breeding for resistance

The ecological and economical impacts of agrochemistry have caused a shift of emphasis from chemistry to biology in plant production. Since genetic disease resistance is the most effective, economical and prophylactic way of plant protection, research on breeding for resistance and the cultivation of resistant

varieties is taking over priority to research on chemical pesticides (Wenzel, 1997). Although resistant cultivars will always be the best method for controlling leaf rust disease, an effective chemical control would be valuable for situations when new races of leaf rust develop and new resistant cultivars are not available. Chemical control has been successfully used in Europe where prices for wheat are supported and where high yields are possible (Buchenauer, 1982). Chemicals can be applied when they are needed and there is no necessity to monitor the rust population except for appearance of the pathogen in the field. Resistance breeding would be unnecessary if the control of fungal pests was exclusively done by application of fungicides. Efforts in breeding have only been made to increase yield and quality (Roelfs et al. 1992). In the recent years, however, the disadvantages of applying fungicides such as costs for the farmer and the possible negative impact on the environment have become apparent. Using fungicides to protect susceptible cultivars against leaf rust attack is no guarantee to prevent yield losses. Many other fungal pathogens have developed resistance to chemicals and this may occur with the rusts as well (Roelfs et al. 1992). In addition, most available fungicides provide inadequate control on susceptible cultivars when environmental conditions are favorable for disease development. Although the application of fungicides has clear advantages, the disadvantages make breeding for resistance an attractive alternative.

Depending on the crop, the acreage occupied and environmental conditions different breeding goals and breeding strategies are existing. Concerning the breeding to achieve durable resistance, a basic question rises: should breeding focus on major genes or on minor genes (QTLs)? Breeding for resistance with single major resistance genes has been shown in exceptional cases to be effective, long lasting and to prevent the appearance of new races. In a crop which is only grown on a few hectares in one region the pressure to a biotic pathogen is not so high to select for new, virulent races. For example, in the Netherlands, flax is a minor crop and all the cultivars carry monogenic, race-specific resistance genes to flax rust. Since 1962 these genes have protected

the flax crop effectively. Four cultivars were grown for periods between 16 and 20 years without loss of resistance. The same type of genes appeared to be non-durable in the USA, especially in the period 1930-1950 when flax was an important crop (Parlevliet, 1997). In general, single race-specific resistance genes are overcome after their introduction into breeding material and release to the fields. Of course, the durability of single resistance genes can vary. It can last from zero years, when the resistance is neutralized already in the last stages of the breeding program, to over 130 years as for instance with the woolly aphid resistance in some apple cultivars (Parlevliet, 1997). In the case of rust resistance genes in wheat, the effectiveness of single resistance genes was on the average less than 10 years (Kilpatrick, 1975). The APR resistance gene *Lr13* as a single gene is not sufficient anymore to protect a line from leaf rust attack (McIntosh et al. 1995). It is, however, still an important gene in resistance breeding as it is often involved in gene combinations (McIntosh et al. 1995) leading to enhanced resistance. In contrast, the introgressed resistance gene *Lr35*, which derived from *T. speltoides*, is still protecting lines from leaf rust attack. Until now, this resistance gene is not exploited in plant breeding (McIntosh et al. 1995). Therefore, it is a question of time until this resistance gene is overcome by the pathogen when it is introgressed as a single gene.

Until recently, small-grain breeders concentrated on the use of single race-specific genes for resistance to the cereal rusts (Mundt and Browing, 1985). This breeding strategy was always very attractive to breeders as the incorporation of single genes into elite lines confers complete resistance and was easy to handle in breeding programs. In the long term, it is not a promising approach to use these resistance genes individually, because one resistance gene after another has to be incorporated successively into the plant to overcome the problem of pathogen adaptation to monogenic resistance. Considering that the number of useful resistance genes is limited, they should be employed carefully (Dyck and Kerber, 1985). Several possible ways to prevent the breakdown of resistance genes exist: (I) through a guided distribution in space and/or time (gene deployment at the regional level, gene-

for-gene use, multilines) or (II) a combination of several resistance genes in one cultivar. Gene deployment is not practicable, because it would require good agreement among and discipline of the farmers and breeders (Roelfs et al. 1992). It was proven that multilines work under great disease pressure (Fischbeck, 1997). Why did not everyone try to develop multilines? One answer is that the variety selected for the recurrent parent may be the best agronomic variety when the program is started, but probably will not be by the time the backcrossing is finished (Knott, 1989). Another possible risk is that many genes are individually exposed to the pathogen population, which probably increases the risk of selection a super virulent race against all the resistance genes used (Roelfs et al. 1992).

The combination or pyramidisation of several resistance genes against the same pathogen appears to be another solution. It is assumed that two or three effective major genes together in the same cultivar are a barrier that is not easily overcome by the pathogen as two or three simultaneous changes in the virulence are needed (McIntosh and Brown, 1997). Several winter wheat cultivars remained resistant to yellow rust for periods of 15 years or longer because of such multiple gene barriers. The resistance became ineffective because the resistance genes were also used separately in other cultivars, enabling the pathogen to increase its complexity stepwise. In all these cases the multiple barrier was obtained by accident (Parlevliet, 1997). It was shown in leaf rust that several gene combinations exist which are supposed to be durable. Among these combinations, the resistance gene *Lr13* plays a major role. (Samborski and Dyck, 1982; Ezzahiri and Roelfs, 1989). The combination of resistance genes can not always be tested via classical virulence tests with leaf rust isolates due to the lack of virulence of some isolates. Marker-assisted selection can overcome this problem. In this case, the presence of several resistance genes in one line could be already detected at the molecular level. The use of molecular markers in wheat breeding would help to prevent the pathogen to overcome the combined resistance genes. In the case of the non-exploited single resistance gene *Lr35*, the application of our new molecular marker with markers for other genes would guarantee that this effective gene is

not introgressed alone in an elite breeding line. Resistance genes would remain effective for a long time and contribute to durable resistance. Therefore, breeding with major resistance genes only make sense in terms of pyramiding these genes. Until today, 17 molecular markers for different major leaf rust resistance genes have been developed (Tab. 6/1). With these markers, the effective combination of several resistance genes in advanced breeding lines should be possible.

Where no molecular markers exist or gene pyramiding fails, breeding should focus on quantitative resistance. Also with this strategy, however, there are some problems:

- Not all quantitative resistances are durable. There are race-specific, non-durable, major genes with an incomplete expression such as the *R10* resistance gene in potato against late blight (Turkesteen, 1993).
- In pathosystems such as wheat/leaf rust, many cultivars carry one or more major genes that are partially overcome because the pathogen population consists of a mixture of races corresponding to the various resistance genes in the cultivars grown. In breeding lines in the experimental fields this shows up as quantitative resistance.
- In the presence of fully effective, non-durable major genes quantitative resistance is masked (Parlevliet, 1997).

These problems of breeding for horizontal, quantitative resistance can be overcome by:

- A selection strategy which is eliminating all fully resistant plants after infection. This selection discards all major resistance genes and resistance selected in this way would probably be polygenic (Knott, 1989).

Table 6/1: Molecular markers for different leaf rust resistance genes in wheat and the source of the resistance genes.

Gene	Source	Marker	Reference
Lr1	*T. aestivum*	RFLP/STS	Feuillet et al. 1995
Lr3	*T. aestivum*	RFLP	Sacco et al. 1998
Lr9	*Ae. umbellulata*	RFLP/STS	Schachermayr et al. 1994 Autrique et al. 1995
Lr10	*T. aestivum*	RFLP/STS	Schachermayr et al. 1997
Lr13	*T. aestivum*	Microsatellite	Seyfarth et al. 1999b
Lr18	*T. timopheevi*	N band	Yamamori, 1994
Lr19	*Thinopyrum*	Isoenzyme RFLP	Winzeler et al. 1995 Autrique et al. 1995
Lr23	*T. turgidum*	RFLP	Nelson et al. 1997
Lr24	*A. elongatum*	RAPD/RFLP	Schachermayr et al. 1995 Autrique et al. 1995 Dedryver et al. 1996
Lr25	*S. cereale*	RAPD	Procunier et al. 1995
Lr27	*T. aestivum*	RFLP	Nelson et al. 1997
Lr28	*T. speltoides*	RAPD/STS	Naik et al. 1998
Lr29	*A. elongatum*	RAPD	Procunier et al. 1995
Lr31	*T. aestivum*	RFLP	Nelson et al. 1997
Lr32	*T. tauschii*	RFLP	Autrique et al. 1995
Lr34	*T. aestivum*	RFLP	Nelson et al. 1997
Lr35	*T. speltoides*	RFLP/STS	Seyfarth et al. 1999a
Lr47	*T. speltoides*	RFLP	Dubcovsky et al. 1998

- Linkage with other, more easily detectable traits should be used. E. g., the stem rust resistance gene *Sr2* gives incomplete resistance and it is partially effective since 60 years. It is linked with head and stem melanism (false black chaff) and can be easily selected at the phenotypic level (Van Ginkel and Rajaram, 1993). Another example would be the leaf rust resistance gene *Lr34* which shows also incomplete resistance and is linked with leaf tip necrosis (Singh, 1992).
- The application of molecular markers to select indirectly for the desired QTLs via MAS.

6.4 Breeding for resistance or tolerance?

Resistance is simply a state of "less disease" and "no disease" is rarely a realistic objective. Four kinds of resistance can be distinguished: (I) Major gene, race-specific, vertical resistance, non-durable; (II) polygenic, race-non-specific, horizontal resistance, durable; (III) race-non-specific, major gene resistance and (IV) interaction or mixture of the cited resistance mechanisms (Simmonds, 1988). No resistance mechanism lasts forever, but there are large differences in the durability of these mechanisms (Parlevliet, 1997). Together with enhanced quality and yield, breeding for resistance is still the most important breeding goal. To breed for increased tolerance is an alternative strategy to reduce yield losses.

The term tolerance is often used incorrectly for partial or incomplete resistance. According to Durbin (1984) tolerance can be defined in plant protection as the ability of a plant to yield more than would normally be expected considering the amount of disease present. Under this general heading a number of diverse physiological phenomena are grouped that contribute to disease tolerance in different ways. Some of them involve interactions with the mechanisms governing translocation of photosynthates. In some cases tolerance appears to be due to the host's ability to continue to "fill" the developing grain in spite of a moderate to high number of pustules of an infection type that ordinarily would

categorize the cultivar as susceptible. In some cultivars the flag leaf, glumes and/or awns contribute substantial amounts of photosynthates to the developing grain. Because of their proximity to the developing grain, they develop very strong source-sink relationships with the grain (Durbin, 1984). More emphasis needs to be placed on exploiting this important type of tolerance. The genetics of tolerance to disease is not understood; neither its relationship, if any exists, to horizontal resistance. The mechanisms of tolerance appear to be independent of the mechanisms of resistance, but similarities are evident (Heitefuss, 1997). Tolerant plants probably exist in most host-parasite combinations (Agrios, 1997).

Thus, breeding for tolerant plants, either for biotic or abiotic stress, should also be an important and interesting goal to achieve high yield and quality under suboptimal growing conditions. In the future, this potential breeding goal could become more important, as single resistance genes in the different gene pools are not available in unlimited numbers.

7. Outlook

Plant breeding is widely recognized for making a substantial contribution to long-term improvement of agricultural productivity. With the help of molecular biology it became possible to shift conventional plant breeding from "art" to "science". It is clear that the use of MAS will not replace the classical breeding process but will support it. Genetic engineering and biotechnology can help classical plant breeding by providing new and novel sources of variation, speeding up the breeding cycle, increasing the efficiency of selection and simplifying the work in the field nursery. Screening of large numbers of plants coming up in breeding programs could be done on the molecular level. Traits could be evaluated independently from climatic or environmental conditions. Therefore, genetic markers could potentially be a valuable tool in accomplishing many plant breeding objectives. Marker-assisted breeding is already showing its value in breeding programs such as introgression of traits from wild species or the combination of different genes in one line. But the impact of molecular biology does not stop at the applied level. The contribution lies as well on the genetic analysis as a first requirement for cloning economically important genes. Once genes are isolated it will be possible to develop a much greater understanding of gene structure and function and to relate this to plant phenotype. This, in turn, will then lead to genetic manipulation at the molecular level to produce new alleles and allelic combinations.

The STS marker BCD260F1/BCD260R2 for the APR gene *Lr35* is ready to be used in MAS. The resistance gene *Lr35* is a very valuable gene as it shows resistance to all leaf rust races. The use of this gene as single source of resistance in a line would most probably be short-lived, because new virulent leaf rust races would evolve. As the number of resistance genes is limited, the careful use of *Lr35* to ensure its long effectiveness is of crucial importance. Therefore, the strategy of combining other resistance genes with *Lr35* should be applied. As we have developed and published a STS marker for *Lr35*, pyramiding of different leaf rust resistance genes should be possible, at least at breeding stations that have the financial and technical resources.

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