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GENETIC ANALYSIS OF CROWN RUST RESISTANCE IN RYEGRASSES (*LOLIUM* SPP.) USING MOLECULAR MARKERS

GENETISCHE ANALYSE VAN KROONROESTRESISTENTIE IN RAAIGRASSEN (*LOLIUM* SPP.) DOOR MIDDEL VAN MOLECULAIRE MERKERS

door

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Variatie maakt het leven boeiend.

Voorwoord

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
ATP	Adenosinetriphosphate
BC	Back-cross marker
BCD	Prefix of RFLP probes isolated from barley
bp	Base pairs
BSA	Bulk Segregant Analysis
CAPS	Cleaved Amplified Polymorphic Sites
cDNA	Complementary DNA
CDO	Prefix of RFLP probes isolated from oat
CET	Conditional Empirical Threshold
CI	Confidence Interval
CIM	Composite Interval Mapping
cM	Centimorgan
CSU	prefix of RFLP probes isolated from maize
CTAB	N-cetyl-N,N,N-trimethylamoniumbromide
DIAS	Danish Institute of Agricultural Sciences
DH	Doubled haploid
DTT	dithiothreitol
EGRAM	European *Gramineae* Mapping project
EST	Expressed Sequence Tag
F	Forward
HD	Heteroduplex
HR	Hypersensitive Reaction
IBF	Prefix of RFLP probes isolated from *Festuca*
IGER	Institute of Grassland and Environmental Research
ILGI	International *Lolium* Genome Initiative
IM	Interval mapping
INRA	Institut National de Recherche Agronomique
LD	Linkage Disequilibrium
LG	Linkage Group
LOD	Likelihood of the odds
MAS	Marker Assisted Selection
Mire	Powdery mildew resistance gene in wheat
Mla	*Powdery mildew resistance gene in barley*

MQM	Multiple QTL mapping
NBS	Nucleotide Binding Site
PAA	Poly-acryl amide
PC	Primer combination
PCR	Polymerase Chain Reaction
PRI	Plant Research International
PSR	Prefix of RFLP probes isolated from wheat
QTL	Quantitative Trait Loci
R	Resistant or Reverse
RAPD	Random Amplified Polymorphic DNA
REC	Recombination frequency
RET	Residual Empirical Threshold
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance Gene Analogue
RGC	Prefix of RFLP probes isolated from rice
RGG	Prefix of RFLP probes isolated from rice
RGR	Prefix of RFLP probes isolated from rice
RH	Relative Humidity
RZ	Prefix of RFLP probes isolated from rice
S	Susceptible
SD	Standard Deviation
SNP	Single nucleotide polymorphism
SSC	Saline sodium citrate buffer
SSCP	Single strand conformation polymorphism
SSR	Simple Sequence Repeat
STS	Sequence Tagged Site
TBE	Tris-borate-EDTA buffer

Content

LIST OF ABBREVIATIONS
CONTENT

Chapter 1

General introduction

1.1 Ryegrasses

Grasslands occupy about 52 million ha in Europe. On at least 12 million ha, ryegrass cultivars (*Lolium* spp.) are used (http://europa.eu.int/comm/agriculture/envir/report/en/). *Lolium* spp. are important forage species, used mainly as pasture, hay crop or ground cover. They are either grown alone or in mixture with legumes such as clovers (Prine, 1991; Reed et al., 2000).

The *Lolium* spp. belong to the family *Poaceae*, subfamily *Pooideae* and tribe *Poeae*. *L. perenne* and *L. multiflorum* are two of the most important species belonging to the genus *Lolium*. They are cool-season perennial bunchgrasses native to Europe, temperate Asia, and North Africa but are widely distributed throughout the world, including North and South America, Europe, New Zealand, and Australia. High palatability and digestibility make these species highly valued for dairy and sheep forage systems (Balasko et al., 1995; Hannaway et al., 1999a & b).

Perennial ryegrass or *L. perenne* is important in forage/livestock systems. It has a high yield and fast establishment potential. It has relatively good resistance to drought stress, is winter hard and persistent. As a result, it is often the preferred forage grass in temperate regions of the world. *L. multiflorum* or Italian ryegrass has lower stress resistance and lower persistence than *L. perenne* but has a higher yield potential. It is an important short-duration grass. It is used in many environments when fast cover or quick feed is required. Both species can be crossed and result in a hybrid called *L. x boucheanum*, which shows intermediate characteristics such as intermediate persistence and intermediate yield (Hannaway et al., 1999a & b; Reed et al., 2000).

1.2 Ryegrass breeding

Ryegrasses are obligate out-breeders with a gametophytic self-incompatibility system. As a consequence the breeding programs for ryegrasses involve the

recurrent selection of multiple parental clones. Selected clones are polycrossed and generate synthetic varieties (Reheul and Ghesquiere, 1996; Van Bockstaele, 1998; Forster et al., 2001). This breeding scheme is an effective way of recombining desirable genes in an out-breeding gene pool to give sufficiently uniform cultivars based on diverse genetic resources. Over the last fifty years, great advances have been made in ryegrass cultivar development. Traditional forage grass breeding programs have succeeded in improving a wide range of valuable traits, by exploiting a wide range of genetic resources, by recombining and selecting new genotypes, by polyploidisation, … (Hayward, 2000).

As a result of the breeding scheme described above, synthetic populations are genetically heterogeneous and show a high degree of developmental buffering capacity. However, this hampers the rapid improvement of ryegrass varieties. Much of the breeding so far has relied on the accumulation of additive gene effects in synthetic varieties with little exploitation of the non-additive genetic variation associated with heterosis (Humphreys, 2000). It is expected that within the next few years, the application of current developments in molecular breeding will enhance the efficiency and effectiveness of traditional breeding methods and objectives.

Reproducible and efficient genetic transformation protocols are available for *Lolium* spp. (Forster and Spangenberg, 1999). This opens up opportunities to evaluate novel approaches to ryegrass improvement. However, due to the outbreeding system and the fact that grasses are a major component of natural ecosystems in many parts of the world, the release of genetically modified grasses is of public concern (Hayward, 2000).

A second development in molecular breeding, which can be of use in breeding programs is marker technology. The incorporation of genetic markers into selection indices will help to increase the efficiency of multi-trait selection, avoid undesirable correlated responses and identify components of specific and general adaptation (Humphreys, 2000).

1.3 Marker technology in ryegrasses

Use of markers in applied breeding programs can range from facilitating appropriate choice of parents for crosses, to mapping/tagging gene blocks

associated with economically important traits. Gene tagging and Quantitative Trait Loci (QTL) mapping in turn permit Marker Assisted Selection (MAS) in backcross, pedigree and population improvement programs. Plant numbers required to obtain the desired segregants can be readily predicted, allowing structured plant breeding programs to be designed. MAS appears especially useful for crop traits that are otherwise difficult or impossible to deal with by conventional means (Hash et al., 2000).

Two major applications of molecular markers are currently being developed in ryegrasses: DNA profiling and MAS. The first involves the use of markers for the evaluation of genetic diversity and for cultivar identification. DNA profiling can be used, among others, to select genetically divergent parents, to evaluate the dynamics of population structure in natural and managed pastures, or to distinguish cultivars (Huff, 1997; Forster et al., 2001; Kubik et al., 2001; Roldán-Ruiz et al., 2001; Wilkins et al., 2002). The second area of application involves the use of markers to dissect the genetic complexity of key agronomic characters identified by breeders (Forster et al., 2000; Humphreys et al., 2000). In this case, the genetic factors controlling the trait are identified by linkage mapping or association studies and associated markers are subsequently used for marker assisted selection.

In contrast to important agricultural crops such as rice, wheat or maize, molecular research in ryegrasses has lagged behind during years. Hayward et al. (1994) were the first to publish a linkage map for ryegrasses. This map was based on restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNAs (RAPDs) and isozyme markers. Later on, more efficient PCR-based marker techniques such as amplified fragment length polymorphisms (AFLPs), sequence tagged sites (STS) and simple sequence repeats (SSRs) became available also for ryegrass and this led to the construction of more advanced linkage maps. Currently, a *Lolium* reference map (the ILGI – International Lolium Genome Initiative- map) containing RFLP, SSR, AFLP, isozyme and STS markers is publicly available (Jones et al., 2002a). Although this map represents an important tool for the understanding of the ryegrass genome and for the analysis of the syntenic relationships with other grass species, the challenge at this moment is to identify the genomic regions involved in the determination of agronomic traits, in order to either implement MAS-programs or to isolate the genetic factors responsible.

1.4 Crown rust

In this study, molecular markers are used to analyze a specific trait of relevance in *Lolium* breeding. We have chosen crown rust resistance, as this is a major selection criterion in current breeding programs. Hayward (1977) studied resistance to crown rust in perennial ryegrass, by means of a diallel cross, in a series of natural populations. The narrow sense heritability was 58%. Comparable narrow-sense heritability values were also reported by Reheul & Ghesquiere (1996). They evaluated parental clones of perennial ryegrass and their offspring over 2 years. The narrow sense heritability estimate based on parent-offspring regression was 46%. Despite this heritability, the levels of resistance in recently released cultivars have shown only limited increase (Fig. 1.1). Reasons for the slow improvement can be found in the comments made by Hides and Wilkins (1978), Schmidt (1980) and Reheul & Ghesquiere (1996). The heritability of crown rust resistance allows good progress in resistance, however, this progress has its price: the negative correlation between resistance and yield performance. According to these authors, a gain of 1 unit rust resistance costs on average 1 % Dry Matter Yield. It is unknown whether this negative correlation is due to linkage or pleiotropism or are associated with the relaxation of selection pressure for agronomic characteristics during selection for rust resistance (Kimbeng, 1999).

Breaking this association should be possible as during the last decade newly released varieties are more productive while their level of crown rust resistance stays at almost the same level (Fig. 1.1). By using molecular markers, the trait 'crown rust resistance' can be genomically dissected and MAS can be used to break the association between low yield performance and high crown rust resistance.
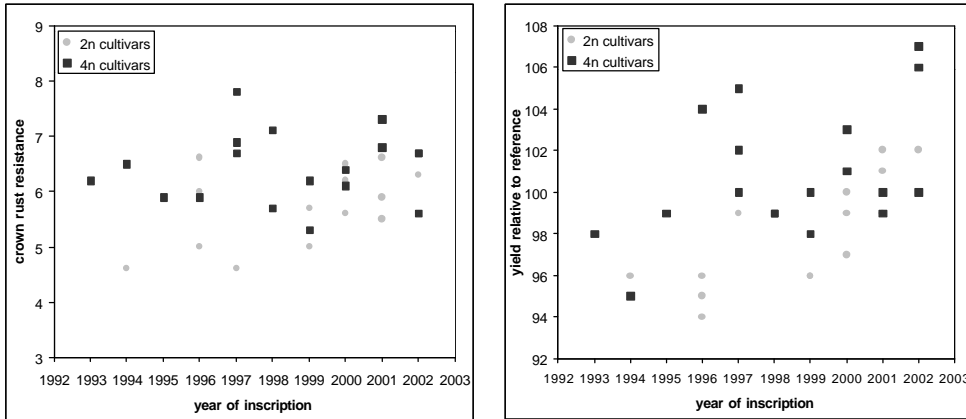
Fig. 1.1 : Crown rust resistance (from 4=susceptible till 8=resistant) and total yield relative to cv. Condesa (100) of cultivars included in the French list of 2002 in function of year of inscription on the list.

1.5 Objectives of current study

The main objective of this study was to identify and characterize genomic regions involved in crown rust resistance in *Lolium*. More concrete objectives were :

1. to construct populations of the studied species (*Lolium perenne*, *L. multiflorum* and their interspecific hybrid *L. x boucheanum*), suited for the phenotypic and genotypic analysis of the studied trait.

2. to use phenotypic analysis to formulate hypotheses about the number and nature of genomic regions involved in the determination of the trait in each of the populations studied.

3. to use genotypic analysis to identify the genomic regions involved in crown rust resistance.

4. to analyze the correspondence between genomic regions involved in crown rust resistance in the *Lolium* species studied.

5. to align the constructed *L. perenne* map with other published *Lolium* maps and with maps of other *Gramineae* and to analyze the

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correspondence between the genomic regions identified in the present study and genomic regions known to be involved in disease resistance in other *Gramineae.*

To achieve these goals, different F_1 populations were constructed by pair crossing resistant and susceptible *L. perenne*, *L. multiflorum* and *L. x boucheanum* plants. The analysis of the phenotypic segregation patterns in each of the populations, allowed us to formulate hypotheses about the number and nature of the genomic regions involved in the determination of the studied trait. These results are presented in Chapter 2. Afterwards, two different approaches were followed to identify genomic regions involved in crown rust resistance in each population. In a first approach, a bulk segregant analysis (BSA) was carried out to identify in a quick way AFLP markers associated with major genes involved in crown rust resistance (Chapter 3). Secondly, a map-based approach was followed to identify minor genes involved in crown rust resistance. Different marker types were evaluated for their use in map construction and QTL analysis (Chapter 4). Especially markers of known map position on the reference ILGI map were considered. This was in order to align the maps produced in this study with the reference *Lolium* map. In Chapter 5, the construction of the linkage map for the *L. perenne* population is described. Also in this chapter, the alignment of the constructed *L. perenne* map with other *Lolium* and *Gramineae* maps is discussed. The results of the QTL analysis in the *L. perenne* population by different approaches are presented in Chapter 6. Finally, in Chapter 7, the map construction and QTL analysis in the *L. multiflorum* and *L. x boucheanum* populations are discussed.

Chapter 2

Inheritance of crown rust resistance in different F$_1$ populations of *Lolium*

2.1 Introduction

2.1.1 Crown rust : economic relevance

Ryegrasses are susceptible to different biotic stresses; the most common diseases found in ryegrasses are listed in Table 2.1. In this study, the focus is on crown rust (Fig. 2.1) because it is a common and major fungal disease of ryegrasses in temperate regions of the world, to which the main part of Europe belongs (Potter et al., 1990; Roderick & Thomas, 1997; Reheul et al., 2000).

Table 2.1 : Bacterial and fungal diseases affecting *Lolium* spp.

Disease	Causal agent
Crown rust	*Puccinia coronata* Corda
Stem rust	*P. graminis* subsp. *Graminicola*
Brown rust	*Puccinia loliina* Sydow.
Leaf spot	*Drechslera (Helminthosporium)* spp.
Bacterial wilt	*Xanthomonas campestris* pv. *Graminis*
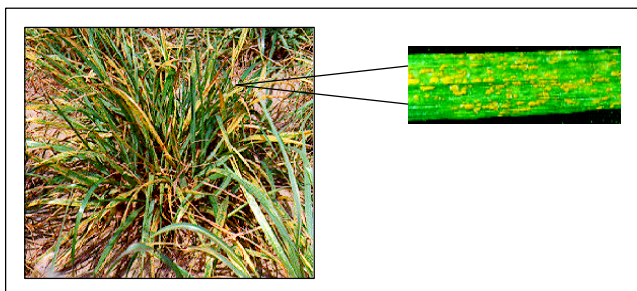
Fig. 2.1 : Disease symptoms of crown rust caused by *Puccinia coronata* on an infected *L. multiflorum* plant

Consequences of crown rust infection can be found at short and long-term level. Short-term losses are related to yield, palatability and nutritive value. Reduced long-term productivity and reduced persistence are among the most important long-term losses (Plummer et al., 1990).

At short-term, the quality of the herbage is affected. The fungus parasites the host and depletes the carbohydrate reserves, resulting in early senescence of the leaves. Infection invokes a higher concentration of crude fibres, lignin and ashes, while the concentration of soluble carbohydrates and proteins decreases (Potter, 1987). As a consequence, crown rust infected ryegrass is less palatable and is of lower nutritional quality. Schmidt (1980) reported that in the last cut of the season (late September – mid October) crown rust can cause yield losses of about 40 to 60% and even 100% for very susceptible varieties. This represents losses of about 10-20% for the yearly production. In South Sweden, yield losses int eh magnitude of 500-800 kg/ha of dry matter have been recorded (Jonsson et al., 1998).

Crown rust affects the long-term productivity of grass swards. Severe infections detract ryegrasses from growth and recuperative potential, and may predispose plants to winter injury. The number of tillers and roots decreases, which has an influence on the overwintering capacity, and the regrowth in the following spring (Kopec et al., 1983). Increased tiller death is most pronounced among young tillers, resulting in alterations of sward age structure. This affects seed production (Plummer et al., 1992).

2.1.2 Crown rust : control

Losses caused by crown rust can be reduced by chemical control, but in sustainable agriculture, especially for forage crops, chemical control of pathogens is expensive and not desirable. Good management practices, reducing conditions favourable to crown rust, constitute the best method to prevent epidemics (Kimbeng, 1999).

Crown rust infection becomes most severe on grasses that grow slowly because of stressful conditions, including drought, nutrient deficiency, low mowing height, shade, and other pathogenic attacks. Early inoculation results in the accumulation of spores during autumn, of which the survival rate is higher

with mild winters. This spore mass serves as an inoculum for a new crown rust epidemic the next year (Prine, 1991).

A good management practice to reduce crown rust losses in grassland is the use of resistant cultivars (Thomas, 1991). Especially with the current tendency to minimize the nitrogen input, the use of resistant cultivars is needed as the less nitrogen, the more crown rust invades ryegrass. Therefore, improving genetic resistance is one of the major goals in most ryegrass breeding programs (Wilkins, 1991; Van Bockstaele, 1999; Reheul et al., 2000). But as already shown in the introduction, the improvement of crown rust resistance in the recently released cultivars is limited.

Before dissecting crown rust resistance in our particular populations, an overview is given of the available knowledge on the life cycle of *Puccinia coronata* and of available reports on inheritance of crown rust resistance in *Lolium* spp.

2.1.3 *Puccinia coronata* : life cycle

Crown rust is caused by *Puccinia coronata*, a Basidiomycete belonging to the order Uredinales. It is a macrocyclic fungus, meaning that different kinds of spores are formed, and a heteroecious fungus, indicating that different hosts are necessary to complete the full reproduction life cycle of the fungus. The complete life cycle of the pathogen is illustrated in Fig. 2.2 and spore types are represented in Fig. 2.3 (Agrios, 1997; Smith et al., 1997).

Infected ryegrass foliage serves as the overwintering site for the dikaryotic mycelium and dikaryotic uredospores of crown rust fungi. When weather is conducive to spore germination or mycelial growth, the foliage becomes infected and new uredial pustules are formed, uredospores are produced in abundance and released from the uredia. These uredospores can be transported over long distances by wind. This spore mixture constitutes the asexual reproduction stage of rusts, with new cycles beginning every two weeks (Agrios, 1997).
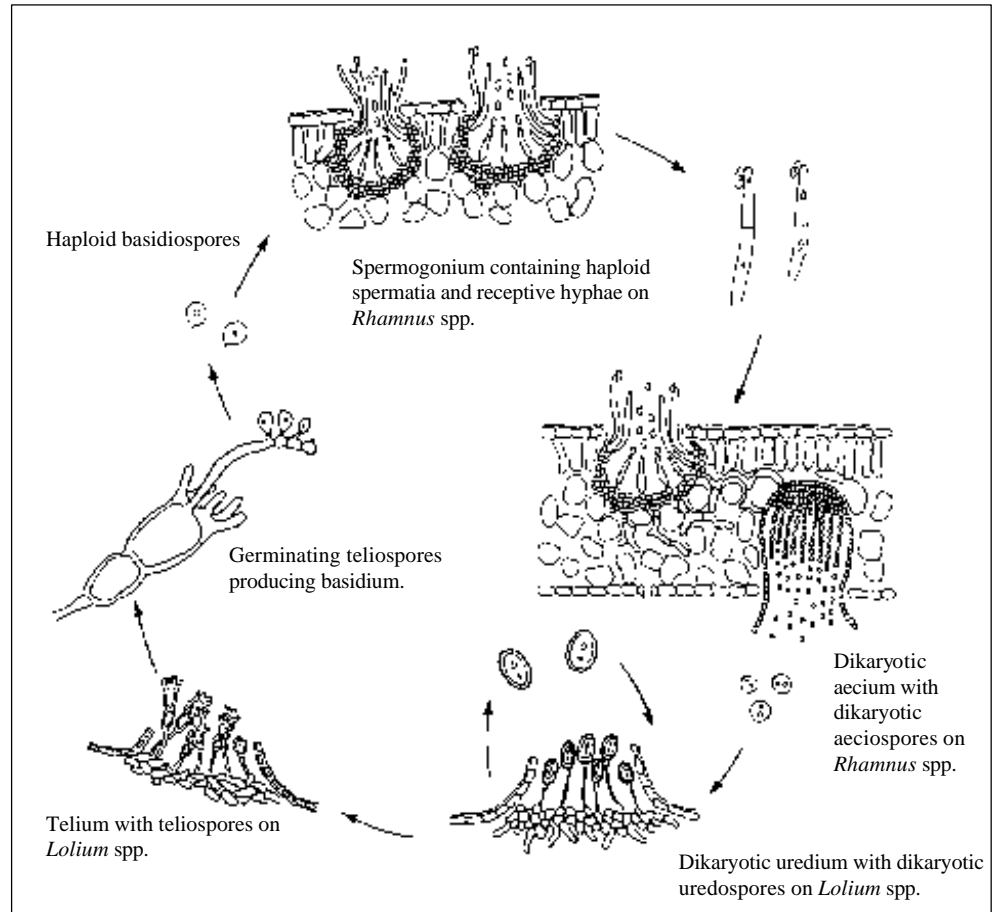
Fig. 2.2 : Sexual and asexual reproduction cycle of *Puccinia coronata* adapted from Agrios 1997).

Rust fungi have in addition to the asexual reproduction cycle, a sexual reproduction cycle. On maturation or drying of the plant foliage, crown rust produces teliospores. Telia occur on abaxial leaf surfaces next to uredia, but are linear and black. The teliospore may overwinter and then germinate to produce a basidium. The basidium, following meiosis, produces four haploid basidiospores. These spores become airborne. By wind they are transported and if they settle down on a susceptible non-grass host, they can germinate and cause a new infection. Basidiospores of *P. coronata* germinate on *Rhamnus* spp.,

producing haploid mycelium that forms spermagonia, containing haploid spermatia and receptive hyphae. Spermatia act as male gametes and are unable to infect plants; their function is the fertilization of receptive hyphae of the compatible mating type and subsequent production of dikaryotic mycelium and dikaryotic spores. This mycelium forms aecia that produce aeciospores, which on infection of *Lolium* plants produce more dikaryotic mycelium that this time forms uredia. The latter produce uredospores, which also infect and produce either more uredia and uredospores or near host maturity telia and teliopores. Thus completing the sexual life cycle. It must be emphasized, however, that spore types other than uredospores are rarely important for the occurrence of rusts on grasses. *Rhamnus* spp. are considered important for genetic recombination within the fungus, as the sexual reproduction cycle takes only place on these non-grass host species (Smith et al., 1997).
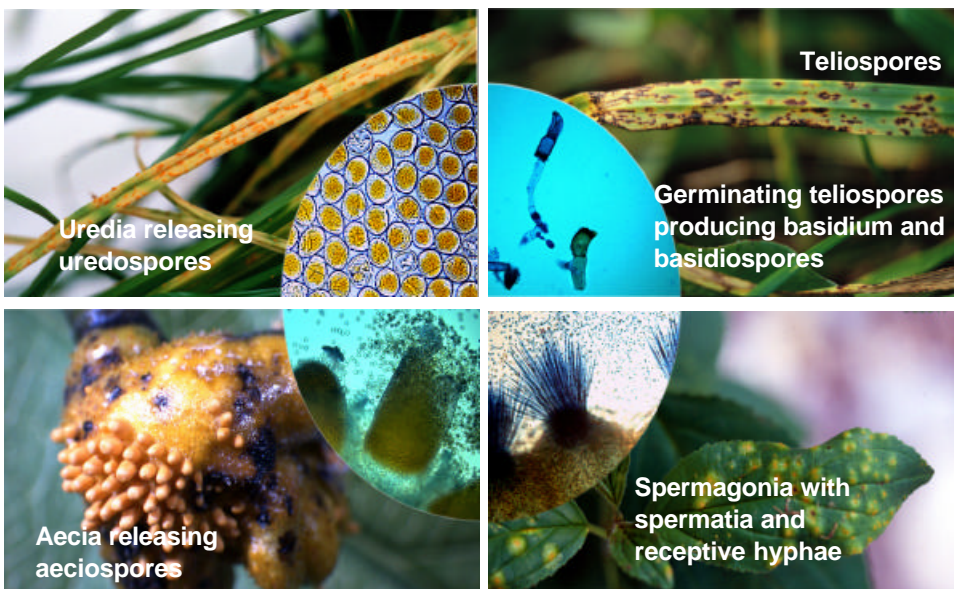
Fig. 2.3 : Spore types necessary for the sexual reproduction cycle of *Puccinia coronata* : uredospores and teliospores on *Lolium* spp.; spermagonia and aeciospores on *Rhamnus* spp. (pictures provided by F. Schubiger, FAL, Zürich, Switserland).

There is evidence of genetic variation among *Puccinia coronata* populations. Publications describe physiological races of *P. coronata* differing in their

virulence on particular perennial ryegrass genotypes (reviewed by Kimbeng, 1999). Given the economic importance of crown rust epidemics, the EUCARPIA Fodder Crops and Amenity Grasses Section decided in 2000 to set up a multisite rust evaluation test, in which different *L. multiflorum* and *L. perenne* cultivars are evaluated at different sites in Europe on a regular time basis. The aim is to determine spatial and temporal diversity in rust susceptibility of a set of ryegrass cultivars. Boller et al. (2002) reported on the first field results of 2001. They obtained a consistent ranking of the cultivars across the different sites. However, using a principal component analysis on the crown rust data obtained on the *L. perenne* cultivars, they could differentiate the field trials performed in Western Europe (France) from the trials conducted in North-Western and Northern regions. This indicates the presence of different strains of crown rust in different regions and/or important interaction with environmental conditions. This dependence of crown rust susceptibility on the testing site was also reported by Reheul et al. (1996). Within the EUCARPIA multisite testing, further research will have to prove the existence of regionally differentiated spore mixtures over the different regions.

2.1.4 Infection process at the cellular level

Puccinias are biotrophic fungi, that parasite the mesophyll cells without killing them. After deposition of the uredospores on the leaf and under favourable conditions, spores germinate. The germinated spore invades the leaf via the stomata. Each appressorium produces a substomatal vesicle which in turn usually gives rise to one or two thin infection hyphae. Infection hyphae elongate and branch. Small round haustoria are observed in mesophyll cells two to three days after inoculation. Subepidermal stromata producing abundant uredospores begin to differentiate in the infected leaves after six to eight days and erupent uredia are detected after eight days (Marte et al., 1994; Roderick & Thomas, 1997).

Marte et al. (1994) found differences at several stages of infection when comparing susceptible and resistant plants. First, a reduced rate of stomatal penetration by fungal appressoria was observed in the resistant plants. Thereafter fungal development stopped in the resistant plant and haustoria remained small and round-shaped whereas they became bigger and lobate in the susceptible plant. Second, they observed that in the resistant plant, haustorium

invaded cells showed more distinct signs of degradation compared to the susceptible control. Some cells adjacent to cells containing a haustorium became necrotic. No further fungal growth was observed in the resistant plant by this time and most of the previously formed infection hyphae appeared empty and were frequently collapsed. This hypersensitive necrosis in response to rust invasion is usually controlled by major genes (Hammond-Kosack and Jones, 1997). Marte et al. (1994) stated that the occurrence of more than one histological response in a single plant-rust fungus interaction may indicate that multiple resistance mechanisms are activated by the plant against the invader organism.

2.1.5 Sources of resistance in ryegrass

Next to knowledge about the pathogen, knowledge about sources of resistance in the host is available. Several publications describe the mode of inheritance of crown rust resistance in *Lolium* spp. Reports exist on either qualitative resistance, conferred by a major gene or on quantitative resistance, conferred by multiple minor genes. In one case, maternal transmission of resistance is reported.

For example, Wilkins (1975) observed two different genetic systems of crown rust resistance in Italian ryegrass: one type explained by a single dominant, major gene and the other by the additive effect of several minor genes. In the case of dominant genes, resistance worked in an ambidirectional way, which means depending on the involved alleles, resistance was dominant or recessive. Wilkins (1975) reported that if a single strain of crown rust was used, major factors were revealed in Italian ryegrass, probably controlled by a single dominant gene. The remaining variation in resistance to the pathogen was controlled by a relatively large number of genes with minor effects, exhibiting neither dominance nor epistasis. These major genes confer complete resistance to the pathogen strains they are active against, and in this way resistance can significantly be improved by selecting for the presence of these genes. He warned, however, for the use of these major genes in breeding programs, as the resistance tends to be lost fairly easily due to genetic evolution of the pathogen. Schmidt (1980) observed a similar pattern: one single dominant gene was responsible for crown rust resistance in Italian ryegrass, but the action of this gene was influenced by several complementary genes with an additive action.

Hayward (1977) reported on crown rust resistance in *L. perenne.* In the studied material, resistance had a quantitative character, where genes were dominant but ambidirectional. McVeigh (1975) found in perennial ryegrass qualitative resistance controlled by two recessive alleles but also quantitative resistance. Lellbach (1999) identified in a F_2 *L. perenne* population two major genes involved in crown rust resistance. Roderick et al. (2000) introgressed resistance factors from *Festuca pratensis* into *L. perenne.* The introduced resistance was temperature insensitive in one of the introgression lines. This was in contrast to the observed increased susceptibility when *L. perenne* plants were incubated at 25°C compared to 10°C.

Adams et al. (2000) described maternally inherited crown rust resistance in a *L. multiflorum* cross. The maternal factor detected is stable and transmitted to the progeny in a predictable way (J. Baert, CLO-DvP, personal communication).

In summary, major and minor genes conferring crown rust resistance seem to be present in ryegrasses. The most effective way of controlling crown rust would be to use resistant ryegrass varieties with specific resistance genes. However the development of new isolates of crown rust by mutation, hyphal anastomosis or sexual recombination in *Rhamnus* often renders these varieties susceptible (Posselt, 1994). Race-specific resistance to crown rust is usually characterized by the host's hypersensitive reaction and is oligogenic in nature. Current interests lie, however, in the development of general or horizontal resistance where quantitative response is, most often, inherited polygenically. In theory, this multigene resistance offers increased stability because the host has an increased buffering capacity against the pathogen (Kopec et al., 1983).

Discussion exists about what kind of inoculum to use for selection and for resistance studies. Wilkins (1975) pointed already to the use of single spore colonies, in order to identify major genes. Other authors like Posselt (1994) emphasise on the use of mixtures in order to obtain non strain-specific resistance, which is more long lasting. Field data seem to correspond better to artificial inoculation data when a crown rust population with a broad genetic basis is used for infection (Posselt, 1994). In this study, a spore mixture will be used. However, we should bear in mind that inoculating with a spore mixture, containing different physiological races, that infection can be due to mainly one dominant physiological race.

2.2 Objectives and rationale

The objectives of the experiments described in this chapter were:

(i) <u>to construct segregating populations suitable for mapping crown rust resistance in ryegrasses.</u> F_1 populations segregating for crown rust resistance were created by crossing a resistant and a susceptible parent plant. *L. multiflorum* and *L. perenne* were included in the analysis as well as the interspecific hybrid, *L. x boucheanum.* This allowed the comparison of the results obtained in these genetically closely related species.

Several crosses of *L. perenne x L. perenne*, *L. multiflorum x L. multiflorum* and *L. x boucheanum x L. x boucheanum* were initially performed, but just one of each kind was finally chosen for marker analysis. Seed yield, seedling survival and the mode of inheritance of the resistance were used as criteria for the selection of the crosses to be analysed thoroughly.

(ii) <u>to determine the mode of inheritance of crown rust resistance in these populations.</u> F_1 populations were phenotyped for crown rust resistance, using an artificial inoculation test. This artificial inoculation test was chosen above field evaluations because crown rust epidemics in the field are unpredictable in time and severity, and are not uniformly spread over the field. In the artificial inoculation test, spore mixtures were used rather than *Puccinia* strains, as the intention of this study was to identify genetic factors controlling broad-based resistance. In order to verify hypotheses concerning inheritance of crown rust resistance in the F_1 populations, F_2 populations obtained by selfing resistant F_1 plants were produced and phenotyped.

This thorough phenotypic characterisation of the populations was considered necessary, as studies presenting different kinds of resistance for crown rust in *Lolium* had been reported (see 2.1.5). On the basis of the results obtained in this analysis, a sound strategy for the identification of markers linked with loci controlling resistance in the selected crosses was developed.

2.3 Material and methods

2.3.1 Plant material

Three different *Lolium* spp. were studied: *L. perenne*, *L. x boucheanum* and *L. multiflorum*. For each species, crosses were made between a diploid, crown rust resistant and a diploid, crown rust susceptible plant. An overview of the different crosses made is given in Table 2.2.

• *L. perenne* crosses: Perennial ryegrass accessions were screened for crown rust resistance at three different field locations in 1997 by Advanta Van der Have (Rilland, The Netherlands). Six resistant genotypes and six susceptible plants were selected and crossed two by two (Table 2.2). Crossing experiments were performed by Advanta Van der Have in the greenhouse under controlled conditions. Flowering stalks of the two crossing partners were placed together and isolated using plastic bags. When seeds were ripe, stalks were harvested separately from each parent (i.e. each cross rendered two seed stocks).

• *L. x boucheanum* crosses: Hybrid ryegrass accessions, originating from a cross of a susceptible *L. multiflorum* plant and a resistant *L. perenne* plant, were screened in 1997 for crown rust resistance by an artificial inoculation method (for method description see below). Three resistant and three susceptible F_1 plants were selected and used for crossing experiments. Three pair crosses between a resistant and a susceptible F_1 *L. x boucheanum* plant were made by Advanta Van der Have (Rilland, The Netherlands) using the same technique as described for the *L. perenne* crossing experiments. F_1 seed was harvested on each crossing partner (i.e. each cross rendered two seed stocks).

• *L. multiforum* crosses: The studied Italian ryegrass crosses were described by Adams et al. (2000). Italian ryegrass accessions were screened for crown rust resistance in the summer of 1993 after a severe natural infection at DvP-CLO (Melle, Belgium). Three resistant plants, Axis-1, Axis-2 and Axis-3 were selected from the cv. Axis, which is known to display moderate to good resistance against crown rust and other diseases (Neusch, 1990). Severely infected plants were selected in the *L. multiflorum* gene pool of DvP-CLO (Melle, Belgium) and named P_1G_3-1, B-1 and B-90. The crosses were made

Table 2.2 : Overview of pair crosses made between resistant (R) and susceptible (S) plants of the three species studied. The first plant name in each cross is the name of the R plant, the second the S plant. The amount of seeds harvested on the respective parent plants is given in grams (diploid seed weighs about 2 g/ 1000 seeds) or in number of seeds. The crosses selected for further analysis are given in bold and the number of seedlings evaluated phenotypically is given in the last column.

Crosses	Harvested seeds			Evaluated seed
	R parent	S parent	Total	# of plants inoculated
L. perenne				
TB1*SA1	**0.1 g**	**0.1 g**	**0.2 g**	**56**
TB2*SA1	0.1 g	0.1 g	0.2 g	
TC1*SA2	10 seeds	0.1 g	0.1 g	
TC2*SA2	5 seeds	0.2 g	0.2 g	
TA1*SB1	0.2 g	0.4 g	0.6 g	
TA2*SB1	0.1 g	0.2 g	0.3 g	
TC1*SB2	**0.2 g**	**0.7 g**	**0.9 g**	**291**
TC2*SB2	0.2 g	0.8 g	1 g	
TA1*SC1	0.1 g	10 seeds	0.1 g	
TA2*SC1	**0.1 g**	**0.1 g**	**0.2 g**	**83**
TB1*SC2	None	0.1 g	0.1 g	
TB2*SC2	None	0.1 g	0.1 g	
L. x boucheanum				
2A2*1B12	**211 seeds**	**77 seeds**	**288 seeds**	**142**
1B6*1H12	161 seeds	4 seeds	165 seeds	
1E6*1H12	None	None	None	
2A1*1F3	158 seeds	50 seeds	208 seeds	
L. multiflorum				
Axis-1*P$_1$G$_3$-1	> 200 seeds	> 200 seeds	> 400 seeds	
Axis-1*B-1	> 200 seeds	> 200 seeds	> 400 seeds	
Axis-2*P$_1$G$_3$-1	> 200 seeds	> 200 seeds	> 400 seeds	
Axis-2*B-1	> 200 seeds	> 200 seeds	> 400 seeds	
Axis-3*B-90	**> 200 seeds**	**> 200 seeds**	**> 400 seeds**	**299**

by planting the plants in the field in pairs of one resistant and one susceptible genotype. Each cross was surrounded by winter rye, which served as barrier for foreign pollen. The crossing partners were not synchronised for flowering time but the difference in mean flowering time was always below fourteen days. When seeds were ripe, stalks were harvested separately from each parent plant (i.e. each cross rendered two seed stocks). The results of the phenotypic evaluation of these crosses have been reported in Adams et al. (2000).

2.3.2 Plant growth conditions

F_1 seed of the different crosses were sown in trays of 96 pots of 4x4x7 cm filled with common soil. They were kept in the greenhouse at 20-25 °C during the day and at 15 °C during the night. When necessary, illumination was supplemented for a total of 14 hours. Plants were infected at least three times. The first inoculation was carried out when the plants were six weeks old and was repeated two times at intervals of one month, using the same plants.

2.3.3 Inoculum

Inoculum for the artificial inoculation tests was harvested in the fields of DvP-CLO (Melle, Belgium) in 1997 on different ryegrass varieties. Spores were harvested using a hover. In front of the vacuum pump, a paper funnel was placed, on which the spores were deposited during hovering. Immediately after harvesting, uredospores were sieved through nylon meshes of 100 µm and 40 µm to remove soil particles. The sieved spore mixture was desiccated at 20% relative humidity for 48 h, and stored at – 80 °C. Aliquots of this spore mixture were used for all artificial inoculations described below. Before each series of inoculations, spore germination rates were estimated on 2% agar-water (wt/vol) plates (Birckenstaedt et al., 1992; Wise et al., 1996). Germination percentages between 60-70% were recorded.

2.3.4 Artificial inoculation test

The uredospores, stored at -80°C, were induced for germination by incubation during two minutes in a water bath at 45°C (Birckenstaedt et al., 1992). The uredospores were diluted in ten times their own volume of talc powder. The mixture was rubbed with a paint-brush on two to three fully expanded leaves of each plant individually at a density of 40 mg uredospores (germination = 100%) per 100 plants. The plants were kept 36 hours at 100% relative humidity (RH) after inoculation. Hundred percent RH was created by submerging the trays with plants in water, by spraying the plants and subsequently covering them with a transparent plastic foil. After breaking the 100% RH, plants were kept as described earlier.

Fourteen days after inoculation, the plants were scored for resistance using a scale derived from the scale of Birckenstaedt et al. (1994). The rating values represent a relative estimate of leaf area occupied by crown rust pustules. The scale of Birckenstaedt varies from 1 = resistant till 9 = susceptible. The scale used in this study was a modified scale using scores varying from 1 = resistant till 6 = susceptible. Score 1 till 5 are identical to these of the Birckenstaedt scale, score 6 comprises score 6 till 9 of the Birckenstaedt scale. This modification was done because it was difficult to differentiate between scores 6 till 9. An example of each rating of the modified scale is given in Fig. 2.4. After each evaluation the plants were cut and allowed to regrow during at least 2 weeks before a new inoculation. This repeated inoculating might cause the induction of systemic acquired resistance or weakening of the plants. Statistics will be used to determine whether this is the case in the studied populations.

2.3.5 Phenotyping in the field

After three inoculation rounds in the greenhouse, all tested plants were transferred to the field at DvP-CLO (Melle, Belgium). Rows of a very crown rust susceptible cultivar (Merlov) were sown between the experimental plant rows (Fig. 2.5). These rows serve as a source of crown rust and favour equal dispersion of spores in the field (Reheul & Ghesquiere, 1996; Van Bockstaele, 1999). Plants were scored for crown rust during the summer of 2000, using the modified scale of Birckenstaedt et al. (1994) described above.
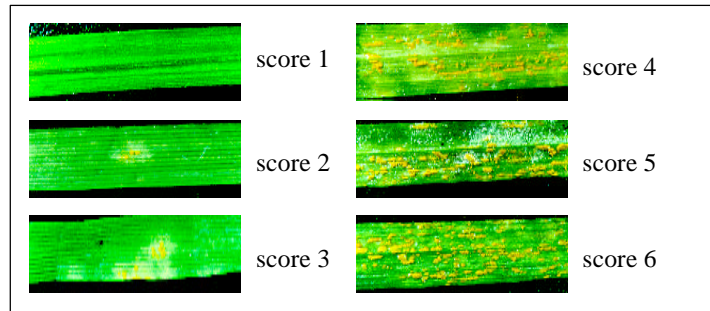
Fig. 2.4. : Modified scale of Birckenstaedt et al. (1994) used for scoring seedlings after artificial inoculation.

Fig. 2.5 : Evaluation of plants for crown rust resistance in the field. Next to the plant rows, a row with a very crown rust susceptible cultivar (Merlov) is sown.

2.3.6 Statistical analysis

The mean crown rust score for each individual plant was calculated by averaging the crown rust scores over the different inoculation rounds. Non-parametric correlation coefficients (Spearman's Rho) between the different rounds were calculated. To identify differences between the three inoculation rounds, the Friedman test was used, which is a non-parametric test that compares three or more paired groups. Mann-Whitney U tests were performed to check for maternal resistance, as these tests identify significant differences in mean

crown rust scores between the two seed stocks. All calculations were performed using the package SPSS (Norusis, 2000).

2.4 Results and discussion

2.4.1 Selection of F_1 populations

An overview of the crosses made and the amount of seed harvested per cross is given in Table 2.2. It was necessary to make several crosses, as parent plants were only selected for crown rust resistance or susceptibility and not for their crossing capability. Low seed set was observed in some *L. x boucheanum* and *L. perenne* crosses. As *Lolium* spp. are highly self-incompatible species (Cornish et al., 1979; Van Daele et al., 2000; Thorogood et al., 2002), incompatibility between the *L. x boucheanum* parent plants was expected. The parent plants were full sibs, selected out of one F_1 population (obtained by crossing a susceptible *L. multiflorum* plant with a resistant *L. perenne* plant).

The *L. perenne* plants were not related, but also for some of these crosses low seed sets were obtained. In these cases, genetic incompatibility between the parents or lack of synchronisation in their flowering dates can be at the basis of the low seed production.

In general, the selection of crosses for phenotypic analysis was based on the amount of seeds harvested, which is an indication of the quality of the cross. For *L. perenne*, three crosses were selected: TB1*SA1, TA2*SC1 and TC1*SB2. The choice was not solely based on the amount of seeds harvested, but also on the origin of the parent plants. In the selected crosses, parents with different genetic backgrounds were involved.

In the case of *L. x boucheanum*, just cross 2A2*1B12 produced enough seed on both parents for further analysis.

In the case of *L. multiflorum*, Adams et al. (2000) detected in four out of the five crosses, a resistance factor maternally inherited. Only in cross Axis-3*b-90, no maternal factors were detected. This cross was selected for further analysis in this thesis.

2.4.2 Correlations between artificial inoculation rounds[1]

The amount of seedlings analysed in the five selected populations is given in column 5 of Table 2.2. Crown rust resistance was determined by means of an artificial inoculation method using the modified scale of Birckenstaedt et al. (1994). Each seedling was scored three times during subsequent rounds of artificial inoculation. In this way, it was possible to determine the agreement of the results obtained for the same plant at three inoculation rounds, to eliminate possible misclassification errors and to identify induction of systemic acquired resistance or weakening of plants.

The variation in crown rust score between the three subsequent rounds of inoculation was studied on the pooled results of the five retained populations and is represented in Fig. 2.6. For each plant, the absolute differences in crown rust score between two inoculation rounds have been calculated and plotted. The scores from the first round of inoculation were compared to the scores from the second (Fig. 2.6a) and third round (Fig. 2.6c). The scores from the second round were compared to the scores from the third round of inoculation (Fig. 2.6b). In the three comparisons, 38% of the plants or more got identical scores at two different inoculation rounds (difference 0). Ninety three percent of the plants or more had a difference of 0 till 2 units in the crown rust score obtained in two different rounds. Taking into account the three comparisons, 12% of the plants had at least once a difference higher than two. These plants were omitted during further phenotypic analysis (i.e. mode of inheritance in this chapter, BSA analysis in Chapter 3 and QTL analysis in Chapters 6 and 7).

These results indicate high consistency between the score assigned to single plants at two different rounds of artificial inoculation. This impression was confirmed by the significant non-parametric correlation coefficients (Spearman's Rho) that were found between inoculation rounds 1 & 2, 2 & 3 and 1 & 3. Values were 0.666, 0.648 and 0.714 respectively. All values were significant at the 0.01 level (2-tailed). When outliers were omitted even higher correlation values were obtained (0.770, 0.754 and 0.802 respectively).

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[1] During marker analysis (Chapter 4), we identified plants which were not true F_1 individuals, meaning that they were not the result of a cross between the two parent plants. Contamination with foreign pollen during the crossing experiment or mixing of seed must have occurred. These 'contaminants' were omitted for the rest of the analyses.
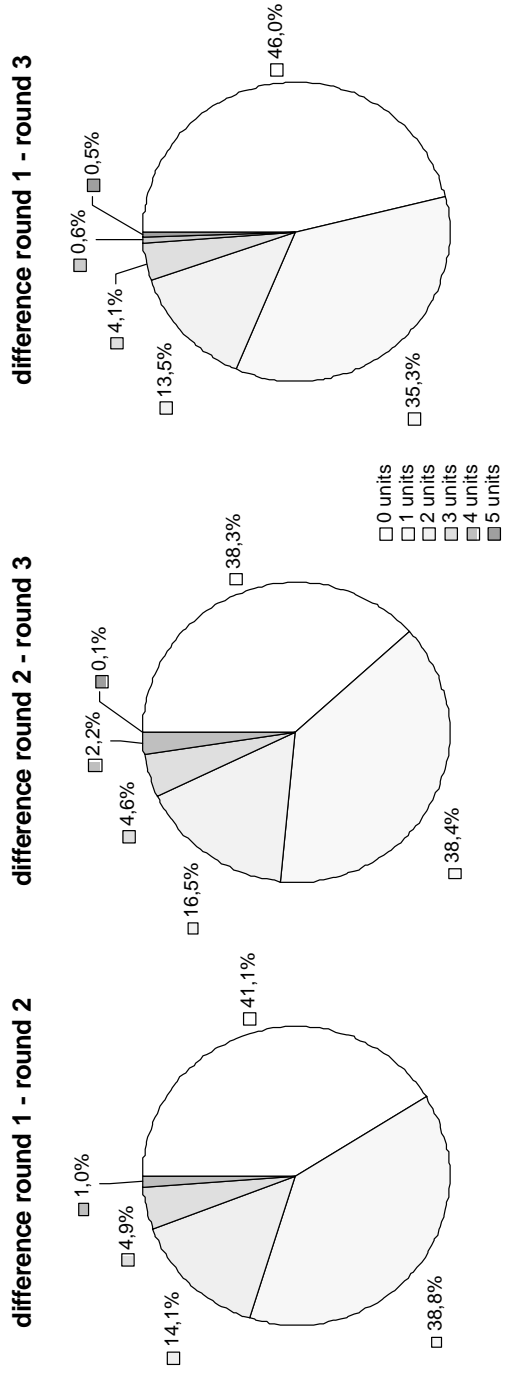
Fig. 2.6 : Percentage of plants with 0, 1, 2, 3, 4 or 5 units absolute difference between the crown rust score obtained in a) inoculation round 1 and 2; b) inoculation round 2 and 3 and c) inoculation round 1 and 3.

The main risk of infecting plants repeatedly is the induction of systemic acquired resistance. This would be seen as a significant decrease of the mean population scores during the three inoculation rounds. On the other hand, repeated infection can result in weakening of the plants, what would be seen as an increase in susceptibility in time. To identify differences or heterogeneity in resistance level during the three inoculation rounds, Friedman tests were used. Tests were performed per selected population separately as these effects might be genetically related (Table 2.3). In the *L. perenne* population TC1*SB2, no significant heterogeneity was observed. In the other populations, significant heterogeneities at the 0.05 level were found. In the populations Axis-3*B-90 and 2A2*1B12, heterogeneity was significant but no clear increasing or decreasing trend was observed. This led us to conclude that the heterogeneity observed was not due to systemic acquired resistance or weakening of the plants. In the TB1*SA1 and TA2*SC1 populations, an increasing trend was observed, but this should be interpreted with care as the number of plants in the populations is rather low (52 and 73 plants respectively). However the significant result in these populations was taken into consideration during selection of the populations. Therefore, they were not retained for further analysis.

Table 2.3 : Mean crown rust score of each selected F_1 population for round 1, 2 and 3. Significance of heterogeneity between population mean scores was calculated using the Friedman test.

	Population mean				
	TC1*SB2 (265 plants)	TB1*SA1 (52 plants)	TA2*SC1 (73 plants)	2A2*1B12 (92 plants)	Axis-3*B-90 (203 plants)
Round 1	1.98	1.77	1.87	1.96	1.87
Round 2	2.05	2.04	1.94	1.91	2.11
Round 3	1.98	2.19	2.19	2.13	2.02
Sign. Friedman Test	0.401	0.028*	0.049*	0.043*	0.012*

* = significant at the P = 0.05 level

2.4.3 Maternally inherited resistance

Adams et al. (2000) reported that a maternal factor may play a role in crown rust resistance in some *L. multiflorum* plants of the cv. Axis. In order to verify whether a maternal factor was present in any of the five populations studied, the F_1 populations were divided into two seed lots according to the parent they were harvested on. Mann-Whitney U tests were used to verify if a significant difference was found between the mean crown rust score of the parental seed lots (Table 2.4). Significant differences at the 0.05 level were found for rounds 1 and 3 in the TB1*SA1 cross and for round 3 in the TA2*SC1 cross, indicating that the seed lot harvested on the R parent contained more resistant plants than the seed lot harvested on the S parent. These significant differences should be interpreted with caution as these populations were very small. TB1*SA1 consisted of 52 plants (15 out of the R seed lot and 37 out of the S seed lot) and the population TA2*SC1 of 73 plants (48 out of the R seed lot and 25 out of the S seed lot). These populations were not retained for phenotypic and DNA-marker analysis as the populations were too small and maternally inherited resistance factors might be present.

Table 2.4 : Mean crown rust score of the R and S seed lot in the five populations studied and results of the Mann-Whitney U tests testing for significant differences between mean crown rust scores of the subpopulations.

Round	1			2			3		
Seed lot	R	S	Sign.	R	S	Sign.	R	S	Sign.
L. perenne									
TC1*SB2	1.97	1.75	0.182	1.81	1.94	0.197	2.07	1.75	0.087
TB1*SA1	1.59	2.67	0.010[*]	2.05	2.27	0.656	2.00	2.87	0.020[*]
TA2*SC1	2.48	3.12	0.056	2.75	2.92	0.886	2.69	3.52	0.015[*]
L. x boucheanum									
2A2*1B12	1.68	1.64	0.776	1.59	1.61	0.419	1.86	1.72	0.591
L. multiflorum									
Axis-3*B-90	2.90	2.79	0.694	3.17	3.03	0.528	2.89	3.09	0.346

[*] significant at the P = 0.05 level (2-tailed)

In the other populations (*L. multiflorum* Axis-3*B-90, *L. x boucheanum* 2A2*1B12 and *L. perenne* TC1*SB2) no maternal effect was identified. These populations were retained for further analysis.

2.4.4 Field observations

Two of the three selected populations (*L. perenne* cross (TC1*SB2) and the *L. x boucheanum* cross (2A2*1B12)) were evaluated for crown rust resistance under field conditions after a natural infection in the summer of 2000. The scores assigned in the field were compared to the scores assigned in the artificial inoculation test using a non-parametric correlation coefficient (Spearman's Rho).

In the *L. x boucheanum* population and the *L. perenne* population the Spearman correlation coefficients were respectively 0.249 with a significance of $P = 0.005$ and 0.578 with a significance of $P \leq 0.000$. The correlation, although significant, was quite low for the *L. x boucheanum* population. This might be due to the late observation date, at which the plants of this population might have already recovered from crown rust infection. The correlation coefficient found in the *L. perenne* population is higher and corresponds with the values (0.5-0.6) obtained by Reheul & Ghesquiere (1996).

2.4.5 Inheritance of resistance

In Fig. 2.7., the frequency distribution of the mean crown rust score of individual F_1 plants is given for each of the three retained populations. The frequency distribution for the total population is given next to the frequency distributions for two subpopulations : for the R seed lot (harvested on the resistant parent) and the S seed lot (harvested on the susceptible one). These frequency distributions illustrate the inheritance of resistance in the three different populations.

When putting forward hypotheses on the inheritance of resistance in these populations, 'genes' is used as a term referring to a genomic region involved in resistance. We should bear in mind that this region can contain more than one gene.

a) *L. perenne* **TC1*SB2**

b) *L. x boucheanum* **2A2*1B12**

c) *L. multiflorum* **Axis-3*B-90**
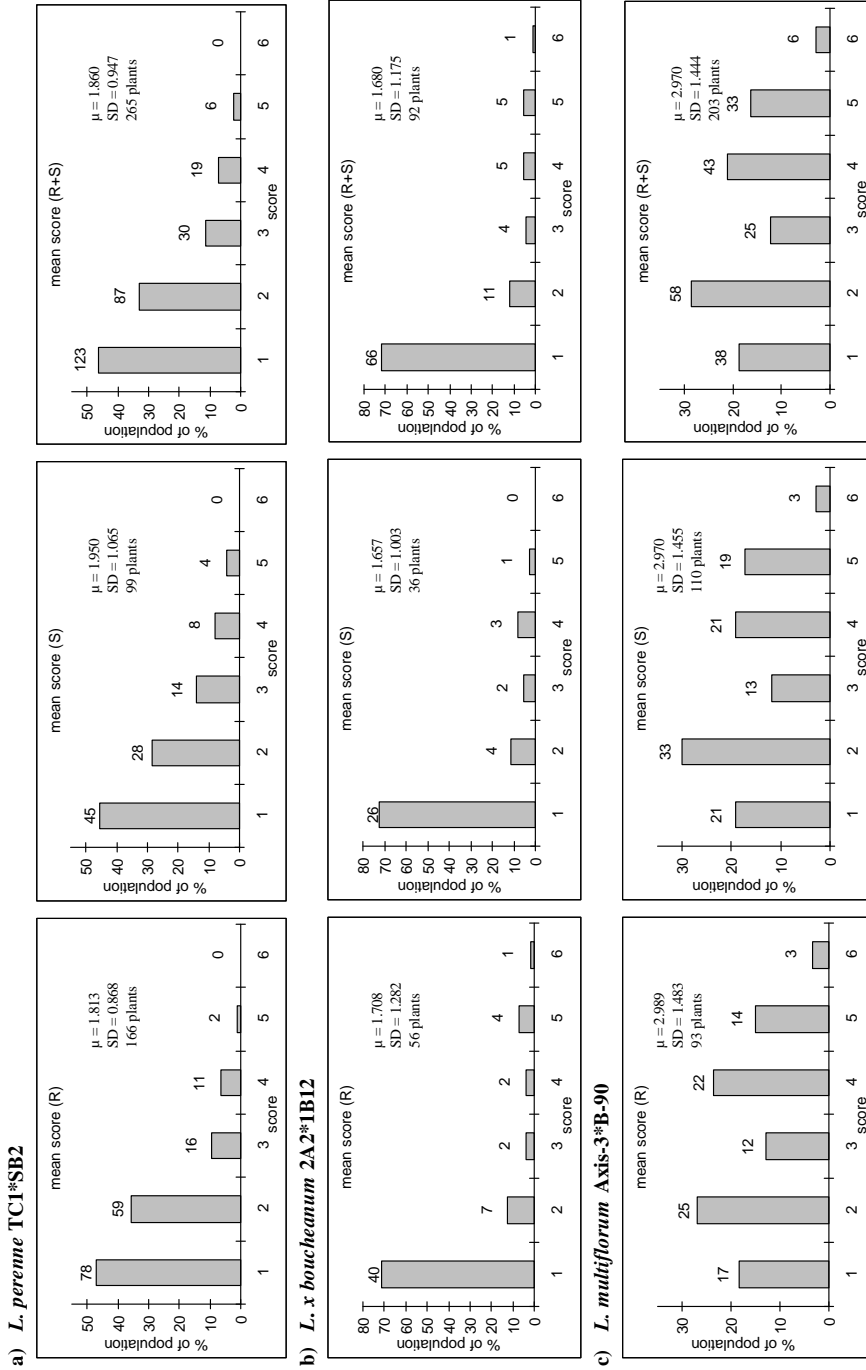
Fig. 2.7 : Frequency distribution of mean scores of a) *L. perenne*, b) *L. x boucheanum* and c) *L. multiflorum* population. Crown rust scores vary from 1= resistant till 6=susceptible. Distributions are given for the whole population (R+S) and for the R and S seedlot. R is the subpopulation derived from the seed harvested on the R parent. S is the subpopulation harvested on the S plant. The number of plants representing the percentage are noted on top of the bar. The population mean, the standard deviation and the number of plants is given for each (sub)population.

Based on the design of the populations, we can just observe the segregation of genes which are heterozygous in the parent plants. Taking into account that the parent plants are very heterozygous, we supposed that for each gene 4 alleles were involved in the cross. The resistant parent contains one dominant resistant allele and one recessive allele at each locus involved and that the susceptible parent has two recessive alleles at each locus involved (Fig. 2.8). Three hypotheses for crown rust resistance were tested using χ^2 test statistics:

A. If one gene confers resistance and the resistance allele (present in the resistant parent) acts in a dominant way, we expect to find a 1:1 segregation of resistant and susceptible F_1 plants.

B. If two genes are involved and their alleles which code for resistance do not show interaction, we expect to find a 3:1 segregation of resistant and susceptible plants.

C. If two genes are present and if they interact in an additive way, we expect to find a 1:2:1 ratio of resistant, intermediate and susceptible plants.

The more genes are influencing the trait in an additive way, the more a normal distribution will be found, and the trait will not be qualitatively but quantitatively inherited.

The frequency distributions of the *L. perenne* and *L. x boucheanum* population are not normally distributed but skewed (Fig. 2.7). The skewness is towards the score of the resistant parent. This might indicate that several major genes are involved in resistance. The non-discrete nature of the distribution can be explained by the presence of minor genes that influence the action of the major genes. Inspecting the frequency distribution of the *L. multiflorum* population, a normal distribution is found, indicating that several genes, with minor effects, are influencing the trait.

HYPOTHESIS A	HYPOTHESES B & C

HYPOTHESIS A

Parental phenotypes :

R x S

Parental genotypes :

R_1r_2 x r_3r_4

Gametes:

	r_3	r_4
R_1	R_1r_3	R_1r_4
r_2	r_2r_3	r_2r_4

Phenotype F_1 population :

50% R / 50% S

HYPOTHESES B & C

Parental phenotypes :

R x S

Parental genotypes :

$R_1r_2/R'_1r'_2$ x $r_3r_4/r'_3r'_4$

Gametes:

	r_3/r'_3	r_3/r'_4	r_4/r'_3	r_4/r'_4
R_1/R'_1	$R_1r_3/R'_1r'_3$	$R_1r'_3/R'_1r'_4$	$R_1r_4/R'_1r'_3$	$R_1r_4/R'_1r'_4$
R_1/r'_2	$R_1r_3/r'_2r'_3$	$R_1r_3/r'_2r'_4$	$R_1r_4/r'_2r'_3$	$R_1r_4/r'_2r'_4$
r_2/R'_1	$r_2r_3/R'_1r'_3$	$r_2r_3/R'_1r'_4$	$r_2r_4/R'_1r'_3$	$r_2r_4/R'_1r'_4$
r_2/r'_2	$r_2r_3/r'_2r'_3$	$r_2r_3/r'_2r'_4$	$r_2r_4/r'_2r'_3$	$r_2r_4/r'_2r'_4$

Phenotype F_1 population :

no interaction $R_1 - R'_1$ (Hypothesis B):

75% R / 25% S

additive interaction $R_1 - R'_1$ (Hypothesis C):

25% R / 50% I / 25% S

Fig. 2.8 : Representation of the hypotheses on the mode of inheritance of crown rust resistance. Hypothesis A: one gene involved, resistance allele is dominant; hypothesis B : two genes, resistance alleles are dominant and do not show interaction; and hypothesis C : two genes, resistance alleles are dominant and act in an additive way. The four alleles present at gene x are represented by R_x or r_x depending on the dominant or recessive character of the allele. The expected phenotypic distribution in the F_1 population is calculated. In hypotheses A and B: two different phenotypes are expected : R (resistant, at least one dominant resistance allele is present) and S (susceptible, solely susceptibility alleles are present). In hypothesis C: three phenotypes are expected : R (at both genes the dominant resistance allele is present), I (intermediate : just at one gene a dominant resistance allele is present) and S (no resistance alleles are present).

2.4.5.1 Number of genes present in the L. perenne *population*

In order to identify the number of resistance genes involved in the *L. perenne* cross, two different approaches to categorize the plants were used. In the first approach, F_1 individuals with score 1 or 2 were considered as resistant and the rest as susceptible. In the second approach plants with score 1 were considered as resistant, plants with scores 2 and 3 were considered intermediate and the rest as susceptible.

According to approach 1, 79% of the *L. perenne* F_1 population belongs to the resistant group while 21% to the susceptible (Table 2.5). This discrete distribution might be the result of the segregation of two major resistance genes of which the resistance alleles are dominant and do not show interaction (hypothesis B). According to the χ^2 test, this hypothesis can be retained (χ^2 = 2.547 with a P–value of 0.110). The variability within the discrete classes, can be explained by the action of minor genes.

In approach 2, in which plants with score 1 are considered resistant, 46% of the plants in the *L. perenne* population are resistant, 44% intermediate plants and 10% susceptible plants. Within this approach, the explanation can be the action of one major gene with a dominant resistance allele (hypothesis A). The observed distribution resembles the expected segregation (χ^2 = 1.362 with a P–value of 0.243).

For the *L. perenne* population, we hypothesize that depending on the interpretation of the phenotypic scores, one or two genes with major effect are involved in the resistance trait. Other minor factors might explain the rest of the variability observed.

2.4.5.2 Number of genes present in the L. x boucheanum *population*

For the *L. x boucheanum* population, the same approaches as for the *L. perenne* population were used.

In the first approach (plants with score 1 or 2 were considered resistant), 80% of the *L. x boucheanum* population belongs to the resistant group while 20% to the susceptible. This discrete distribution refers to an identical situation as for the *L. perenne* population (hypothesis B). This segregation can be explained by

the action of two major genes of which the resistance alleles do not show interaction ($\chi^2 = 1.500$ with a P–value of 0.221).

Table 2.5 : Testing of hypotheses on mode of inheritance of crown rust resistance in the *L. perenne* (LP), *L. x boucheanum* (LB) and *L. multiflorum* (LM) F_1 populations. Three hypotheses were tested using the χ^2 tests (Fig. 2.8). Classification of plants into a resistant (R), intermediate (I) and susceptible (S) group was done using two different approaches. In approach 1, plants with mean score 1 and 2 were considered as R and the rest as S. In approach 2, plants with score 1 were seen as R, plants with score 2 and 3 as I and plants with score 4 till 6 as S. P values indicate the significance of the null hypotheses. Retained hypotheses are indicated in bold.

Hypothesis		Approach 1 (R=1-2/S=3-6)			Approach 2 (R=1/I=2-3/S=4-6)		
		L. perenne	LB	*L. multiflorum*	*L. perenne*	LB	LM
A :	Exp.	R=50/S=50			R=50/I+S=50		
	Obs.	R=79/S=21	R=80/S=20	R=82/S=121	R=46/I+S=54	R=65/I+S=35	R=36/I+S=167
	c^2	90.660	45.125	109.192	1.362	11.281	84.537
	P	0.000	0.000	0.000	**0.243**	0.001	0.000
B :	Exp.	R=75/S=25			R=75/I+S=25		
	Obs.	R=79/S=21	R=80/S=20	R=82/S=121	R=46/I+S=54	R=65/I+S=35	
	c^2	2.547	1.500	129.67	115.483	7.042	355.049
	P	**0.110**	**0.221**	0.000	0.000	**0.008**	0.000
C :	Exp.	R=25/S=75			R=25/I=50/S=25		
	Obs.	R=79/S=21	R=80/S=20	R=82/S=121	R=46/I=44/S=10	R=65/I=20/S=15	R=36/I=99/S=68
	c^2	415.881	204.167	25.657	76.109	109.547	10.212
	P	0.000	0.000	0.000	0.000	0.000	**0.006**

In the second approach, in which plants with crown rust score 1 are considered resistant, 65% of the population belongs to the resistant group, 20% to the intermediate susceptible group and 15% to the susceptible group. It was difficult to accept one of the three hypotheses based on a χ^2 test. The highest p-value obtained (0,008), was in the case of hypothesis B : resistance explained by two genes of which the alleles do not show interaction. This was the same hypothesis selected using approach 1.

For the *L. x boucheanum* population, we hypothesize the presence of two major genes of which the resistance alleles do not show interaction. As in the

case of the *L. perenne* population, residual variability can be explained by the action of minor genes.

2.4.5.3 Number of genes present in the L. multiflorum *population*

The same approach as employed in two previous populations was used to identify the number of genes involved in resistance. None of the proposed hypotheses fits with the phenotypic data collected in the *L. multiflorum* population (Table 2.5). The highest significance was found in hypothesis C (2 additive dominant genes) indicating that resistance in the *L. multiflorum* population is inherited quantitatively; we considered that a number of minor additive genes confers resistance in this population.

2.4.6 Further dissection of inheritance of resistance in the *L. perenne* and *L. x boucheanum* population

In order to confirm the stated hypotheses on the inheritance of resistance in the *L. perenne* and *L. x boucheanum* populations, selfings and crossings between resistant F_1 plants were made (Table 2.6).

Although the plants used in the crosses were highly related, a good seed set was obtained in most cases. However, a high percentage of mortality within the seedlings was observed. In Fig. 2.9, the frequency distribution of the mean crown rust score observed in the viable plants is given. Number of viable plants obtained from 96 seeds is given.

Table 2.6 : Crossings and selfings made between resistant F_1 individuals of the
L. perenne (19528, 19698, 19588 and 19574) and *L. x boucheanum* population
(19807, 19907, 19784, 19785). Seed yield is indicated.

	Pair-cross		Selfing	
	Plants	Seed yield	Plants	Seed yield
L. perenne	19528*19698	2.8 grams	19528*19528	0.3 grams
	19588*19574	4.9 grams	19698*19698	7.3 grams
L. x boucheanum	19807*19907	1.2 grams	19807*19807	0.5 grams
	19784*19785	0.6 grams	19907*19907	0.6 grams
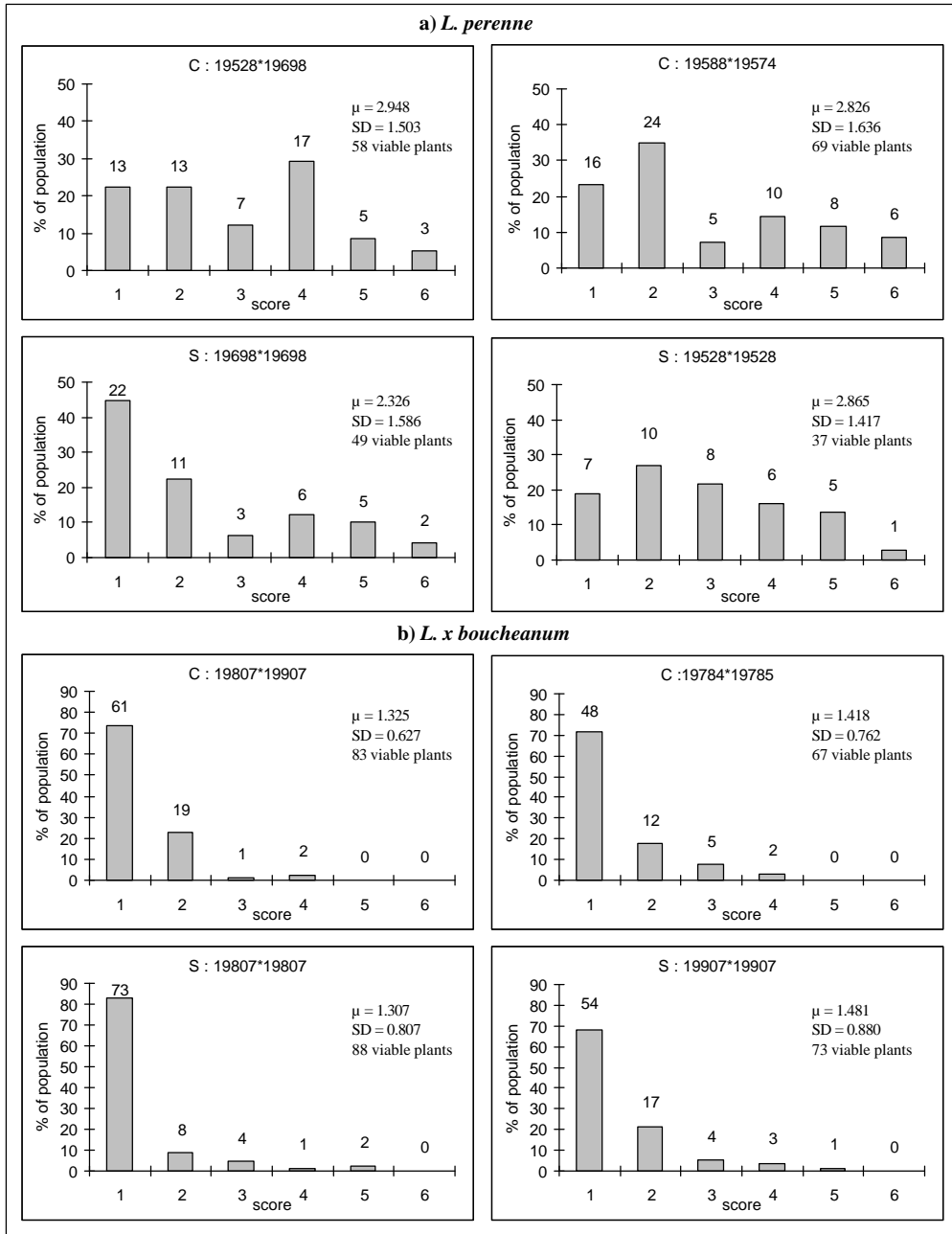
Fig. 2.9 : Frequency distribution of mean crown rust scores of populations obtained by crossing (C) or selfing (S) resistant F_1 plants of the *L. perenne* or *L. x boucheanum* populations. The crown rust score varies from 1 (resistant) till 6 (susceptible). The number of plants representing the percentage are noted on top of the bars. The number of viable plants obtained from 96 seeds is represented.

In the *L. perenne* crosses, the survival of the offspring was too low to draw conclusions about the inheritance of resistance. The high rates of mortality, ranging between 28% (19588*19574) and 61% (19528*19528), were probably due to inbreeding depression and low viability of the plants. However, if the conclusion of the phenotypic analysis of the F_1 population was that one or two dominant genes, showing no interaction were involved in resistance, we can speculate about the segregation in the selfings. If one gene is involved, resistant F_1 plants contain at that gene one dominant resistance allele and one susceptibility allele. Selfing this F_1 individual leads to a segregation of three resistant plants to one susceptible plant. If two genes are involved, then two types of resistant F_1 individuals can be found : individuals containing at both genes the dominant resistance allele and individuals containing just at one gene the resistance allele. In the first case, selfing will lead to a population segregating in 15 resistant plants to one susceptible plant. In the second case, a 3 to 1 ratio is expected. Looking at the frequency distributions, a 15 to one ratio is never reached. The ratio obtained in 19698*19698 (2.06:1) is the closest to a 3 to one ratio; all the other ratios obtained are less than 3:1. One explanation can be that plants with two resistance alleles at one gene (homozygous for the resistance gene), do not display a resistant phenotype or that they possess a reduced viability or reduced fitness. This reduced viability could be caused by associations between resistance alleles and viability genes in this population. This means that the 15:1 and the 3:1 ratios change to a 1:1 ratio. This ratio fits more to the frequency distributions found. However, these speculations should be taken with care as a high mortality rate in the four crosses was observed.

For the *L. x boucheanum* crosses, the offspring displayed a good viability. In all cases, the frequency distributions of the mean crown rust score are skewed towards resistance. This was as expected, as crosses and selfings were made with resistant plants. As we could not retain a hypothesis in approach 2 in the *L. x boucheanum* F_1 population, only approach 1 is used to explain the frequency distributions obtained in these F_2 populations.

According to approach 1, in which plants with score 1 or 2 are considered resistant, we hypothesized that in this *L. x boucheanum* population two genes, of which the resistance alleles do not show interaction were present (see 2.4.5.2). Following this hypothesis, and as already explained for the *L. perenne* population, we expect after selfing a F_1 individual a segregation of 3:1 or 15:1. For 19907, the observed ratio is 9:1 and for 19807, the ratio is 11.5:1. The

observed segregations of the selfing of 19907 and 19807 fit well the expected 15:1 segregation ($\chi^2 = 0.436$ and 2.026 with P-values 0.509 and 0.105) and we can thus conclude that for both selfed plants (19907 and 19807) at the two genes a resistance allele is present.

In the cross 19907*19807, we then expect to find again a 15:1 segregation. The observed segregation is 24:1, which fits the proposed model ($\chi^2 = 0.984$ with a P-value of 0.321). This confirms again our previous conclusion of both plants having a resistance allele at the two major genes.

2.5 Conclusions

Three F_1 populations, consisting of an acceptable number of plants for statistical analysis were created. No maternal effect on crown rust resistance was present in the three selected populations. A good correlation was found between the three scoring dates but, as expected, lower correlation was found between the score observed in the field and the mean score obtained using the artificial inoculation test. We should bear in mind that the results of this study are based on phenotypic analysis on seedlings using one spore mixture in an artificial inoculation method. This is a good starting point to analyse crown rust resistance in *Lolium* populations, as the environmental conditions are controlled and an uniform spore mixture is used. However, it is very interesting to compare these results with phenotypic data obtained on adult plants, using different spore mixtures or single spore isolates and in different environments. This was however beyond the scope of this thesis.

For each F_1 population, hypotheses on the number of genomic regions involved in crown rust resistance were put forward. In the *L. perenne* population, we found that resistance was conferred by one or two genes, of which the resistance alleles are dominant and do not interact. For the *L. x boucheanum* population, we could conclude that the resistance was conferred by two major genes, of which the resistance alleles are dominant and do not show interaction. After considering the results of the crossings and selfings of F_1 plants in the *L. x boucheanum*, the proposed hypothesis was confirmed. As *Puccinia* spp. are biotrophic fungi, we expected to find genes that display characteristic gene-for-gene specificity. Wise et al. (1996) stated that this kind of genes are often found in the resistance of monocotyledonous species to obligate

fungal biotrophs, such as *Zea mays* to *Puccinia sorghi*, *Triticum aestivum* to *Puccinia* spp. and *Hordeum vulgare* to *Erysiphe graminis*. However, phenotypic data for the two studied populations demonstrate that the action of minor genes should be taken into account. These genes are responsible for the spreading within classes.

In the *L. multiflorum* population, a normal distribution of the mean crown rust score was found, indicating that the resistance present in this population was probably conferred by several additive genes with no major effects.

The resistance found in the *L. perenne* and *L. x boucheanum* population resembles most to the resistance reported by Schmidt (1980). The resistance found in the *L. multiflorum* population resembles more the resistance reported by Wilkins (1975) and Hayward (1977). No recessive resistance alleles were identified in the constructed populations.

In order to confirm and to study the genetic background of the resistance mechanisms found more in detail, DNA markers will be used to identify genomic regions involved in the trait. Identification of markers linked to resistance genes combines the use of detailed genetic maps (QTL analysis) and targeted mapping strategies (BSA). For QTL analysis, the trait should follow a Gaussian distribution. BSA, on the other hand, is limited to monogenic traits or to major genes of which the action is modified by minor genes (Lefebvre & Chevre, 1995). These two approaches could be complementary in the search for molecular markers linked to nuclear factors involved in crown rust resistance. Three scenarios are possible:

1) we are dealing with an oligogenic trait : a BSA approach should be appropriate to identify molecular markers linked to the gene responsible for the resistance;

2) we are dealing with a trait controlled by one or a few major genes modified by numerous minor genes : a BSA approach where just the plants with the most extreme crown rust scores are included could help to identify markers linked to major resistance genes. A QTL approach could help to identify the chromosome regions where minor genes are located;

3) in the case we are dealing with a pure polygenic trait, a BSA approach should be unsuccessful and only a QTL analysis should help to identify the location of the genes involved.

As we are dealing with scenario 2 in the *L. x boucheanum* and *L. perenne* population, a BSA and QTL analysis will be applied on both populations. For the *L. multiflorum* population, scenario 3 is appropriate, however, a BSA analysis is used as confirmation of the hypotheses formulated in the present chapter and to select the most informative AFLP primer combinations for linkage map construction.

Chapter 3

Identification of markers linked with crown rust resistance using Bulk Segregant Analysis

3.1 Introduction

In the previous chapter, inheritance of crown rust resistance in populations of *L. perenne*, *L. multiflorum* and *L. x boucheanum* was discussed. The trait was semi-quantitatively or quantitatively inherited, depending on the population. In this and the following chapters, DNA-marker technology is used for confirmation of the observed inheritance and further dissection of the genetic components involved in crown rust resistance in the three populations selected.

3.1.1 Use of molecular marker technology

Plant breeders typically have little information on the number of genetic factors controlling the expression of relevant agronomic traits, the chromosomal location of these loci, the relative contribution of individual loci to trait expression, pleiotropic effects, epistatic interactions among genetic factors in different environments etc. (Stuber et al., 1999). The advent of molecular marker technologies has opened a door for the dissection and genetic characterisation of complex traits and for the identification of beneficial allelic variants. Marker assisted selection (MAS) helps breeders to manipulate more efficiently and effectively. Introgression programmes can benefit greatly from MAS. Indeed, the use of markers allows significant gains in terms of generations required to reduce the donor genotype to a minimum in the introgressed line and allows the introgression of shorter chromosome segments. MAS enables also the accumulation of multiple beneficial alleles for example to pyramid disease resistance or improve traditional traits which have a comparatively long history selection (Humphreys, 2000). MAS becomes effective when phenotyping in conventional breeding is costly, time-consuming and dependent on environmental conditions (William et al., 1997; Chantret et al., 2000) and when the trait is influenced by different genetic components whose individual detection is labour-intensive (Kumar, 1999). Marker

technology has already been extensively used to dissect disease resistances by means of QTL analysis (for an overview Kover & Caceido, 2001). These QTLs can be readily used for practical breeding and selection.

Next to identifying the different genetic components and practical breeding applications, markers are used for fine mapping of the genes responsible for the trait (Chantret et al., 2000). In major crops with genomes well characterized with molecular markers, it is possible to select markers that are evenly dispersed over the genome. This framework serves as a starting point in linkage analysis between markers and trait. Once the linked markers are found, one can situate the responsible genes on a genetic map and saturate the genomic region of interest with markers to fine-map the gene(s) involved in the trait expression. The precise location of genes enables the study of the homoeoallelic relationships between disease resistance genes among genomes (Chantret et al., 2000). Several resistance genes with specific functions have already been isolated using molecular markers (for an overview Kumar, 1999). For example, Schwarz et al. (1999) were able to isolate the *Mla* locus which determines resistance to the powdery mildew pathogen *Erysiphe graminis* in barley.

In the first reports on tagging resistance genes, RFLPs (restriction fragment length polymorphism) were used. Later on, PCR-based marker techniques such as RAPD (random amplified polymorphic DNA) were introduced (Michelmore et al., 1991; Chagué et al., 1997). SSR (simple sequences repeats) markers amplifying short sequence repeats, are now the most preferred in linkage studies. Chantret et al. (2000) located the powdery mildew resistance gene *MIRE* on the genome of wheat using SSRs. However, only a small number of primer sequences to amplify SSR loci in ryegrasses are publicly available (Kubik et al., 1999; Jones et al., 2001; Kubik et al., 2001). We will use thus AFLPs (amplified fragment length polymorphisms; Vos et al., 1995) as this PCR-based marker technique enables the analysis of a large number of marker loci in a single PCR without the necessity of prior knowledge about genomic sequences. This universally applied technique in linkage and association studies provides dominant markers with polymorphisms detected as the presence or absence of amplified fragments (f.e. Cervera et al., 1996; Bai et al., 1999; Tang et al., 2000).

3.1.2 Bulk Segregant Analysis

In order to find a marker-trait linkage, extensive genotyping is required. To reduce the amount of genotyping work, Bulk Segregant Analysis (BSA) is the classical way to find markers linked to a trait under monogenic control (Michelmore et al., 1991). The method involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross (see Fig. 3.1). Within each pool, or bulk, the individuals are identical for the loci of interest but are arbitrary for all other loci. Or in other words, each pool contains individuals selected to have identical genotypes for particular loci but random genotypes at unlinked loci. These two pools are analysed to identify markers that distinguish them. Markers that are polymorphic between the pools are candidate markers to be linked to the loci involved in the trait of interest (Michelmore et al., 1991).
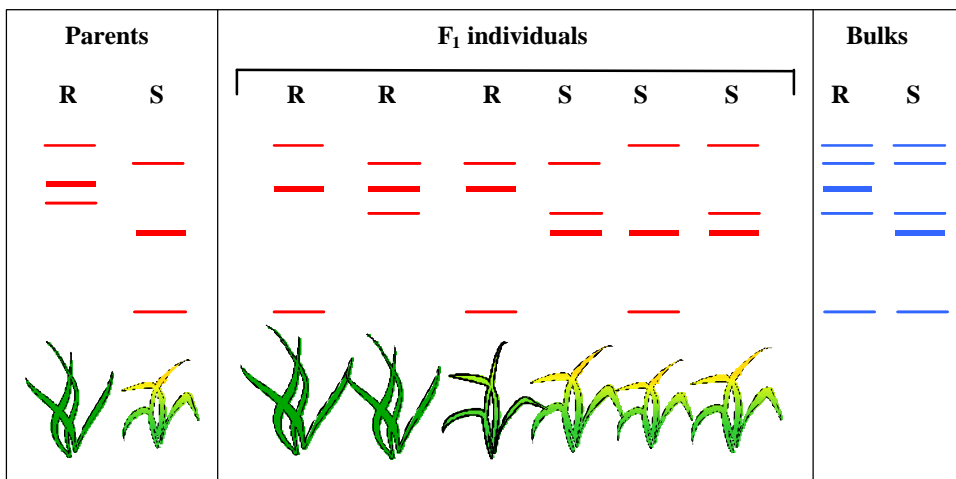
Fig. 3.1 : Bulk Segregant Analysis is used to identify markers linked with the characteristic of interest, f.e. disease resistance. Fingerprints of the resistant (R) and susceptible (S) parent are compared with the fingerprints of a bulk derived from resistant F_1 individuals (R bulk) and a bulk derived from susceptible F_1 individuals (S bulk). Markers potentially linked with resistance or susceptibility are indicated in bold.

BSA is a valuable approach that avoids the necessity to genotype each member of a population, which is time consuming when no beforehand knowledge on good candidate markers exists. The only prerequisite is the

availability of a population resulting from a cross that segregates for the trait of interest. The success of the approach will depend on the genetic divergence between the parents in the target region.

BSA assumes that markers adjacent to the targeted locus will be in linkage disequilibrium among themselves and with respect to the trait (i.e., recombination will not have randomised these markers with respect to the targeted locus). As the genetic distance increases, more recombinants will be present in each bulk, culminating in 50% recombinants, no linkage disequilibrium, and therefore resulting in no differences between the bulks. Several factors should be taken into consideration when planning and interpreting a BSA experiment:

- The size of the bulk is related to the probability for unlinked loci (false positives) to be detected as polymorphic between the bulks. This in turn will depend on the type of marker being screened (dominant or co-dominant) and the type of population used to generate the bulks (F_2, backcross, full sib, etc.). For example, for a dominant marker segregating in a F_2 population the probability of a bulk of n individuals having a band and a second bulk of equal size not having a band will be $2(1-(1/4)^n)(1/4)^n$, with n= bulk size. In this case, the probability to misclassify an unlinked marker as linked marker using bulks of 10 individuals is 2×10^{-6}. Thus, the chance of detecting a polymorphic unlinked locus (false positive) is small, even when many loci are screened. The smaller the bulks, the higher the frequency of false positives (Michelmore et al., 1991). The *Lolium* populations used in this study, were created by crossing two heterozygous plants. Using a dominant marker system like AFLP, only the back cross markers (BC), heterozygous in one parent plant and absent in the other parent are interesting for BSA. The chance to detect an unlinked locus being polymorphic between bulks of 10 individuals is for a BC marker in this kind of segregating population $2(1-(1/2)^n)(1/2)^n$ or 2×10^{-3}. Therefore, the chance of misclassification (false positives) is higher in the *Lolium* populations used in the present study than in a standard F_2 population. This factor should be taken into account during the interpretation of the results.

- The width of the genetic window around the target locus, from which markers will be detected as linked with the target, depends on the sensitivity of the marker technique to detect rare alleles in the bulks. In

practice, alleles as prevalent as a proportion of 0.1 of a mixture were barely detectable using RAPD and were never detected if they constituted a proportion of 0.04 or less of the total (Michelmore et al., 1991). Depending on the band, polymorphisms have been detected when the rarer allele constituted proportions up to 0.2-0.4 of the mixture, at least as differences in band intensities. Therefore, in a F_2 population using RAPD markers, loci within 15cM of the target loci are likely to be identified by BSA. Many markers within a 30% recombination window will also be detectable, at least as bands of unequal intensity (Michelmore et al., 1991; Quarrie et al., 1999); with AFLP comparable results were obtained (I. Roldán-Ruiz, CLO-DvP, personal communication), however the interpretation of differences in band intensities was not always reliable.

The width of the genetic window will also depend on the segregating population used. Bulks made from backcross populations provide greater focus around the region of interest than F_2 populations, which provide maximal genetic width of the region screened for polymorphism. If sufficient individuals are pooled to form each bulk, the genetic window will be symmetrical around the target locus; this is in contrast to the region around a locus selected during the generation of NILs, which may be extremely asymmetrical (Michelmore et al., 1991). In the *Lolium* populations used in the present study, a maximal genetic width of the region is expected, as the parents are unrelated and highly heterozygous.

3.1.3 BSA for QTL identification

BSA was originally used to detect markers linked with a trait under monogenic control, but it can also be used to identify QTLs (O'Donoughue et al., 1996; Chagué et al., 1997; William, 1997). If a quantitative trait is controlled by a few major genes, comparison of bulks of individuals with extreme phenotypes could rapidly identify markers linked to these QTLs. This could be made more powerful by progeny testing the extreme phenotypes and discarding those that do not show heritable variation.

Using BSA as a technique to identify markers linked to a QTL, one should take into account that the QTL has a sufficiently large effect. Hill (1998) stated that BSA is likely to produce uniform selected groups for the QTL only if the magnitude of its effect approaches two times the standard deviation. In this

way, there is a high probability that the high and low bulks each comprise almost all individuals of the same genotype; then the two bulks differ clearly in genotype profile for markers closely linked to major QTLs.

3.2 Objectives and rationale

The objectives within Chapter 3 were to identify markers linked with crown rust resistance in the three selected *Lolium* populations (*L. perenne* TC1*SB2, *L. x boucheanum* 2A2*1B12 and *L. multiflorum* Axis-3*b-90). In the previous chapter, the inheritance of crown rust resistance was found to be semi-quantitative or quantitative, depending on the population considered. To identify markers linked with the major genes involved in crown rust resistance, a BSA approach was applied in each of the selected populations. For this purpose, bulks were constructed with individuals displaying extreme phenotypes. The fingerprints of these bulks were compared with the fingerprints of the parents used to perform the cross. AFLP was the chosen marker technique. Markers selected in this comparison were validated by testing their segregation on the samples used to construct the bulks, individually. Promising markers were tested on the whole F_1 population.

As these data sets are non-parametric, alternative statistical analyses were used to evaluate the degree of linkage between the markers and the trait. Finally, markers were evaluated for their use in MAS.

3.3 Material and methods

3.3.1 Plant material and DNA extraction

The three populations selected and described in Chapter 2 were used for BSA (offspring of the crosses *L. perenne* TC1*SB2, *L. x boucheanum* 2A2*1B12, *L. multiflorum* Axis-3*b-90). The results of the phenotypic evaluation were described in Chapter 2. Before phenotypic evaluation, young leaf material was harvested, frozen in liquid nitrogen, lyophilised and stored under vacuum conditions. About 100 mg of lyophilised material was grinded using a mill (Retsch MM200). Genomic DNA was prepared using a modified CTAB protocol (Weising et al., 1991).

3.3.2 AFLP analysis

AFLP analysis was performed according to Vos et al. (1995) with available kits. Two different enzyme combinations were tested: *Eco*RI/*Mse*I and *Hind*III/*Mse*I. 250 ng of genomic DNA was digested for 2 h at 37 °C in a final volume of 25 µl containing 10 mM MgOAc, 50 mM DTT pH 7.5, 2.5 U *Mse*I (Gibco BRL) and 2.5 U *Eco*RI (or *Hind*III) (Gibco BRL). Two adaptors, one for the *Eco*RI ends (or *Hind*III ends) and one for the *Mse*I ends, designed to avoid the reconstruction of the restriction sites, were ligated to the restriction fragments by adding 25 µl of a mix containing 5 pmol *Eco*RI adaptor (or *Hind*III adaptor), 50 pmol *Mse*I adaptor, 8 mM ATP, 10 mM Tris-HCl, 10 mM MgOAc, 50 mM DTT pH 7.5 and 1.4 U DNA ligase (Gibco BRL). The ligation mixture was incubated for 2 h at 37 °C. Ligation mixtures were diluted to 1/10 their initial concentration.

A preamplification step was performed with primers complementary to the *Eco*RI (or *Hind*III) and *Mse*I adaptors with an additional selective 3' nucleotide. The PCR reactions were performed in a 50 µl volume of 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 25 ng of each primer (Gibco BRL), 1 U *Taq* DNA polymerase (Boehringer) and 5 µl of the diluted ligation mixture. The PCR amplifications were carried out in a Hybaid Omni Gene cycler using 20 cycles consisting of 30 sec at 94 °C, 60 sec at 56 °C and 60 sec at 72 °C. The preamplification products were diluted to 1/10 their initial concentration to be used as starting material for the fluorescent selective amplification.

The PCR amplification mixture was composed of 3 µl diluted preamplification, 1 µl *Mse*I primer at 5 µM, 1 µl *Eco*RI (or *Hind*III) primer at 1 µM (fluorescent labelled) and 15 µl AFLP Core Mix (Perkin Elmer). The selective amplification was carried out with the following parameters : 1 cycle of 2 min at 94 °C, 30 sec at 65 °C, 2 min at 72 °C, followed by 9 cycles in which the annealing temperature decreases 1 °C per cycle, followed by 23 cycles of 1 min at 94 °C, 30 sec at 56 °C, and 2 min at 72 °C. At the end of the selective fluorescent PCR, the samples were denatured by adding 20 µl of formamide buffer and heating for 3 min at 90 °C. 1.5 µl of each sample was loaded on 5 % polyacrylamide/bisacrylamide 19:1 (Biorad), 7.5 M urea (Gibco BRL) and 1x TBE gels and analysed with an ABI Prism 377 DNA sequencer (ABI377; Perkin Elmer). GS-500 Rox labelled size standard was loaded in each lane in order to allow the automatic analysis of the data. Genescan Analysis Software 2.1 was

used to translate the information collected by the ABI377 into fragment sizing information and Genotyper 2.5 was used to score the fingerprints (Roldán-Ruiz *et al.*, 2000). Only AFLP fragments in the range 75 bp to 450 bp were used for analysis.

3.3.3 Bulk Segregant Analysis

For each population, resistant and susceptible bulk samples were constructed by pooling of preamplified DNA of at least ten F_1 individuals displaying extreme phenotypes. Prior to bulking, the quality of the preamplification products of each plant was checked by performing one selective amplification. Intensity of the obtained fingerprints after selective amplification had to be identical over the different individuals of the bulks. In this way we ensured that the contribution of each individual component of the bulk was equal.

The composition of the bulks for each population is given in Table 3.1. Resistant bulks consisted of at least 10 plants with score 1 in each of three subsequent inoculation rounds (see Chapter 2 for details on phenotyping). Susceptible bulks consisted of at least 10 plants having a mean phenotypic score differing two times the standard deviation of the population from the score of the plants selected in the resistant bulk. The threshold for the susceptible plants was set in this way to be able to identify markers linked to QTLs with major effects (Grattapaglia et al., 1996; Hill W., 1998).

For the *L. x boucheanum* and *L. perenne* populations, three bulks were made: two resistant bulks and one susceptible bulk. One resistant bulk consisted of 12 (*L. perenne*) or 10 (*L. x boucheanum*) resistant plants harvested on the resistant parent. The other bulk consisted of 12 (*L. perenne*) or 10 (*L. x boucheanum*) resistant plants harvested on the susceptible parent. The susceptible bulk consisted of the 12 (*L. perenne*) or the 9 (*L. x boucheanum*) plants from both seed lots showing the highest susceptibility.

For the *L. multiflorum* population, only two bulks were constructed. The resistant bulk consisted of 13 resistant plants having a mean crown rust score of 1. The susceptible bulk consisted of 10 susceptible plants showing the highest susceptibility in the population.

Table 3.1: Composition of BSA bulks for each population. The crown rust score is given per plant and per subsequent round of inoculation (crown rust score 1, 2 and 3).

Population	Resistant bulk 1 = R_1				Resistant bulk 2 = R_2				Susceptible bulk = S			
	Plant number	Rust score			Plant number	Rust score			Plant number	Rust score		
		1	2	3		1	2	3		1	2	3
L. perenne	19484	1	1	1	19665	1	1	1	19476	4	3	4
(TC1*SB2)	19502	1	1	1	19676	1	1	1	19498	4	4	4
	19519	1	1	1	19685	1	1	1	19508	4	2	6
	19528	1	1	1	19695	1	1	1	19512	3	4	2
	19539	1	1	1	19698	1	1	1	19527	2	4	5
	19560	1	1	1	19703	1	1	1	19535	5	3	4
	19570	1	1	1	19713	1	1	1	19684	5	4	5
	19588	1	1	1	19716	1	1	1	19702	6	2	4
	19603	1	1	1	19738	1	1	1	19711	4	4	5
	19622	1	1	1	19747	1	1	1	19720	3	4	6
	19645	1	1	1	19754	1	1	1	19746	3	3	5
	19651	1	1	1	19762	1	1	1	19757	4	3	5
L. x boucheanum	19775	1	1	1	19773	1	1	1	19774	4	5	5
(2A2*1B12)	19799	1	1	1	19779	1	1	1	19786	6	4	5
	19822	1	1	1	19784	1	1	1	19788	3	6	4
	19824	1	1	1	19785	1	1	1	19805	6	5	6
	19864	1	1	1	19791	1	1	1	19814	5	6	6
	19872	1	1	1	19807	1	1	1	19815	6	4	5
	19873	1	1	1	19819	1	1	1	19837	4	6	5
	19879	1	1	1	19832	1	1	1	19899	6	4	5
	19895	1	1	1	19845	1	1	1	19900	6	5	5
	19907	1	1	1	19849	1	1	1				
L. multiflorum	19159	1	1	1	Only one R bulk was				19161	6	6	6
(Axis3*b-90)	19181	1	1	1	made				19163	6	5	6
	19211	1	1	1					19177	5	5	5
	19268	1	1	1					19220	5	5	5
	19279	1	1	1					19286	6	6	6
	19299	1	1	1					19293	5	6	5
	19308	1	1	1					19295	5	6	6
	19312	1	1	1					19310	6	6	6
	19319	1	1	1					19418	5	6	6
	19329	1	1	1					19459	5	6	6
	19344	1	1	1								
	19408	1	1	1								
	19409	1	1	1								

AFLP fingerprints of the bulks and the parents were compared. The primer combinations yielding candidate markers were subsequently tested on each individual plant of the bulks. The most promising markers were further analysed on all the F_1 individuals of the population to confirm the marker-trait linkage.

3.3.4 Statistical analysis

The data generated in the BSA analysis is non-parametric; marker information is discrete binary data and crown rust information is categorical data (from 1 till 6). As a consequence, statistics for normally distributed data like one-way analysis of variance or linear regression cannot be applied, in strict statistical terms. Permutation testing as described by Churchill and Doerge (1994) is a nonparametric approach suited for genetical analyses when classical assumptions of normality or large sample sizes are violated. Permutation tests as described in Churchill and Doerge (1994) were designed by Moerkerke et al. (University of Gent, personal communication) in S-Plus 6.0 professional release 2 (Insightful Corp.).

For each candidate marker tested on the whole population, the marker – trait linkage was analysed. Per marker, an original T_0 test statistic (the standardized regression coefficient from a simple linear regression model, equivalent to a t-test) was calculated. Then, the trait values were randomly permuted among the progeny, destroying the relationship between the trait values and the genotype of the marker locus in the observed data. A new test statistic was estimated on the permuted data set and the test statistic obtained in each permutation was recorded. This procedure was repeated numerous times on several such randomly permuted data sets, giving a distribution of permuted test statistic values expected if there was no linkage between the marker and the trait. Values at appropriate percentile points of the empirical distribution can be used as test statistic threshold values to establish significance of the observed T_0. We tested at the two-sided significance level $\alpha=0.05$. Thus the 2.5 and 97.5 percentile value are the empirical thresholds for significance. Since it is computationally impossible to perform all permutations in order to obtain the exact null distribution of the test statistic, we used the approach as presented by Nettleton and Doerge (2000) and construct 95% confidence intervals (CI) for the 2.5 and 97.5 percentile values. Based on a sample of 800 permutations, markers having a T_0 statistic > 97.5 percentile or < 2.5 percentile and situated outside the CI of the critical threshold values, display a significant linkage with crown rust resistance or susceptibility. These markers were retained for further analysis.

Simple linear regression was used to calculate the coefficient of determination, R^2. For each selected marker, this coefficient provides a measure of the proportion of the total variance explained by each candidate marker or provides a description of the magnitude of the marker-linked phenotypic effect.

Multiple linear regression, including in the model all the markers identified as linked to crown rust resistance, was used to calculate partial R^2, to confirm which loci may be tagging the same genomic region, and the total R^2, to measure the total proportion of phenotypic variation explained by those loci (William et al., 1997). Regression analyses were performed using the package SPSS (Norusis, 2000).

Finally, recombination frequencies between candidate markers were calculated using the linkage software Joinmap version 3.0 (van Oijen and Voorrips, 2001).

3.4 Results and discussion

3.4.1 Phenotypic data

To identify QTLs for disease resistance much attention should be paid to the accurate phenotyping of the plant material used. Consistent disease pressure is critical for accurate assessment of the resistance potential of plant genotypes and for determination of the magnitude of the genetic factors that contribute to resistance (Bai et al., 1999). This is especially important for crown rust, since environment is one of the major determining factors for initiation and development of crown rust infection. Phenotyping of the three populations studied here was discussed in Chapter 2. In this study, favourable temperature and moisture conditions were provided in greenhouses under controlled conditions to minimize environmental effects on crown rust initiation and development. A high degree of consistency, as shown in Chapter 2, makes the scoring method suitable for generating the phenotypic data for QTL analysis.

3.4.2 Bulk segregant analysis

The AFLP marker technique was used in the BSA analysis, as it is a rapid technique, generating in an efficient way a high number of markers with the use of small amounts of DNA. In this study, more than 60 AFLP fragments were amplified per primer combination (PC). Each PC that amplified a clear band profile, revealed polymorphisms between the two parents. These results indicate that the AFLP is a suitable technique for the purposes of our study.

Two combinations of restriction enzymes were tested (*Hind*III-*Mse*I and *Eco*RI-*Mse*I). Per restriction enzyme combination, two preamplifications were performed, using different selective nucleotides. A summary of primer combinations (PC) tested in the different populations is given in Table 3.2. Parents and bulks of the *L. perenne, L. x boucheanum* and *L. multiflorum* populations were screened using in total 187, 94 and 224 PC respectively (third column in Table 3.2).

Table 3.2 : Number of primer combinations (PC) tested in the 3 populations during BSA. Number of selected markers putatively linked to crown rust resistance (R) or susceptibility (S) is given.

a) *Lolium perenne* **TC1*SB2**

Restriction enzyme combination	Preamplification	Nr. of PC tested	Nr. of selected markers			Nr. of selected markers/PC
			R-linked	S-linked	total	
*Eco*RI-*Mse*I	*Eco*RI+A-*Mse*I+C	43	42	23	65	1.51
	*Eco*RI+A-*Mse*I+G	44	26	19	45	1.02
*Hind*III–*Mse*I	*Hind*III+T-*Mse*I+C	48	74	37	111	2.31
	*Hind*III+T-*Mse*I+G	52	35	11	46	0.88
Total		187	177	90	267	1.43

b) *Lolium x boucheanum* **2A2*1B12**

Restriction enzyme combination	Preamplification	Nr. of PC tested	Nr. of selected markers			Nr. of selected markers/PC
			R-linked	S-linked	total	
*Eco*RI-*Mse*I	*Eco*RI+A-*Mse*I+C	24	27	5	32	1.33
	*Eco*RI+A-*Mse*I+G	24	34	20	54	2.25
*Hind*III–*Mse*I	*Hind*III+T-*Mse*I+C	22	25	51	76	3.45
	*Hind*III+T-*Mse*I+G	24	31	6	37	1.54
Total		94	117	82	199	2.12

c) *Lolium multiflorum* **Axis-3*b-90**

Restriction enzyme combination	Preamplification	Nr. of PC tested	Nr. of selected markers			Nr. of selected markers/PC
			R-linked	S-linked	total	
*Eco*RI-*Mse*I	*Eco*RI+A-*Mse*I+C	58	107	88	195	3.36
	*Eco*RI+A-*Mse*I+G	64	51	76	127	1.98
*Hind*III–*Mse*I	*Hind*III+T-*Mse*I+C	49	143	113	256	5.22
	*Hind*III+T-*Mse*I+G	53	121	82	203	3.83
Total		224	422	359	781	3.49

As an average over the three populations, 35 AFLP fragments/PC were polymorphic between the parents. This high amount of polymorphisms can be explained by the heterozygosity and heterogeneity of the parent plants. This

means, e.g for the *L. perenne* population that 187 PC, generating 35 polymorphic markers/PC, screen 6545 polymorphic markers (187 PC * 35 markers/PC).

Markers linked with resistance or susceptibility were selected (Fig. 3.2). An overview of the marker selection procedure in the BSA analysis is given in Table 3.3. The main difference between selection in the *L. multiflorum* and the other two populations is that in the *L. perenne* and *L. x boucheanum* population, markers had to be present in two different R bulks while in the *L. multiflorum* population only one R bulk was constructed (Table 3.3).

Fig. 3.2 : AFLP PC 42 (E-ACG-M-CTA) generating markers putatively linked with resistance or susceptibility in the *L. multiflorum* population. Four fingerprints are compared: a) the resistant parent Axis3, b) the susceptible parent b-90, c) the resistant bulk and d) the susceptible bulk. Retained fragments are given three labels : first the fragment size (bp), second the peak height and third the peak modulation. A fragment of 91 bp is putatively linked with resistance as it is present only in fingerprints a and c. Three fragments of 191 bp, 286 bp and 313 bp are putatively linked with susceptibility as they are present only in fingerprints b and d.

Table 3.3 : Marker selection procedure used in the BSA analysis. ('+': AFLP fragment present; '-': AFLP fragment absent; '/': not applicable.)

Population	Linkage	Presence of AFLP-marker				
		R parent	S parent	R_1 bulk	R_2 bulk	S bulk
L. perenne	R	+	-	+	+	-
	S	-	+	-	-	+
L. x boucheanum	R	+	-	+	+	-
	S	-	+	-	-	+
L. multiflorum	R	+	-	+	/	-
	S	-	+	-	/	+

In Fig. 3.3, the frequency distribution of the number of informative markers selected per PC is represented for each population. The strategy in the *L. multiflorum* population was less stringent and resulted in the detection of more candidate markers (including a high percentage of false positives) in comparison to the other two populations. The average number of markers selected in the *L. perenne* (1.4 markers/PC) and *L. x boucheanum* populations (2.1 markers/PC) was lower than in the *L. multiflorum* population (3.5 markers/PC). Indeed, by using two bulks, a more stringent criterion is applied for the selection of markers. This controls for false positives. However Grattapaglia et al. (1996) reported a reduction of detection power, when using more bulks.

The efficiency of a BSA to identify putatively linked markers can be measured by the frequency at which markers, polymorphic in the parent plants, show differences between bulks. Under our conditions, we screened in the *L. perenne*, *L. x boucheanum* and *L. multiflorum* population, respectively 6545, 3290 and 7840 markers that were polymorphic between the parents. If we consider that 1) AFLP markers are distributed uniformly throughout the genome, 2) the length of the *Lolium* genome is 1190 cM (Naylor, 1960; Hayward et al., 1998) and 3) we are looking for a marker within a window of 10% recombination either side of the target locus, we expect 1.9% of the polymorphic loci to fall within a window of 20 cM around the target locus, and to be linked to the target gene. The observed percentages for the *L. perenne*, *L. x boucheanum* and *L. multiflorum* population were 9.96%, 4.07% and 6.04% respectively. Several factors can contribute to these higher percentages than theoretically expected. A first reason can be the high level of heterozygosity and heterogeneity in the parent plants. In this specific kind of cross the chance of detecting false positives is 2×10^{-3}. This is higher than in a BSA analysis on a F_2 population generated from two homozygous parent plants. Secondly, the

number of markers does not always reflect the number of loci analysed. This is especially the case when dominant markers are used. As AFLP is a dominant marker system, different AFLP markers may be derived from the same locus. Finally, these higher levels of polymorphism can be due to the fact that more than one locus is segregating for resistance in the populations, as demonstrated in the previous chapter. In the *L. multiflorum* population, one more reason has contributed to the higher percentage of markers selected. This is the fact that only one R bulk was used in the BSA, instead of two in the *L. perenne* and *L. x boucheanum* population.
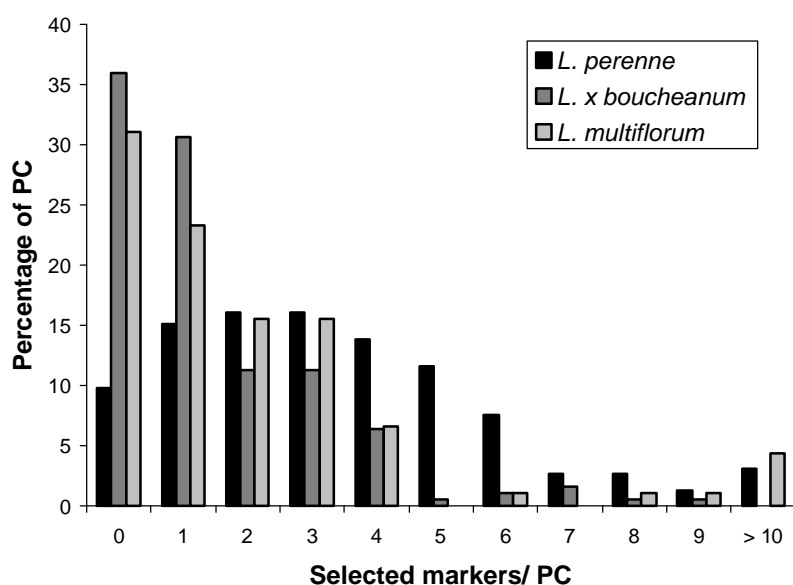
Fig. 3.3 : Frequency distribution of number of selected markers/PC in the three studied populations.

3.4.3 Analysis of selected markers on bulk individuals and all individuals.

In a second screening, the most promising polymorphisms observed in the parents and bulks were confirmed by testing their behaviour in all the individuals used to construct the bulks. For each preamplification series (*Eco*RI+A/*Mse*I+C, *Eco*RI+A/*Mse*I+G, *Hind*III+T/*Mse*I+C and

*Hind*III+T/*Mse*I+G), at least 3 PC were tested. Per population, a minimum of 18 PC were tested on the bulk individuals.

The results of the testing of PC on all bulk individuals are summarized in Table 3.4. A R-linked marker was retained when it was present in less than 40% of the S bulk individuals and present in more than 60% of the R bulk individuals. The reverse was used for S markers. In total, 40, 49 and 125 markers were tested in the *L. perenne*, *L. x boucheanum* and *L. multiflorum* populations respectively and 20, 32 and 7 markers were retained in the *L. perenne*, *L. x boucheanum* and *L. multiflorum* populations respectively. These results confirm our suspicion that most of the markers initially selected by the BSA analysis (see above) were false positives. Out of these, 4, 4 and 2 PC amplifying in total 6, 8, and 3 putatively R-linked markers in the *L. perenne*, *L. x boucheanum* and *L. multiflorum* cross respectively were chosen to be tested on all F_1 individuals (last column of Table 3.4).

Table 3.4 : Second round of marker selection by testing on all bulk individuals. R markers were retained if presence in the R bulk was higher than 60% and lower than 40% in the S bulk and vice versa for a S marker. A subsample of the retained marker was selected to be tested on the whole F_1 population.

Population	Marker type	# markers tested	# linked markers	markers tested on the whole population
L. perenne	R	34	18	PC008-R2 PC026-R2, PC026-R3, PC026-R4 PC106-R2 PC168-R1
	S	6	2	-
L. x boucheanum	R	45	31	PC008-R2, PC008-R3 PC078-R2, PC078-R3 PC157-R1, PC157-R2 PC400-R2, PC400-R3
	S	4	1	-
L. multiflorum	R	95	5	PC065-R1, PC065-R2 PC175-R2
	S	30	2	-

3.4.4 Linkage of markers with genetic factors determining crown rust resistance

As the BSA data are non-parametric (marker data is 1/0 and crown rust score data is categorical from 1 till 6), permutation tests, as described in materials and methods, were used to test for significant linkages between the markers and crown rust score. An example of a permuted test statistic distribution obtained after 800 permutations is given in Fig. 3.4. In this case, the linkage between crown rust resistance and marker PC106-R2 in the *L. perenne* population was tested for. The T_0 test statistic for this marker is –8.861, and falls outside the 95% CI of the 2.5 percentile of the permuted test statistic distribution. This indicates a significant (P=0.05) and positive linkage with crown rust resistance.
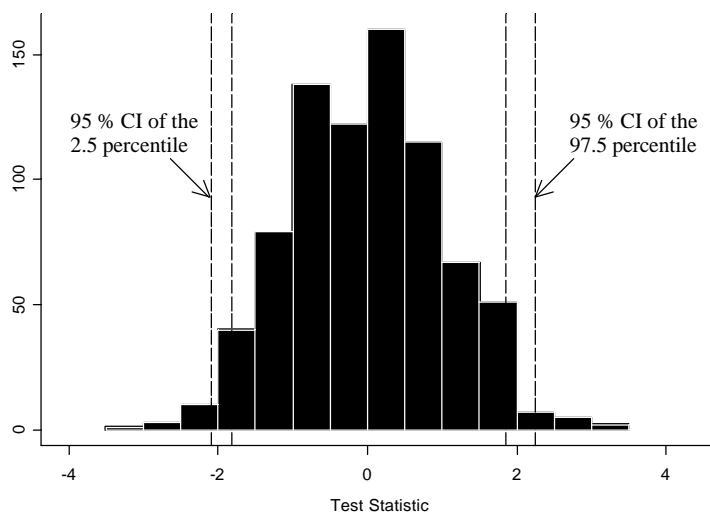
Fig. 3.4 : Statistical analysis of linkage between marker PC106-R2 and mean crown rust score in the *L. perenne* population. The null distribution of the permuted test statistics (800 permutations) is represented. The boundaries of the 95% CI of the 2.5 percentile and the 97.5 percentile (critical threshold values) are indicated. The T_0 test statistic of PC106-R2 is –8.861 and is more extreme than the CI of the 2.5 percentile of the distribution. This means that there is a positive and significant (P<0.05) linkage between the marker and the mean crown rust score in the *L. perenne* population.

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In Table 3.5, the original T_0 test statistics are given for all tested markers, as well as the 95% confidence intervals of the critical threshold values (2.5 percentile value and 97.5 percentile value). Linkage was considered to be significant only when in all cases (using the score of individual inoculation rounds and the average score) significant linkage was found.

Table 3.5 : Statistical analysis of the linkage between tested markers and crown rust scores (scores of the three inoculation rounds and the average score). Original test statistic values (T_0) of all selected markers are given. The upper and lower boundaries of the 95 % CI of the 2.5 and the 97.5 percentile value of the null distribution of the permuted test statistics obtained after 800 permutations are represented.

a) *L. perenne* **TC1*SB2**

Marker	Inoculation round	T_0value	Boundaries of 95% CI of the 2.5 percentile		Boundaries of 95% CI of the 97.5 percentile	
			Lower	Upper	Lower	Upper
PC008-R2	1	-4.072[**]	-2.233	-1.824	1.916	2.188
	2	-2.684[**]	-2.112	-1.830	1.915	2.198
	3	-3.799[**]	-2.215	-1.778	1.765	1.983
	Average	-3.726[**]	-2.220	-1.821	1.694	1.958
PC026-R2	1	-3.243[**]	-2.541	-1.854	1.901	2.312
	2	-2.078	-2.507	-1.936	1.965	2.250
	3	-3.357[**]	-2.342	-1.901	1.984	2.315
	Average	-3.458[**]	-2.496	-1.959	1.968	2.370
PC026-R3	1	-3.920[**]	-2.215	-1.940	1.695	2.106
	2	-3.211[**]	-2.193	-1.766	1.727	2.153
	3	-3.728[**]	-2.247	-1.805	1.767	2.098
	Average	-3.673[**]	-2.157	-1.889	1.788	2.055
PC026-R4	1	-3.387[**]	-2.126	-1.852	1.774	2.048
	2	-2.079[**]	-2.078	-1.795	1.831	2.258
	3	-3.067[**]	-2.170	-1.840	1.724	2.054
	Average	-2.737[**]	-2.062	-1.929	1.608	2.007
PC106-R2	1	-7.411[**]	-2.288	-1.876	1.746	2.156
	2	-6.288[**]	-2.093	-1.809	1.955	2.382
	3	-7.872[**]	-2.187	-1.857	1.813	2.142
	Average	-8.861[**]	-2.082	-1.816	1.850	2.251
PC168-R1	1	-7.413[**]	-2.452	-1.906	1.831	2.103
	2	-6.609[**]	-2.362	-1.936	1.946	2.229
	3	-7.223[**]	-2.065	-1.847	1.911	2.239
	Average	-7.981[**]	-2.174	-1.909	1.868	2.400

[**] = Value outside the 2.5 and 97.5 percentiles when testing for a 2-sided association at the á=5% significance level

Table 3.5 *continued*

b) *L. x boucheanum* 2A2*1B12

Marker	Inoculation round	T_0 value	Boundaries of 95% CI of the 2.5 percentile		Boundaries of 95% CI of the 97.5 percentile	
			Lower	Upper	Lower	Upper
PC008-R2	1	-3.395[**]	-2.245	-2.031	1.934	2.145
	2	-2.795[**]	-2.103	-1.881	1.848	2.292
	3	-3.434[**]	-2.208	-1.833	1.847	2.223
	Average	-3.569[**]	-2.208	-2.000	1.873	2.289
PC008-R3	1	-3.413[**]	-2.482	-2.051	1.695	2.113
	2	-3.205[**]	-2.482	-2.031	1.689	2.126
	3	-3.982[**]	-2.287	-1.910	1.944	2.322
	Average	-3.816[**]	-2.208	-2.000	1.861	2.276
PC078-R2	1	-1.183	-2.082	-1.851	1.827	2.057
	2	-0.483	-2.347	-1.858	1.534	1.770
	3	-0.470	-2.237	-1.826	1.622	1.823
	Average	-0.858	-2.167	-1.718	1.656	1.878
PC078-R3	1	-1.618	-2.270	-1.832	1.838	2.277
	2	-1.409	-2.080	-1.852	1.780	2.237
	3	-2.308[**]	-2.111	-1.727	1.701	2.084
	Average	-1.844	-2.057	-1.845	1.741	2.166
PC157-R1	1	-0.877	-2.315	-1.888	1.878	2.305
	2	-0.917	-2.205	-1.981	1.759	2.202
	3	-1.327	-2.445	-1.873	1.819	2.195
	Average	-1.266	-2.283	-1.866	1.813	2.228
PC157-R2	1	-3.232[**]	-2.315	-1.888	1.878	2.305
	2	-2.668[**]	-2.205	-1.762	1.759	2.202
	3	-3.487[**]	-2.060	-1.873	1.819	2.195
	Average	-3.415[**]	-2.073	-1.866	1.813	2.228
PC400-R2	1	-3.786[**]	-2.361	-1.934	1.821	2.245
	2	-3.510[**]	-2.292	-1.847	1.881	2.102
	3	-4.399[**]	-2.223	-1.847	1.833	2.019
	Average	-4.188[**]	-2.289	-1.873	1.795	2.208
PC400-R3	1	-1.158	-2.419	-1.984	1.818	2.246
	2	-2.027	-2.486	-2.027	1.748	2.194
	3	-2.737[**]	-2.536	-1.954	1.772	2.150
	Average	-1.902	-2.542	-1.903	1.811	2.445

[**] = Value outside the 2.5 and 97.5 percentiles when testing for a 2-sided association at the á=5% significance level

Table 3.5 *continued*

c) *L. multiflorum* **Axis-3*b-90**

Marker	Inoculation Round	T_0value	Boundaries of 95% CI of the 2.5 percentile		Boundaries of 95% CI of the 97.5 percentile	
			Lower	Upper	Lower	Upper
PC065-R1	1	-1.533	-2.196	-1.753	1.706	2.037
	2	-0.144	-2.057	-1.687	1.759	2.224
	3	-1.352	-2.311	-1.828	1.843	2.423
	average	-1.441	-2.179	-1.861	1.865	2.290
PC065-R2	1	-0.876	-2.306	-1.860	1.943	2.504
	2	-0.111	-2.500	-1.843	1.894	2.362
	3	0.319	-2.140	-1.850	1.834	2.417
	average	-0.215	-2.199	-1.880	1.860	2.286
PC175-R2	1	-0.858	-2.047	-1.828	1.812	2.251
	2	-0.418	-2.322	-1.860	1.823	2.284
	3	-1.814	-2.100	-1.814	1.912	2.295
	average	-1.366	-2.095	-1.781	1.801	2.220

[**] = Value outside the 2.5 and 97.5 percentiles when testing for a 2-sided association at the á=5% significance level

In the *L. perenne* population, all tested markers showed linkage with crown rust resistance for all three individual crown rust scores and for the average score, except marker PC026-R2. This marker did not show significant linkage with the score of inoculation round 2. Four of the eight selected markers in the *L. x boucheanum* population displayed significant linkages with crown rust resistance (PC008-R2, PC008-R3, PC157-R2 and PC400-R2). For the other four markers some significant values were obtained, but were not consistent over the different rounds of inoculation. Finally, in the *L. multiflorum* population no significant linkage was detected between crown rust resistance and the three markers selected in the previous round (PC065-R1, PC065-R2 and PC175-R1).

To estimate the proportion of variance explained by each marker, a linear regression model was fitted to the data (Table 3.6). The coefficient of determination R² was used as a measure of the magnitude of the marker-linked phenotypic effect (Chagué et al., 1997; William et al., 1997; Bai et al., 1999). In the *L. perenne* population, two R^2 values of more than 20% were found (PC106-R2 25.1% and PC168-R1 21.3%). For the *L. x boucheanum* population, R^2 values varied from 0% (PC157-R1 and PC078-R2) till 20% (PC008-R3), 17% PC008-R3 and 15% (PC008-R2). The markers selected in the *L.*

multiflorum population had a very low R^2. This was expected as the permutation-based linkage test already indicated a low linkage between the markers and crown rust resistance.

Table 3.6 : Simple linear regression of selected markers tested on all individuals of the respective population.

a) *L. perenne* **TC1*SB2**

Marker	Simple linear regression			
	R	R^2	Adjusted R^2	Std Error of the estimate
PC008-R1[**]	0.239	0.057	0.053	0.987
PC026-R2	0.222	0.049	0.045	0.991
PC026-R3[**]	0.235	0.055	0.051	0.998
PC026-R4[**]	0.178	0.032	0.027	1.001
PC106-R2[**]	0.504	0.235	0.251	0.878
PC168-R1[**]	0.466	0.217	0.213	0.900
All markers	0.606	0.367	0.350	0.818

[**] = T_0 test statistic outside the 2.5 and 97.5 percentiles when testing for a 2-sided association at the á=5% significance level (see Table 3.5)

b) *L. x boucheanum* **2A2*1B12**

Marker	Simple linear regression			
	R	R^2	Adjusted R^2	Std Error of the estimate
PC008-R2[**]	0.410	0.168	0.155	1.206
PC008-R3[**]	0.433	0.188	0.175	1.192
PC078-R2	0.108	0.012	-0.004	1.315
PC078-R3	0.226	0.051	0.036	1.288
PC157-R1	0.158	0.025	0.009	1.306
PC157-R2[**]	0.395	0.156	0.134	1.215
PC400-R2[**]	0.467	0.218	0.205	1.170
PC400-R3	0.233	0.054	0.039	1.286
All markers	0.511	0.261	0.156	1.206

[**] = T_0 test statistic outside the 2.5 and 97.5 percentiles when testing for a 2-sided association at the á=5% significance level (see Table 3.5)

c) *L. multiflorum* **Axis-3*b-90**

Marker	Simple linear regression			
	R	R^2	Adjusted R^2	Std Error of the estimate
PC065-R1	0.107	0.011	0.006	1.450
PC065-R2	0.016	0.000	-0.005	1.468
PC175-R1	0.101	0.010	0.005	1.450
All markers	0.123	0.015	-0.001	1.455

[**] = T_0 test statistic outside the 2.5 and 97.5 percentiles when testing for a 2-sided association at the á=5% significance level (see Table 3.5)

The total percentage of the phenotypic variance explained by the selected loci is still rather low compared to the study of Kover and Caceido (2001). They found after summarizing different resistance QTL studies that on average 51% of the phenotypic variance was explained by QTLs. Several explanations can be proposed for the low phenotypic variance explained in the present study. Firstly, the distance between the markers and the QTL locus may still be large, and thus the QTL effect can be underestimated. Secondly, detection of only one of several putative QTLs involved in this resistance may also be due to our use of starting material or as we only phenotyped in one environment, the environment masks the effect of unidentified loci. This consists of the most contrasting individuals in the population. When a phenotype is influenced by multiple genetic loci and environment, individuals can exhibit extreme phenotypes due to different sets of QTLs or due to non-genetic factors. However thirdly, several QTLs of lesser effect but accounting together for a non-negligible part of the genetic variation of the resistance trait have probably escaped detection. The undetected QTLs in the current study may result from the limitation of the BSA strategy. This technique may directly target the resistance QTL with major effects, but not those with minor effects. A QTL analysis based on a genetic map can help to identify those other genomic regions involved in resistance.

3.4.5 Linkage analysis of R linked markers

In the *L. perenne* population, multiple linear regression including all six markers resulted in a R^2 of 35% (Table 3.6). In the *L. x boucheanum* population, a similar result was seen: multiple linear regression including eight markers resulted in a R^2 of 15.6% (Table 3.6). This indicates that in both populations the combination of loci results in a small increase of the proportion of phenotypic variance explained or in no increase at all. This is expected when some markers are detecting the same loci. Therefore, the recombination frequencies between the selected markers were calculated.

Linkage analysis in the *L. perenne* population revealed that some of the tested markers were linked (Table 3.7). Two clusters were identified: PC026-R3 and PC026-R4 are tightly linked (6.03 cM map distance), and PC106-R2 and PC168-R2 are tightly linked (9.05 cM). This explains the result obtained with

the multiple regression analysis in which only a slightly higher R^2 value was seen compared to the different individual R^2 values.

In the *L. x boucheanum* population, the four significant markers are loosely linked to each other. PC157-R2-PC008-R2 (1.45 cM) and PC008-R2-PC400-R2 (5.8 cM) are tightly linked, and both couples of markers are also linked to each other (10-12 cM apart). Most of the other markers are also linked to the four significant markers. In fact, the markers detect all the same region involved in resistance. This explains that the R^2 obtained by multiple linear regression is of the same order as the R^2 obtained for each of the markers when tested individually.

Table 3.7 : Recombination percentages between the selected markers in the *L. perenne* and *L. x boucheanum* population.

a) *L. perenne* **TC1*SB2**

	PC008-R1	PC026-R2	PC026-R3	PC026-R4	PC106-R2
PC026-R2	45.69	-	-	-	-
PC026-R3	22.41	42.24	-	-	-
PC026-R4	28.45	43.10	6.03*	-	-
PC106-R2	48.71	17.67	46.98	45.26	-
PC168-R1	46.55	21.55	46.55	47.04	9.05*

* = recombination percentage is less than 10%

b) *L. x boucheanum* **2A2*1B12**

	PC008-R2	PC008-R3	PC078-R2	PC078-R3	PC157-R1	PC157-R2	PC400-R2
PC008-R3	15.94	-	-	-	-	-	-
PC078-R2	33.33	37.68	-	-	-	-	-
PC078-R3	15.94	28.99	31.88	-	-	-	-
PC157-R1	13.04	26.09	34.78	17.39	-	-	-
PC157-R2	1.45*	17.39	34.78	14.49	11.59	-	-
PC400-R2	10.14	5.80*	34.78	23.19	23.19	11.59	-
PC400-R3	21.74	28.99	43.48	28.99	26.09	20.29	23.19

* = recombination percentage is less than 10%

3.4.6 Evaluation of significant markers for MAS

The possibility to use the selected markers for MAS was explored by dividing a segregating population into subpopulations based on the presence/absence of a specific marker. Differences in mean crown rust score and the frequency distribution of crown rust score were compared between subpopulations (Fig. 3.5 and Table 3.8). For example if the presence/absence of PC106-R2 was used as a criterion to subdivide the *L. perenne* population, a shift in frequency distribution and mean crown rust score was observed. An improvement of 0.48 units (crown rust score) was achieved with respect to the average score of the complete population (1.86). Within the subpopulation of plants that display this marker, 71% have a crown rust score of 1 meaning that they did not display any crown rust symptom. Only 7% of the plants with this marker had crown rust scores of 3, 4 or 5, while the rest of the individuals displayed an intermediate phenotype (score 2). For marker PC168-R1 also 71% of the plants that displayed the marker were highly resistant (score 1). In this case, the proportion of plants with the marker that were susceptible (score 3, 4 or 5) was 5%.

MAS within the *L. perenne* population   **MAS within the *L. hybridum* population**
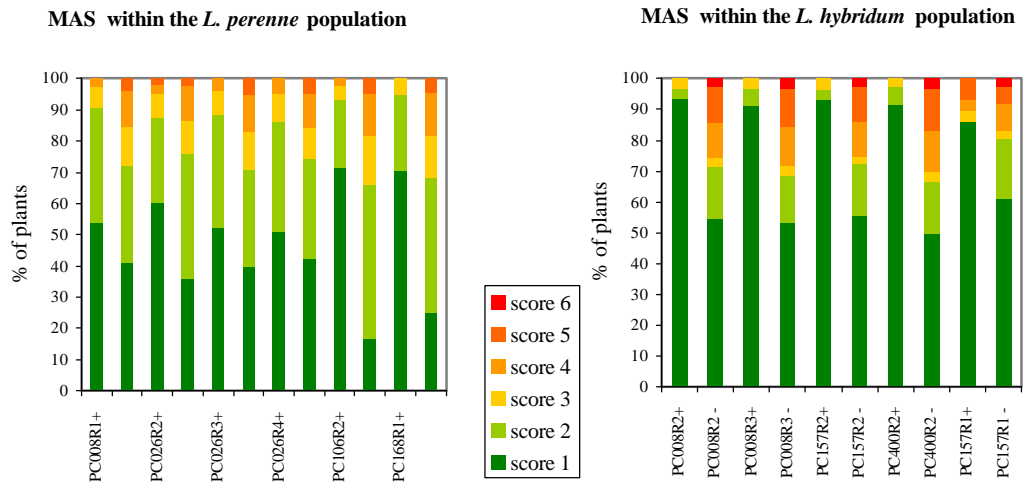
Fig. 3.5 : Evaluation of selected markers for use in MAS : F_1 populations are divided according to the presence(+) absence (-) of the marker. Average crown rust score distribution is given for the two subpopulations. Rust score varies from 1=resistant till 6=susceptible.

In the *L. x boucheanum* population, identical analyses were performed. For example, in the subpopulation in which marker PC157-R2 was used : a gain of 0.54 units in crown rust resistance is made compared to the mean crown rust score of the whole population. In this subpopulation, no plants were susceptible, while in the subpopulation without the marker, 25% of the plants were susceptible.

Table 3.8 : Characteristics of subpopulations made on the basis of presence (M+) / absence (M-) of selected markers in a) the *L. perenne* and b) the *L. x boucheanum* population. (R = resistant, score 1; I = intermediate, score 2 till 3; S = susceptible, score 4 till 6)

a) *L. perenne* **population TC1*SB2 (population mean rust score = 1.86)**

Marker	Mean rust score		% of plants					
	M+	M-	M+			M-		
			R	I	S	R	I	S
PC008-R1*	1.58	2.07	54	37	9	41	31	28
PC026-R2*	1.59	2.04	61	27	12	36	40	24
PC026-R3*	1.63	2.11	52	36	12	40	31	29
PC026-R4*	1.68	2.04	51	35	14	42	32	26
PC106-R2*	1.38	2.41	71	22	7	17	50	33
PC168-R1*	1.35	2.29	71	24	5	25	43	32

* mean crown rust score of subpopulations are significantly different at the 0.05 level

b) *L. x boucheanum* **population 2A2*1B12 (population mean rust score = 1.64)**

Marker	Mean rust score		% of plants					
	M+	M-	M+			M-		
			R	I	S	R	I	S
PC008-R2*	1.10	2.17	93	7	0	54	20	26
PC008-R3*	1.12	2.25	91	9	0	53	20	28
PC157-R2*	1.10	2.14	93	7	0	56	19	25
PC400-R2*	1.11	2.33	91	9	0	50	20	30

* mean crown rust score of subpopulations are significantly different at the 0.05 level

3.5 Conclusion

The use of AFLP as a marker technique in combination with BSA was a good choice as a high number of markers putatively linked with the studied trait were revealed. However, the number of identified putatively linked markers was higher than expected. This was due to the high level of heterozygosity and heterogeneity present in the parent plants, resulting in more false positives than

theoretically expected. A second reason for the higher number can be the use of dominant markers. Some markers might be derived from the same loci. The percentage of tested markers which were retained in the BSA was lower in the *L. perenne* and *L. x boucheanum* population than in the *L. multiflorum* population. The reason was the higher stringency applied in these two populations, in which two different R bulks were used instead of one R bulk in the *L. multiflorum* population.

After screening the markers on all F_1 individuals, six, eight and three markers were retained in the *L. perenne*, *L. x boucheanum* and *L. multiflorum* population respectively.

In the *L. perenne* population five out of six selected markers were clearly linked with crown rust resistance, the sixth one being less clearly linked. All six selected markers together explained 35% of the phenotypic variance in the *L. perenne* population. Two markers detect a genetic factor explaining more than 20% of the phenotypic variance. The markers selected were located at two genomic regions. This is in agreement with the hypothesis postulated in Chapter 2 that two unlinked loci can explain the segregation found (Table 2.5 – Chapter 2).

In the *L. x boucheanum* population, four markers showed significant linkage with crown rust resistance. All four markers explained 15% of the phenotypic variance. Linkage analysis revealed that the four selected markers were closely linked and that they detect the same locus involved in crown rust resistance. In the previous chapter, we hypothesized the presence of two major loci involved in resistance (Table 2.5 – Chapter 2). BSA might not have detected all loci involved in the *L. x boucheanum* population.

In the *L. multiflorum* population, no significant linkages were found between the selected markers and crown rust resistance. These results are in line with the conclusion drawn for this population in Chapter 2, that resistance is encoded by minor genes, which are not easily detected by BSA.

The value of the selected markers for MAS was further demonstrated. A shift towards a more resistant population was seen if e.g. presence of marker PC106-R2 was used as selection criterion in the *L. perenne* population. The improvement of the population achieved is sufficient for breeding purposes, and offers as advantage that the selection is carried out already at the seedling stage. However, before the selected markers can be used on a breeding pool with a

broad genetic basis, the strength of the linkage needs to be evaluated. As *Lolium* species are very heterozygous plants, associations between one marker allele and the trait might hold true in one population, but not in another. Linkage Disequilibrium (LD) between marker allele and trait might vary among crosses due to several reasons including the coancestry between the parents of the crosses and difference in the frequency of recombination events due to structural features of the genome. Therefore, the behaviour of the selected markers need to be evaluated in breeding gene pools and the positive associations between marker-alleles/traits need to be confirmed before a reliable MAS procedure can be applied. Another important condition that has to be fulfilled before applying the marker in breeding is its transformation into a co-dominant high throughput marker.

A direct MAS application is to use these markers to select plants with specific allele combinations (homozygous - heterozygous). The effect of the allele combinations on crown rust resistance can be studied and specific genotypes can be introduced into a breeding pool as donors of crown rust resistance in a similar way as in introgression programs of other traits.

The set of markers, identified in this chapter, explain an amount of phenotypic variance : 35% in the *L. perenne* population and 15%, in the *L. x boucheanum* population. In the *L. multiflorum* population, we were not able to identify markers with significant effect on crown rust resistance. There are different reasons for the low percentages obtained, like, that the environment masks the genetic effect of the identified loci or the markers are still a large distance away from the resistance locus or some loci may not be detected as BSA only detects loci with a major effect (> 2*SD), … In the next chapters, the construction of a genetic map for each of the populations will be discussed. This map will be used to further dissect the resistance trait and to verify if more loci than those detected in the BSA or involved in crown rust resistance in these studied populations. This will be done by a QTL analysis using map information.

Chapter 3

Chapter 4

Screening of co-dominant marker systems for mapping in *Lolium*

4.1 Introduction

Different marker techniques are available for molecular and genetic linkage map construction. Restriction fragment length polymorphisms (RFLPs) were the first DNA markers used for linkage studies. Later on, PCR-based marker techniques were developed. They are well adapted to efficient non-radioactive DNA fingerprinting. These include among others random amplified polymorphic DNA (RAPD), AFLP, Sequence tagged sites (STS), cleaved amplified polymorphic sites (CAPS) and simple sequence repeats (SSRs). In Table 4.1, an overview is given of the advantages and disadvantages of the different techniques. In this chapter, the different marker techniques are discussed and evaluated for their use in *Lolium* mapping studies.

Table 4.1 : Advantages and disadvantages of available molecular marker techniques (adapted from Forster et al., 2001).

Molecular marker system	Advantage	Disadvantage
RFLP	Co-dominant High reproducibility	Low multiplex ratio High time/labor requirements Big amounts of DNA required
RAPD	Low time/labor requirements Medium multiplex ratio	Dominant Low reproducibility
AFLP	High reproducibility High multiplex ratio	Dominant Moderate time/labor
SSR	Co-dominant High reproducibility Low time/labor requirements	High development costs Low multiplex ratio

4.1.1 Amplified Fragment Length Polymorphisms (AFLPs)

In the previous chapter, AFLP was used to identify DNA-markers linked with crown rust resistance in *Lolium* spp. and was particularly useful for BSA as it

generates a high number of markers without the need of big amounts of DNA. AFLP can detect single nucleotide changes and amplifies in *Lolium* spp. around 95 DNA-fragments in a single experiment, in the absence of prior sequence knowledge (Roldán-Ruiz et al., 2000). In mapping studies, AFLP is commonly used to generate in an efficient way a dense backbone of markers on which other markers (mostly co-dominant) are placed.

There is one important disadvantage of using this technique for mapping. AFLPs have low information content due to their dominant character. This precludes the distinction between homozygously and heterozygously inherited AFLPs.

Due to the nature of the mapping populations used in this study (crosses derived from two highly heterozygous and heterogeneous plants), co-dominant markers were required for reliable map construction. For map integration over the three crosses (TC1*SB2, 2A2*1B12 and Axis-3*b-90) and for map comparison with published maps, anchor markers, transferable between different mapping populations are needed. Co-dominant markers, in which both alleles at one locus can be detected, are most suitable for these purposes. The evaluation of different techniques to generate co-dominant markers in the *Lolium* populations studied will be discussed below.

4.1.2 Restriction Fragment Length Polymorphisms (RFLPs)

A first co-dominant marker technique, suitable for comparative genetics, is RFLP. RFLP markers have the ability to detect homologous DNA sequences in genomes. They give information about gene families and about the genomic distribution of genes (Kumar, 1999; Yu & Wise, 2000).

Firstly, RFLPs, if derived from cDNA-sequences, provide information about the map location of certain genes. This is useful information that can be assigned to QTLs coinciding with these RFLPs on a linkage map.

Secondly, RFLPs, developed in one species, can detect heterologous sequences in related species, elucidating information for comparative genetics (Yu and Wise, 2000). For example, RFLP probes isolated from barley, rice, oat, maize and other *Poaceae* have been used to study the synteny between *Gramineae* species (Devos and Gale, 1997; Van Deynze et al., 1998; Devos and Gale, 2000; Hash et al., 2000), resulting in strong evidence that till some degree,

genome organization has been conserved during plant evolution. Although other marker systems have been tested for their use in synteny studies, RFLP markers remain the system of reference (Davierwala et al. 2000).

Despite these advantages, RFLPs are not practical for routine mapping or MAS. In particular the large genome size of *Lolium* makes the generation of RFLP-markers labor intensive, technically demanding, requires substantial quantities of good quality genomic DNA and the use (in most cases) of radioactivity. However, in order to align the *Lolium* maps produced in the present study to the other available *Gramineae* maps and to identify syntenic regions, a set of RFLP probes mapped in several *Gramineae* spp. was tested in the *L. perenne* population. Especially loci located on the group 1 homologous chromosomes of the *Gramineae* were envisaged as this group is associated with a variety of disease resistance genes (Yu et al., 1996).

4.1.3 Simple Sequence Repeats (SSRs)

Another technique generating co-dominant DNA-markers is Simple Sequence Repeats (SSRs). The SSR technique involves the use of specific primers to PCR-amplify genomic regions containing tandems of short sequence motifs (1-6 nucleotides), or SSR loci. Di-, tri-, and tetra nucleotide repeats are the most common (Peakall et al., 1998; Holton, 2001; McCallum et al., 2001). Polymorphisms at SSR loci arise due to changes in the number of repeats, insertion of motifs and imperfect motifs, to variations in the adjoining non-repetitive region or to interruption of perfect repeats (Davierwala et al., 2000).

Several features make SSRs useful genetic tools: (i) they are widely dispersed throughout eukaryotic genomes, (ii) can be assayed on automatic DNA sequencers (making them relatively easy to score), (iii) they are co-dominant and multiallelic and (iv) they are highly polymorphic, robust, transferable and highly reproducible (Forster et al., 2001; Holton, 2001). Powell et al. (1996) examined the utility of RFLP, RAPD, AFLP and SSR markers for soybean germplasm analysis by evaluating for each marker system the information content (expected heterozygosity), number of loci simultaneously analyzed per experiment (multiplex ratio) and effectiveness in assessing relationships between accessions. SSR markers had the highest expected heterozygosity (0.60) while AFLP markers had the highest effective multiplex ratio.

SSR's ease of use and high information content has ensured that SSRs have largely replaced RFLPs as mapping technology. The creation of skeletal genetic maps with SSRs is becoming an achievable goal in more species, where SSRs can provide anchor points for specific regions of the genome. Gaps between the SSR markers may be filled in with other markers such as AFLPs (Holton, 2001). However a dense SSR map is not achievable for most minor crops due to the initial isolation and characterization, which can be expensive and time-consuming.

Three main reports on publicly available SSR markers in *Lolium* appeared during the time-span of this project. Kubik et al. (1999&2001) isolated SSR sequences in *L. perenne.* They suggested that SSRs may be less abundant in perennial ryegrass than in some other plant species. For example, one SSR occurs every 104 and 150 kb in wheat and rice respectively, while in ryegrass roughly one GA/GT SSR per 350 kb is present. Jones et al. (2001) isolated SSR sequences from *L. perenne* and published primer sequences for detection of ten SSR loci. The SSR loci contained mostly di or tri-nucleotide repeats, perfect or imperfect. In a second publication, Jones et al. (2002) presented a genetic map of *Lolium* including 92 SSRs, however these SSRs are not freely available. This set is licensed to Advanta Van der Have (Rilland, The Netherlands) and they tested the set on the *L. perenne* population.

Although a few SSRs in *Lolium* are published, several institutes are presently developing SSRs (f.e. INRA, Lusignan, France; DIAS, Slagelse, Denmark; IGER, Aberystwyth, UK). At the DvP, a set of SSRs was developed of which eleven were tested in this study (Dendauw J., personal communication).

A disadvantage of SSRs is that primers designed to amplify a SSR locus are often species-specific and cannot be used for comparative genetics. This is due to the high variability in the sequences surrounding the SSR sites (Peakall *et al.*, 1998). However, some publications are available on successful cross-amplification of SSRs. Jones et al. (2001) reported high levels of cross-amplification of SSR primers designed in *L. perenne* in the two closely related ryegrass species *L. rigidum* (80%) and *L. multiflorum* (71%). Amplification levels were slightly lower in *Festuca* species, Kentucky bluegrass, *Phalaris* and oats. Davierwala et al. (2000) studied the conservation of one SSR, isolated from rice, in other cereals (oat, barley, maize, pearl millet, rye and wheat). In all species studied, the SSR was conserved, proving that this SSR was useful for comparative genetics. On the other hand, Peakall et al. (1998) tested cross-

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amplification of SSRs isolated in *Glycine max*. They had to use touch down PCR conditions to get 60% of amplification of the SSRs in other species belonging to the subgenus *Glycine* and just 3 to 13% of amplification in species outside the genus. In an intent to enlarge the available set of SSRs for *Lolium*, we tested cross-amplification in *Lolium* of three SSRs isolated in wheat (Röder et al., 1995).

4.1.4 Sequence Tagged Sites (STSs)

A third frequently used co-dominant marker technique is based on the development of primers from characterized expressed sequences and targets loci with gene information. Sequences of cDNAs from economically significant and model plant species are accumulating at a massive rate. Markers derived from these databases resulted in a method called candidate gene approach, which is a promising approach to identify genes for monogenic characters and quantitative trait loci (QTL) in plants. However, for minor crops with a complex genome, a major challenge is to use these growing numbers of expressed sequence tags (ESTs) as markers for genetic mapping (McCallum et al., 2001; Wang et al., 2001).

Earlier methods for mapping ESTs involved RFLP analysis using cDNA fragments as hybridization probes. This approach has since been improved by more efficient PCR-based approaches. EST-specific primers are used to amplify coding sequences. As introns are less well conserved than exons, primer sets spanning introns have a higher probability for polymorphism detection (McCallum et al., 2001). Polymorphisms in the PCR fragments can be detected as differences in length, restriction sites or conformation. Conformational polymorphisms can be detected using hetero-duplex (HD) analysis or single-strand conformational polymorphism (SSCP) analysis. Both methods visualize the polymorphisms as an electrophoretic mobility shift in polyacrylamide gels. Whereas SSCP analysis is restricted to PCR products of 100-300 bp in length, the optimal size of PCR products for HD analysis ranges from 300 to 900 bp (Schneider et al., 1999).

There are a number of advantages to the use of ESTs instead of 'anonymous' markers. A first advantage of EST markers is that the resulting transcriptional map provides a preliminary description of the organization of expressed genes and insights about genome evolution (McCallum et al., 2001). ESTs integrated

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into a linkage map, can be tested for their association with QTLs. In maize, this approach has been successful in assessing the role of genes encoding key enzymes in carbohydrate metabolism during the early growth of the plant (Causse et al., 1995).

Secondly, in contrast to AFLP and SSR markers, EST markers are more likely to be conserved across species (Taylor et al., 2001). The reason for this assumption is that the primer binding sites are designed in coding DNA, that generally has a high degree of sequence conservation. As such, EST markers will be especially useful for aligning genome linkage maps and comparing QTLs. If information is lacking for a target species, ESTs derived from other species can be used as the basis for genetic mapping in the species of interest. Genetic mapping with ESTs thus enables a more rapid transfer of linkage information between related species (Cato et al., 2001).

Today, two publications on STS markers tagging potentially expressed sequences in *Lolium* are available. Taylor et al. (2001) assessed a total of 21 primers sets developed in barley, *Triticum tauchii* and *Phalaris coerulescens* for their ability to amplify homologous sequences in *L. perenne*. Eleven primer sets successfully amplified homologous fragments. Other primer sets amplified multiple products, resulting in a profile comparable to a RAPD profile (Taylor et al., 2001). A second report on STS markers in *Lolium* was published by Lallemand et al. (1998). STS markers were based on gene sequences of *Gramineae* species. Consensus zones identified by comparing homologous sequences in different *Gramineae* species, which flank introns were selected for primer design. Thirty primer sets were tested, of which sixteen gave polymorphic patterns in *Lolium* (Lallemand et al., 1998). These sixteen primer sets were tested in this study, as their ability to detect polymorphisms in ryegrass had already been demonstrated (Lallemand et al., 1998; Roldán-Ruiz et al., 2001).

4.1.5 Resistance Gene Analogues (RGAs)

A special class of STS markers used in this study, was based on the knowledge available on resistance genes (R genes) in plants. Resistance to many diseases, particularly those caused by biotrophic fungal pathogens, is determined by individual members of families of dominant genes. R gene specificity is often conferred by a gene-for-gene interaction; for every resistance gene there is a corresponding gene for avirulence in the pathogen (Flor, 1971).

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To date, genes conferring resistance to the major classes of plant pathogens (bacteria, virus, fungi, and nematodes) have been isolated from different plant species. Many resistance genes appear to encode components of signal transduction pathways. Among the cellular events that characterize resistance are oxidative burst, cell wall strengthening, induction of defense gene expression and rapid cell death at the site of infection. The R gene protein products share common structural domains. Based on these common molecular features, the R-genes are classified into 7 classes (for a review see Hammond-Kosack and Jones, 1997; Young, 2000). One of the most common domains is the nucleotide binding site (NBS), of which the conserved motifs are situated within the NBS domain and in a hydrophobic domain downstream the NBS (f.e. in *Arabidopsis RPS2* (Bent et al., 1994); tobacco *N* (Whitham et al., 1994); and flax *L6* (Lawrence et al. 1995)).

By making use of conserved domains, various investigators have designed degenerate primers for amplifying similar regions from genomes of diverse plant species: e.g. rice (Mago et al., 1999), soybean (Kanazin et al., 1996; Yu et al., 1996), barley (Leister et al., 1998), citrus (Deng et al., 2000), maize (Collin et al., 1998), potato (Leister et al., 1996), *Arabidopsis taliana* (Aarts et al., 1998), and lettuce (Shen et al., 1998). Genetic analyses have associated a number of these sequences to genetic loci known to confer resistance to viruses, bacteria, fungi, or nematodes (Kanazin et al., 1996; Leister et al., 1996; Yu et al., 1996; Aarts et al. 1998; Shen et al. 1998; Mago et al., 1999; Pan et al., 2000).

These studies demonstrate that PCR approaches using degenerate primers based on the conserved NBS domains of cloned R genes can provide an attractive strategy to amplify multiple candidate resistance gene sequences. These sequences can be transformed into molecular markers. We tested this strategy in the *L. perenne* population, using the primers developed by Yu et al. (1996) and Mago et al. (1999).

4.2 Objectives and rationale

In the previous chapter, AFLP markers putatively linked with crown rust resistance were identified, but explained just a small part of the phenotypic variance present in the populations. In the *L. multiflorum* population, we were unable to identify AFLP markers explaining a significant percentage of the phenotypic variance. A QTL analysis performed on the basis of genetic maps,

can elucidate the genetic organization of genes influencing crown rust resistance. For mapping studies, more markers than those generated during the BSA analysis are required, especially co-dominant markers are needed as information on linkage phase between markers is needed for map construction. In this chapter, different approaches to generate co-dominant markers suitable for mapping studies in *Lolium* are discussed and evaluated. The efficiency, advantages and disadvantages of available marker techniques such as AFLP, RFLP, SSRs and STS markers in ryegrass mapping will be handled.

4.3 Material and methods

4.3.1 Plant material and DNA extraction

The plant material described in Chapter 2 was used. Marker techniques were evaluated on a subset of plants, including the six parents of the three selected populations (TC1*SB2, 2A2*1B12 and Axis-3*B-90) and a variable number of F_1 individuals of the 3 crosses. DNA was prepared as described in Chapter 3.

4.3.2 RFLP analysis

The set of 51 RFLP probes listed in Table 4.2 was screened in the *L. perenne* mapping population using three restriction enzymes (*Eco*RI, *Hind*III and *Dra*I). The probes were derived from the anchor probe set developed within the European *Gramineae* mapping project (EGRAM). The probes had been previously isolated from oat (CDO), rice (RZ, RGR, RGC, RGG), barley (BCD), maize (CSU), wheat (PSR) and *Festuca* (IBF). RFLP analysis was performed using ^{32}P labeled probes as described by Berry et al. (1995). Probes were hybridized to membranes containing 11 µg digested *Lolium* DNA per lane. Hybridization conditions were 60 °C and 0.6 x SSC. Washing conditions were 60 °C and 0.25 x SSC. Membranes were exposed to X-ray films for 2 to 16 days at –80°C with intensifying screens or were analyzed using a phosphor-imager (Bio-imaging analyzer BAS-2500 Fujifilm) after one overnight exposure.

Table 4.2: Constitution of the EGRAM anchor RFLP probe set tested in the *L. perenne* population. Screening results for mapping are : P = polymorphic, NH = no hybridization, NP = non polymorphic, AH = aspecific hybridization)

Probe prefix	Species	Origin	Clone type	Probes	Screening results for mapping
BCD	*H. vulgare*	Cornell University, Ithaca, NY 14853, USA	cDNA	BCD127	P
			cDNA	BCD135	P
			cDNA	BCD147	P
			cDNA	BCD855	P
CDO	*A. sativa*	Cornell University, Ithaca, NY 14853, USA	cDNA	CDO17	NH
			cDNA	CDO87	P
			cDNA	CDO89	P
			cDNA	CDO98	NP
			cDNA	CDO99	NH
			cDNA	CDO385	P
			cDNA	CDO405	NP
			cDNA	CDO412	P
			cDNA	CDO456	P
			cDNA	CDO459	P
			cDNA	CDO497	P
			cDNA	CDO718	NP
CSU	*Zea mays*	Californian State university	cDNA	CSU6	P
			cDNA	CSU21	AH
			cDNA	CSU25	P
			cDNA	CSU39	NP
			cDNA	CSU68	NP
			cDNA	CSU70	P
			cDNA	CSU95	P
			cDNA	CSU109	NP
IBF	*Festuca*	Agricultural university of Norway, As, Norway	gDNA	IBF64	P

Table 4.2 *continued*

Probe prefix	Species	Origin	Clone type	Probes	Screening results for mapping
PSR	*T. aestivum*	John Innes Centre, Colney, Norwich, Norfolk NR4 7UH,UK	cDNA	PSR8	AH
			cDNA	PSR104	P
			cDNA	PSR119	P
			cDNA	PSR129	P
			cDNA	PSR154	NP
			cDNA	PSR167	NP
			?	PSR580	NP
			gDNA	PSR598	NP
RGC	*O. sativa*	National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, JAPAN	cDNA	RGC390	P
			cDNA	RGC424	P
			cDNA	RGC488	P
			cDNA	RGC496	NP
			cDNA	RGC742	P
			cDNA	RGC1286	P
RGG	*O. sativa*	National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, JAPAN	gDNA	RGG1125	NP
RGR	*O. sativa*	National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, JAPAN	cDNA	RGR77	P
			cDNA	RGR617	NP
			cDNA	RGR662	P
			cDNA	RGR1927	P
RZ	*O. sativa*	Department Plant Breeding, Cornell University, Ithaca, NY 14853-1901, USA	cDNA	RZ141	P
			cDNA	RZ244	P
			cDNA	RZ508	NP
			cDNA	RZ537	NP
			cDNA	RZ816	NH
			cDNA	RZ892	NP

4.3.3 SSR analysis

Five sources of SSRs were used (Table 4.3) : (1) six primer sets from Kubik *et al.* (1999), (2) ten from Jones et al. (2001), (3) eleven developed at DvP (Dendauw *et al.* personal communication), (4) three wheat primer sets tested for cross-amplification in *Lolium* (Röder et al., 1995) and (5) 100 unpublished primer sets developed by Jones et al. (La Trobe University, Australia) and licensed to Advanta Van der Have (Rilland, The Netherlands). This set was then screened and tested at Advanta Van der Have (Rilland, The Netherlands).

Table 4.3 : SSR primer sets used in the *L. perenne*, *L. x boucheanum* and *L. multiflorum* population : a) primer sets developed by DvP (Dendauw et al., personal communication), b) developed by Kubik et al. (1999) and c) Jones et al. (2001). Forward (F) and reverse (R) primer sequences, repeat motif and the hybridization temperature T_h are presented; nucleotides of the pig tail are given in *Italic*.

a) DvP

SSR		Primer sequence	Repeat motif	T_h
Rye001	F	TCA GTG CTC TCA GTT GTG AAC T	AC_{20}	51
	R	CTG TTA TCC GGG AAG TAC AAC		
Rye002	F	ACT GCA CTG TGT TCA ATC ATC C	AC_{20}	55
	R	*GTTT*GAA AGT TGA ACC CTG TTA TCC G		
Rye005	F	GAC ACC TCA CCT GGG TCG	TG_{26}	55
	R	TTG AAC AGG AAG ACA TTT GGG		
Rye008	F	GCG ACA CAG ACA CAC ACA GA	CA_{23}	55
	R	CTA TTG CAT TGA GCG AGC		
Rye009	F	GCG ACA CAA AGG TTT AGG G	CA_{23}	55
	R	*GTTTCTT*TCA GAG AGC AGG ATA GGA GG		
Rye010	F	TGA CGA ACG ATG TGG ATT AG	CA_{29}	55
	R	TTG AAG GAG CAC AAC CAT C		
Rye012	F	GGT CTA ATT GTC GTC CTT TC	CA_{23}	51
	R	*GTTT*GAG TGA TTT GGA GGT GAG AA		
Rye013	F	TGG AAG CAA GAA AGG ACA TC	CA_{21}	51
	R	AGA GAA GTA CAA GTC GGT GCT		
Rye014	F	CTG CTC TGT GTT TGT GTG AC	CA_{26}	51
	R	GCC TTT CAT CGT TAC TGT CT		
Rye016	F	CCT ACA CAA ACT GCC CTC TC	TAG_{6+10}	51
	R	*GTTTCTT*TGC TGC TGC TAC TGC TAC TG		
Uni001	F	AGC CAC ACT TTA CCT AAT GCT G	?	55
	R	*GTTT*CCC GCA AAA CTT ACA ATT AAA		

b) Kubik et al. (1999)

SSR		Primer sequence	Repeat motif	Th
Rye021	F	CAC CTC CCG CTG CAT GGC ATG T	$(GT)_8AGGT$	51/
(M4-213)	R	TAC AAC GAC ATG TCA AGG		55
Rye022	F	GGT CTG GTA GAC ATG CCT AC	$(GA)_5TTAGAGG(GA)_{17}$	51
(M15-185)	R	TAC CAG CAC AGG CAG GTT C		
Rye023	F	TGC TGT GGC TCT TGT GAC	$(GA)_3G(GA)_{18}GG(GA)_7$	51
(M16-B)	R	AGC CGA GGC TCA GCT CGA		
Rye024	F	AGA GAC CAT CAC CAA GCC	$GATT(GA)_{12}GT(GA)_{15}$	51
(M4-13)	R	TCT GGA AGA AGA TTT CCT TG		
Rye025	F	GCA ACT TCT ATC GAG TTG	$(GT)_9(GA)_9$	51
(M2-148)	R	GAG GCT CGA TCT TCA CGG A		
Rye026	F	CTA CAA TGC ATT CGT GCA	$(GA)_9$	51
(M12-52)	R	TAG AGG CAC CCG CGC CCT		

Table 4.3 : *continued*

c) <u>Jones et al. (2001)</u>

SSR		Primer sequence	Repeat motif	Th
Rye031	F	AAA GAC CGC AGA CGA AGT	$(CA)_{27}$	51
(LPSSRH01A02)	R	AAC CAA AGC CGC AAG ACA		
Rye032	F	TGG AGG GCT CGT GGA GAA GT	$(GT)_9$ imperfect	51
(LPSSRH01A07)	R	CGG TTC CCA CGC CTT GC		
Rye033	F	GAG GCA CCCG GCC ATG GAG	$(CTT)_{20}$ imperfect	51
(LPSSRH01A10)	R	AGG ACG AGC CAC TCA CTT G		
Rye034	F	CAA GTG CCA CCA TAG ATA CAA	$(AG)_8$ imperfect	51
(LPSSRH01D09)	R	CGT GAA GAT CAC TAT AAA CAC GA		
Rye035	F	CGC AGC TTA ATT TAG TC	$(CA)_{10}$	51
(LPSSRH01E10)	R	GCT TTG AGT ATG TAA AGT T		
Rye036	F	TCT GTG GGT CCT TCT GGA T	$(TCGC)_6$ imperfect	51
(LPSSRH01F02)	R	TCGGGT GAT GAT GTT GAC TT		
Rye037	F	ATT GAC TGG CTT CCG TGT T	$(CA)_9$	51
(LPSSRHO1H06)	R	CGC GAT TGC AGA TTC TTG		
Rye038	F	TGG AAT AAC GAT GAA AAG	$(CA)_4TA(CA)_4$ interrupted	51
(LPSSRH02C11)	R	CAT CAC GAA TTA ACA AGA G		
Rye039	F	GGA CGA ACT GCC GAG ACA	$(CTT)_7$	51
(LPSSRK01A03)	R	CGG GCA TGG TGA GAA GGA		
Rye040	F	CGG CCA CCC TTG ATA GAG	$(CA)_{21}$ imperfect	51
(LPSSRK01A11)	R	TCG TCA AGG ATC CGG AGA		

PCR amplifications of the three sets were performed using the Geneamp PCR reagent kit of Applied Biosystems. The reaction volume of 20 µl contained 25 ng DNA, 1 x PCR buffer, 0.2 mM dNTPs, 0.075 µM forward and reverse primer, with one primer being fluorescent labeled, 2.5 µg BSA and 1 U of *Taq* polymerase. PCR was performed in a Perkin Elmer 9600 thermocycler. Cycling conditions were 10 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 51°C or 55°C depending on the primer combination and 1 min at 72°C, and a final elongation step of 10 min at 72°C. At the end of the fluorescent PCR, the samples were denatured by adding 20 µl of formamide buffer and heating for 3 min at 90°C. 1.5 µl of each sample was loaded on 5% polyacrylamide/bisacrylamide 19:1 (Biorad), 7.5 M urea (Gibco BRL) and 1x TBE gels and analysed with an ABI Prism 377 DNA sequencer (ABI377; Perkin Elmer). GS-500 Rox labelled size standard was loaded in each lane in order to allow the automatic analysis of the data. Genescan Analysis Software 2.1 was used to translate the information collected by the ABI377 into fragment sizing information and Genotyper 2.5 was used to score the fingerprints.

4.3.4 STS analysis

Sixteen primer pairs developed by Lallemand *et al.* (1998) (Table 4.4) were screened in the three mapping populations. PCR amplifications were performed using the Geneamp PCR reagent kit of Applied Biosystems. A reaction volume of 20 µl contained 15 ng DNA, 1 x PCR buffer, 0.2 mM dNTPs, 0.5 µM forward and reverse primer and 0.8 U of the *Taq* polymerase. PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research). Cycling conditions were 1 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at T_h depending on the primer combination (Table 4.4) and 1 min at 72°C, and a final elongation step of 10 min at 72°C. STS alleles were separated on a 8 % PAA gel and visualized by UV illumination after staining with ethidium bromide.

4.3.5 Resistance gene analogues

The primers used in this study (Table 4.5) were described by Mago et al. (1999) and Yu et al. (1996). Nine primer combinations were tested (s1/nbs1-r, s1/nbs2-r, s1/nbs3-r, s1/nbs4-r, s2/nbs1-r, s2/nbs2-r, s2/nbs3-r, s2/nbs4-r, nbs-f1/nbs-r1). PCR was performed in a total volume of 20 µl containing 75 ng DNA, 0.2 mM dNTPs, 2 µM forward and reverse primer, 1 x PCR buffer and 0.8 U of the *Taq* polymerase of the Geneamp PCR reagent kit of Applied Biosystems. PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research). Cycling conditions were 1 min at 94°C, 40 cycles of 30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C, and a final elongation step of 6 min at 72°C. PCR fragments were separated on a 1.5 % agarose gel using gel electrophoresis and visualized by UV illumination after staining with ethidium bromide.

PCR fragments were excised from agarose gels and purified using the Qiagen Gel Extraction Kit. Fragments were cloned using the TOPO TA cloning kit (Invitrogen) and sequenced using the ABI Prism BigDye Terminator cycle Sequencing kit of Perkin Elmer Biosystems. Sequencing reactions were analyzed using an ABI Prism 377 DNA Sequencer. Sequence analysis was performed using the GCG software (FASTA, MAP, Translate)

Table 4.4 : STS primer sequences developed by Lallemand et al. (1998). Forward (F) and reverse (R) primer sequences, the hybridization temperature of the primers (T_h) and the origin of the consensus sequences on which primers were designed are given.

Primer set	Gene	Origin consensus sequences	T_h		Primer Sequences
ADH	Alcohol dehydrogenase	Maize, barley and rice	60	F	GCG TCA AGA TCC TCT TCA CC
				R	CRC CCT CTC AAA CAC TCT CY
MZE	Triosephosphate isomerase	Maize, rice and rye	60	F	TCA AAG GTC ATT GCA TGT
				R	CNG NGT TGA TGA TGT CGA TGA A
OSW	ADP-Glucose glycosyl transferase	Maize, barley, rice and sorghum	60	F	TTC TGC ATC CAC AAC ATC TCC TA
				R	CTG ACG TCC ATG CCG TTG ACG AT
LP1	Pollen allergen	Ryegrass	60	F	CAC CAA GCC GAC ATT CCA C
				R	CAC CGT GCG AGC AAA GAA AG
PRO	Profilin	Maize and *Phleum*	60	F	TAC CAA GTA CAT GGT CAT CC
				R	ATS GGC TCG TCG TAG ATG C
OSE	Late abundant embryogenesis protein	Maize, barley, rice and wheat	62	F	CGT CGT CCC CGG CGG CAC CG
				R	TTG GAC TCG TCG ATG TCG AT
SCF	RUBISCO	Maize, barley, rice and sugar cane	62	F	GGC TCA AGT CCA CCG CCA GC
				R	AAC ATG GGC AGC TTC CAC AT
OSBR	á-amylase 3	Maize, barley, rice and wheat	60	F	GAC AGC CGC CTC GAC TGG GG
				R	GAT CTC CTG CRTT CAG GTT CC
ADP	ADP glucose phosphorylase	Barley, rice and wheat	60	F	CCT CCG TGA ACA ATT TCC TG
				R	TCC AAT ACG AGC ATT CTT GT
PHOS	Phopholipase	Maize and rice	45	F	AAC CCC AAG GAC TAY CTC AC
				R	AMC CRA TGA TGA TGT ACT CR
PGLU	Prepro glutelin	Rice and oat	43	F	CYG AAR GTC AAA GCC AAA GC
				R	AAK CCA CTR AAT ATG TTT TG
PAL	Phenylalanine ammonialyase	Barley, rice and wheat	62	F	AGC GGA TGG TGG AGG AGT AC
				R	TTG GA GCA TCA TGT AGG AG
CAT	Catalase	Barley and rice	60	F	GAG CGT GGA AGC CCT GAG AC
				R	CCA TGT GCC TGT AGT TGA GT
SER	Serine carboxypeptidase	Barley and rice	50	F	TGG GGT TTA TGT YCC TAC TC
				R	GAS CCA TTC CAT GWG CAA AT
ASP	Aspartic protease	Barley and rice	62	F	GCC TGT GAG ATG GCT GTT GT
				R	ATG GCT GTG AAT CCA CTG AT
CAF	Caffeic acid O-methyl transferase	Ryegrass	62	F	CGC TCA TGGA ACCC AGG ACA AC
				R	GGG ATG CCG CCG TCA AGG AC

Table 4.5 : Degenerate oligonucleotides developed by Mago et al. (1999) and Yu et al. (1996) based on the conserved motifs in the NBS region of tobacco *N* and *Arabidopsis RPS2* and flax *L6* genes. [1] conserved regions used by Mago et al. (1999) for primer design, [2] conserved regions used by Yu et al. (1996) for primer design.

	Amino acid sequence of conserved region			Degenerate primers		
	N	RPS2	L6	name	Sequence (5'-3')	Tm
Sense : P loop region	GGVGKTT[1]	GGVGKTT[1]	GGIGKTT[1]	s1	GGT GGG GTT GGG AAG ACA ACG	55
				s2	GGI GGI GTI GGI AAI ACI AC	55
	GMGGVGKT[2]	GPGGVGKT[2]	GMGGIGKT[2]	nbs-f1	GGA ATG GGN GTN GGN AAR AC	55
Antisense: hydrophobic region	GLPLAL[1]	GLPLAL[1]	GLPTL[1]	nbs1-r	CCA CGC TAG TGG CAA TCC	55
				nbs2-r	IAA IGC IAG IGG IAA ICC	55
				nbs3-r	IAG IGC IAG IGG IAG ICC	55
				nbs4-r	ARI GCT ARI GGI ARI CC	55
kinase3 region	SRIIITTR[2]	CKVMFTTR[2]	SRFIITSR[2]	nbs-r1	YCT AGT TGT RAY DAT DAY YYT RC	55

(I=inosine, N=A+C+T+G, R=A+G, Y=C+T, D=A+G+T)

81

4.4 Results and discussion

4.4.1 RFLP markers

A set of 51 RFLP probes consisting of both cDNA and genomic DNA probes known to be distributed evenly over the chromosomes of rice were screened for RFLPs in the *L. perenne* population. The restriction enzymes *Hind*III, *Dra*I and *Eco*RI were used. Screening results are summarized in Table 4.2. Three probes did not hybridize, three probes showed non-specific hybridization, fourteen probes were non-polymorphic and twenty-nine were polymorphic (an example is displayed in Fig. 4.1). This indicates that the selected set was very suitable for cross-hybridisation in *L. perenne* as just 6 of 51 probes resulted in no or non-specific RFLP signals. Taking into account that these probes have also been mapped in other grass species, they will enable, after mapping, the alignment of the *L. perenne* map with that of other *Gramineae* (Chapter 5).
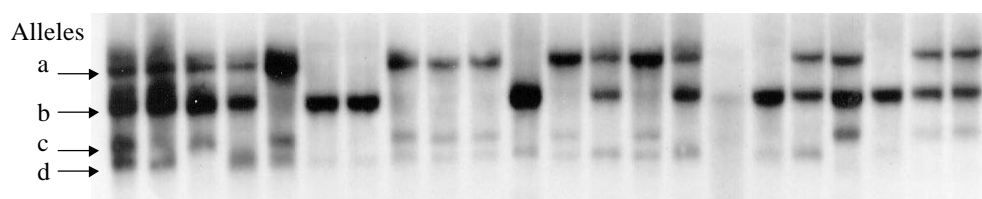
Fig. 4.1 : RFLP profiles obtained with probe CDO87 of the EGRAM anchor probe set. Lane 1 and 2 are the parents of the *L. perenne* population (TC1 and SB2). All other lanes contain F_1 individuals. This RFLP probe detected two loci. One locus consists of allele c and d; the second locus out of a and b. The loci have been scored co-dominantly.

These RFLP markers demonstrated very successful for co-dominant scoring, as 23 out of 29 (polymorphic) probes have been scored co-dominantly. Despite the high value of these markers, the procedure for the generation of RFLPs is very laborious in ryegrass due to its relatively large genome. Big quantities of genomic DNA were necessary for southern blotting.

4.4.2 SSR markers

SSRs are PCR-based markers and easier to generate than RFLPs. They were used as a second source of co-dominant markers. The set of 27 primer combinations developed in *Lolium*, was first tested in the *L. perenne* population. A summary of the results is given in Table 4.6. Four primer combinations did not produce any amplification. Six primer combinations resulted either in non-specific amplification or in weak amplification. Eight primer combinations were monomorphic and nine were polymorphic.

In some cases the amplification profile contained a lot of stutter bands, because of slippage of the *Taq* polymerase during the PCR amplification and the non-templated addition of an extra A to the 3' end of PCR products (Smith et al., 1995). This is a characteristic often seen in SSRs with dinucleotide repeats (Harker, 2001). Addition of a short nucleotide sequence at the end of the primer (pigtailing; Brownstein et al., 1996), resulted in a better profile with less stutter bands (Fig. 4.2: rye005 primer with and without tail).

For three primer combinations (rye005, rye014 and rye023), we observed "mirror" profiles. If one fragment was present, a slightly bigger fragment was also observed (Fig. 4.2). This might indicate a possible duplication of the SSR locus close to the original locus. Two primer combinations (rye005 and rye014) revealed always the same genotype in each F_1 plant, which indicated that both SSRs were derived from the same locus. For two primer combinations (rye012 and rye022), amplification was just observed when ramping conditions were included in the PCR program. The primer combinations rye012, rye014, rye022, rye031 and rye035 displayed null alleles, but four of these five primer combinations could still be scored co-dominantly. Only rye022 could not be scored co-dominantly, as one allele was amplified.

After this first screening, the most promising SSRs were also tested for polymorphisms in the *L. x boucheanum* and *L. multiflorum* populations (Table 4.6). Finally, three polymorphic SSRs were retained for mapping in the *L. multiflorum* population (uni001, rye024 and rye035) and four in the *L. x boucheanum* population (uni001, rye021, rye024 and rye035).

The set of 100 SSRs developed by Jones et al. (La Trobe University, Australia) and licensed to Advanta Van der Have (Rilland, The Netherlands) was tested in the *L. perenne* mapping population at Advanta Van der Have (Netherlands). 37 SSRs with a good amplification pattern were retained for genotyping a set of 60

F$_1$ plants of the *L. perenne* mapping population (performed by Advanta Van der Have, Rilland, The Netherlands).

SSRs isolated in wheat (Roder et al., 1995) were tested in the studied populations without any success (no or weak amplification; results not shown).

Table 4.6 : SSR primer sets used to amplify SSR loci in the *L. perenne*, *L. x boucheanum* and *L. multiflorum* population. The markers selected for mapping are indicated in *Italics*.

Source	SSR	Reaction type[*]		
		L. perenne	*L. x boucheanum*	*L. multiflorum*
DvP	Rye001	NA	-	NA
	Rye002	NA	-	NA
	Rye005	P, primer with tail	NA	NA
	Rye008	A	-	NA
	Rye009	NA	A	NA
	Rye010	A	-	NA
	Rye012	P, with ramping	M	M
	Rye013	NA	-	NA
	Rye014	P	NA	NA
	Rye016	A	A	M
	Uni001	P	P	P
Kubik et al. 1999	Rye021	M	P	A
	Rye022	P, with ramping	A	M
	Rye023	P	M	A
	Rye024	P	P	P
	Rye025	M	M	M
	Rye026	M	A	A
Jones et al., 2001	*Rye031*	P	M	M
	Rye032	A	A	-
	Rye033	A	A	-
	Rye034	M	M	-
	Rye035	P	P	P
	Rye036	A	A	-
	Rye037	M	M	-
	Rye038	M	M	-
	Rye039	M	M	-
	Rye040	M	M	-

[*] NA = no amplification; A = aspecific amplification; M = monomorphic; P = polymorphic; - = not tested
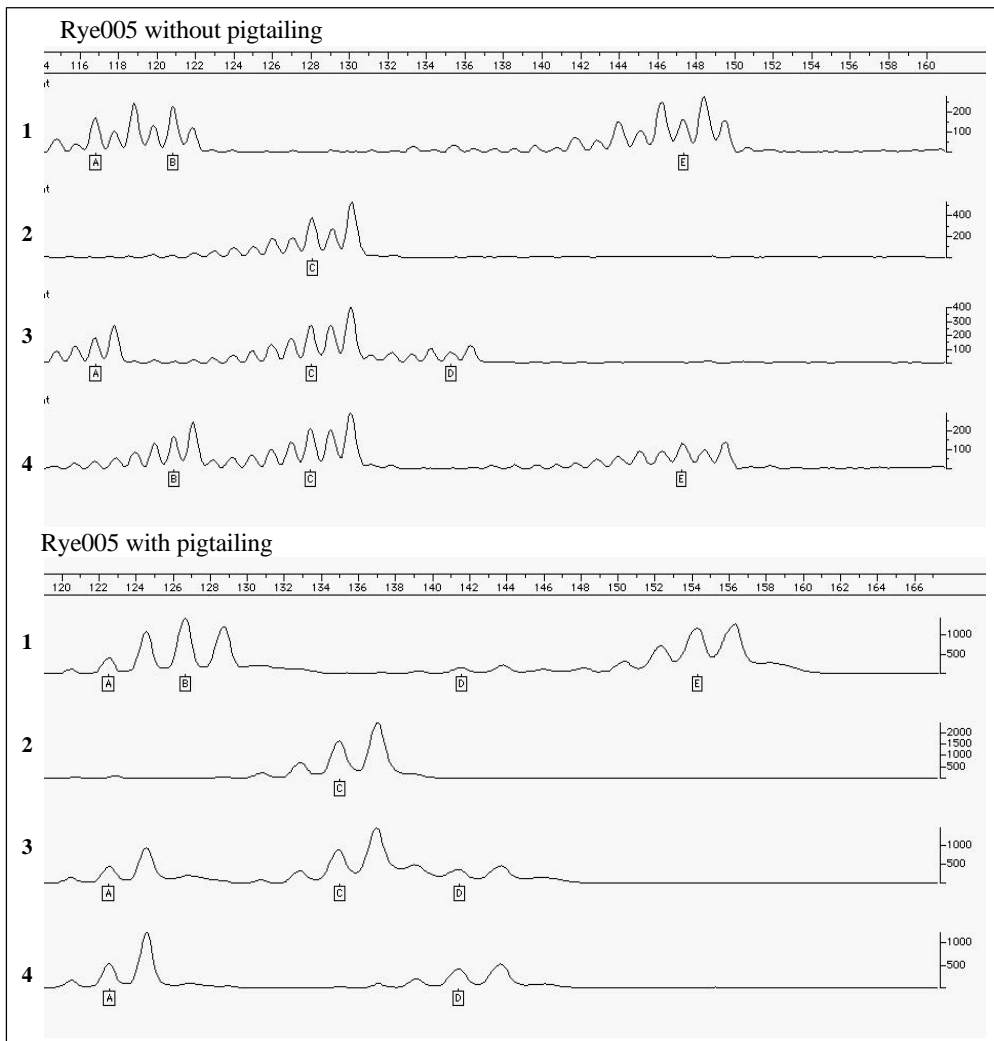
Fig. 4.2. : SSR pattern obtained with SSR Rye005 in the two *L. perenne* parents (lane 1 and 2) and two F₁ individuals (lane 3 and 4). Fingerprint is given as an electroferogram, in which fragments are represented as peaks. Without pigtailing, a lot of stutter bands can be seen; addition of a tail to the primer resulted in a pattern of a higher quality. With this primer combination, the mirror effect was observed. Peaks A and D and peaks B and E were always segregating together.

4.4.3 STS markers

The sixteen STS primer combinations developed by Lallemand et al. (1998) were tested for length polymorphisms in the three mapping populations studied. An

example is shown in Fig. 4.3. The screening results of the three populations are summarized in Table 4.7.
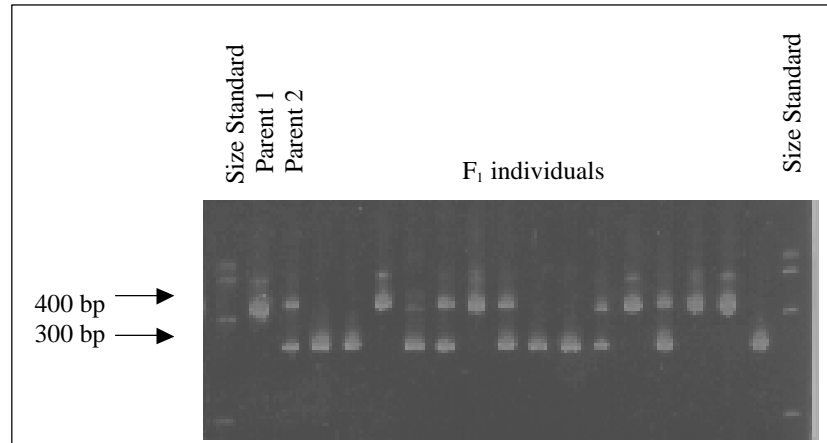
Fig. 4.3: STS marker OSW : amplification products obtained with primer combination OSW. Lane 2 and 3 are parent 1 and 2 respectively; the other lanes contain F_1 individuals. Fragments were separated on a 8% PAA gel and visualized by UV illumination after ethidium bromide staining.

Table 4.7 : Results obtained with the sixteen primer sets developed by Lallemand et al. (1998) in the *L. perenne*, *L. x boucheanum* and the *L. multiflorum* population. (Reaction type: M = monomorphic, P= polymorphic, A= Aspecific).

STS	*L. perenne*	*L. x boucheanum*	*L. multiflorum*
ADH	M	**P**	**P**
MZE	M	M	M
OSW	**P**	**P**	P
LP1	**P**	**P**	**P**
PRO	M	M	M
OSE	**P**	**P**	M
SCF	M	A	M
OSBR	**P**	M	M
ADP	M	P	M
PHOS	A	M	P
PGLU	A	P	M
PAL	M	M	A
CAT	**P**	**P**	**P**
SER	M	M	P
ASP	M	M	P
CAF	M	M	M
Total polymorphic	5	7	7

In the respective *L. perenne*, *L. multiflorum*, and *L. x boucheanum* population, five, seven and seven out of sixteen primer combinations revealed length polymorphisms and were retained for mapping purposes. Furthermore, the markers ADH, OSW, LP1, OSE and CAT were polymorphic in more than one population and are useful for aligning the maps of the three mapping populations.

4.4.4 Resistance gene analogues

A special case of STS markers used in this study, is the amplification of Resistance Gene Analogues. The degenerate primers utilized in this study (Yu et al., 1996; Mago et al., 1999) were designed to include no introns. Therefore, the size of PCR products arising from genomic RGA sequences were predicted from the known R sequences for all primer sets. Of the nine primer combinations tested, just the primer set S1/NBS1-R amplified clear bands of approximately 500 bp (one fragment of 630 bp and one fragment of 540 bp), which is the expected size for RGA fragments (Fig. 4.4). The other primer pairs amplified fragments with lengths not corresponding to the length expected from the primer design. The 540 and 630 bp fragments amplified in the *L. perenne* parents (SB2 and TC1) with the primer pair S1/NBS1-R were cloned and sequenced.

Six clones of the 540 bp fragment amplified in SB2 were sequenced, of which four were identical S1/NBS1-R fragments; two other clones included S1/S1 fragments, which were rejected for further analysis. Ten clones of the 540 bp fragment amplified in the TC1 parent were sequenced of which nine clones contained identical S1/NBS1-R sequences; one clone contained a S1/S1 fragment, which was not of interest. The 540 bp S1/NBS1-R fragments of both parents showed homology with known resistance genes (Table 4.8). One clone of the 630 bp fragment amplified in the SB2 parent was sequenced, but did not show any homology with known resistance genes.

Comparison of the RGA sequences obtained in TC1 and SB2 revealed four single nucleotide polymorphisms (Fig. 4.5). Translation of the RGA sequences of TC1 and SB2 resulted in amino acid sequences without any stop codons. The single nucleotide polymorphisms resulted in 2 amino acid polymorphisms (Fig. 4.6).

Restriction site analysis of the RGA sequences of TC1 and SB2 revealed restriction site polymorphisms situated in the regions of the single nucleotide

polymorphisms (Fig. 4.7). We used these polymorphisms to derive a CAPS marker from this RGA sequence. Specific primers were designed to amplify the 540 bp S1/NBS1-R fragment and not the S1/S1 fragments. Using these primers and the restriction enzyme *MboI* or *TaqI*, we were able to turn the 540 bp RGA fragment into a CAPS marker useful for mapping in the *L. perenne* population (Fig. 4.8).
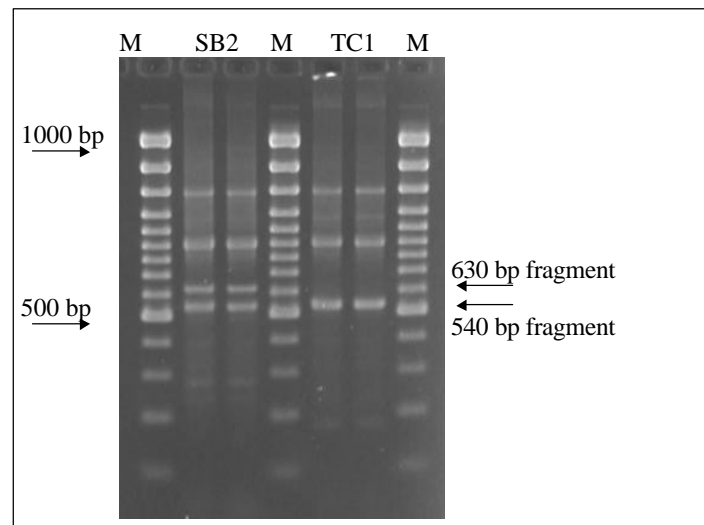
Fig. 4.4 : PCR amplification products obtained with primer combination S1/NBS1-R in the parent plants (SB2 and TC1) of the *L. perenne* population. The 540 and 630 bp fragment were excised for further analysis. (M= 100 bp marker MBI fermentas).

Table 4.8 : Homology results of the 540 bp fragment obtained in both parents of the *L. perenne* population. Homology searches were based on the amino acid sequence using the FASTA algorithm of GCG

Clone	Gene	Organism	Accession number	E-value (FASTA)
Clone18 (isolated in SB2)	*RPP13*	*Arabidopsis thaliana*	Q9M667	1.2e-19
	RPP8	*Arabidopsis thaliana*	L32592	2.6e-17
	I2	*Lycopersicon esculentum*	Q9XET3	9.1e-17
Clone61 (isolated in TC1)	*RPP13*	*Arabidopsis thaliana*	Q9M667	2.1e-19
	RPP8	*Arabidopsis thaliana*	L32592	3.7e-17
	RPM1	*Arabidopsis thaliana*	Q39214	4.8e-17
	I2	*Lycopersicon esculentum*	Q9XET3	8.6e-17

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               .              .              .              .              .
cl 18  1 GGTGGGGTTGGGAAGACAACGCTGGCTAAAAAAGTCTACACATCATCTAG
         ||||||||||||||||||||||||||||||||||||||||||||||||||
cl 61  1 GGTGGGGTTGGGAAGACAACGCTGGCTAAAAAAGTCTACACATCATCTAG
               .              .              .              .              .
      51 AGTCAAACAACACTTTGAAGTAGTTGCATGGGTGACCGTGTCTCAGACAT
         |||||||||||||||||||||||| |||||||||||||||||||||||||
      51 AGTCAAACAACACTTTGAAGTACTTGCATGGGTGACCGTGTCTCAGACAT
               .              .              .              .              .
     101 TCAAGGGCATTGATTTACTCAAGGATATCATGAAACAAATAACAGGGGGC
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     101 TCAAGGGCATTGATTTACTCAAGGATATCATGAAACAAATAACAGGGGGC
               .              .              .              .              .
     151 ACATATGATTCATCGAATCTCATGCAGGAGTTTGATGTCGGAAAGAAGAT
         |||||||||||| |||||||||||||||||||||||||||||||||||||
     151 ACATATGATTCAACGAATCTCATGCAGGAGTTTGATGTCGGAAAGAAGAT
               .              .              .              .              .
     201 TAGGGATTTTTTGTTTACAAAGAGATACTTAGTAGTTCTGGATGATGTGT
         |||||||||||||||||||||||||||||||||||||||| |||||||||
     201 TAGGGATTTTTTGTTTACAAAGAGATACTTAGTAGTTCTCGATGATGTGT
               .              .              .              .              .
     251 GGGAAGCAGACACATGGGACCAATTAAATAGAACAGTTGAAGCCTTTCCA
         ||||||||||||||||||| ||||||||||||||||||||||||||||||
     251 GGGAAGCAGACACATGGGATCAATTAAATAGAACAGTTGAAGCCTTTCCA
               .              .              .              .              .
     301 AATGAAGATAACGGTAGTAGATTACTGCTAACCACACGGAAGGTAGATGT
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     301 AATGAAGATAACGGTAGTAGATTACTGCTAACCACACGGAAGGTAGATGT
               .              .              .              .              .
     351 TGCAAATCATGTTGAAAGGCCAACCCATGTTCATGCTCTGAAGCACTTAA
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     351 TGCAAATCATGTTGAAAGGCCAACCCATGTTCATGCTCTGAAGCACTTAA
               .              .              .              .              .
     401 ACGAAGAGAAAAGTTGGAAGCTATTTTGTAGCAAAGCTTTTCCATCATAC
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     401 ACGAAGAGAAAAGTTGGAAGCTATTTTGTAGCAAAGCTTTTCCATCATAC
               .              .              .              .              .
     451 AAAAGGTCTGTCATGCGTGACGTTGCTGAGTTTCAAAAAATTGGGAGAAA
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     451 AAAAGGTCTGTCATGCGTGACGTTGCTGAGTTTCAAAAAATTGGGAGAAA
               .              .              .              .
     501 ACTAGCAAGCAAATGTGATGGATTGCCACTAGCGTGG 537
         |||||||||||||||||||||||||||||||||||||
     501 ACTAGCAAGCAAATGTGATGGATTGCCACTAGCGTGG 537
```

Fig. 4.5 : Alignment of the 540 bp RGA fragments obtained in the parent plants : clone 18 isolated from SB2 and clone 61 from TC1. The single nucleotide polymorphisms are indicated in bold.

```
            .           .           .           .
cl 18  1 GGVGKTTLAKKVYTSSRVKQHFEVVAWVTVSQTFKGIDLLKDIMKQITGG
         ||||||||||||||||||||||||||·||||||||||||||||||||||||||
cl 61  1 GGVGKTTLAKKVYTSSRVKQHFEVLAWVTVSQTFKGIDLLKDIMKQITGG
            .           .           .           .
      51 TYDSSNLMQEFDVGKKIRDFLFTKRYLVVLDDVWEADTWDQLNRTVEAFP
         ||||·|||||||||||||||||||||||||||||||||||||||||||||||
      51 TYDSTNLMQEFDVGKKIRDFLFTKRYLVVLDDVWEADTWDQLNRTVEAFP
            .           .           .           .           .
     101 NEDNGSRLLLTTRKVDVANHVERPTHVHALKHLNEEKSWKLFCSKAFPSY
         |||||||||||||||||||||||||||||||||||||||||||||||||||
     101 NEDNGSRLLLTTRKVDVANHVERPTHVHALKHLNEEKSWKLFCSKAFPSY
            .           .
     151 KRSVMRDVAEFQKIGRKLASKCDGLPLAW 179
         ||||||||||||||||||||||||||||||
     151 KRSVMRDVAEFQKIGRKLASKCDGLPLAW 179
```

Fig. 4.6 : Translation and comparison of the RGA fragments isolated from TC1 (cl. 61) and SB2 (cl. 18). The single nucleotide polymorphisms resulted in two amino acid sequence polymorphisms (indicated in bold).

```
                    SNP1                  SNP2

                                               TaqI
SB2 clone18 67 GAAGTAGTTGCA 78  157 GATTCATCGAAT 168
               |||||| |||||          |||||| |||||
TC1 clone61 67 GAAGTACTTGCA 78  157 GATTCAACGAAT 168
                    TatI
                    Csp6I
                      RsaI & ScaI


                    SNP3                  SNP4

                                               NlaIV
                                               AvaII
                                               Sau96I
SB2 clone18 234 AGTTCTGGATGA 245   270 CTGGGACCAATT 281
                |||||| |||||           |||||| |||||
TC1 clone61 234 AGTTCTCGATGA 245   270 ATGGGATCAATT 281
                     TaqI                  MboI
                                           DpnI
                                             AlwI
```

Fig. 4.7 : Restriction site analysis of the two RGA fragments isolated from parent TC1 and SB2. The single nucleotide polymorphisms (SNP) are indicated in bold. The enzymes used to generate CAPS markers are also indicated in bold.

Fig. 4.8 : Restriction analysis of RGA fragments amplified in SB2 and TC1. Restriction enzymes used were *Taq*I and *Mbo*I. Next to the parents of the *L. perenne* population (P_1=TC1 and P_2=SB2), four F_1 individuals were analysed, in order to observe the inheritance of the polymorphisms.

4.5 Conclusions

Of the techniques tested for marker generation in *Lolium*, some offer clear advantages above others. Although, 29 out of 51 RFLP probes have been turned into polymorphic markers in the *L. perenne* population, RFLP remains a laborious and very tedious mapping technique, demanding big quantities of good quality DNA. This makes the technique difficult to perform in early seedling stage.

The other techniques tested, all of them PCR-based, are better suited for high throughput analysis and applicable to small quantities of DNA. Nine out of 27 SSRs tested at DvP, were polymorphic in the *L. perenne* population. Out of the set developed by Jones et al. (La Trobe University, Australia) and licensed to Advanta Van der Have (Rilland, The Netherlands), 37 SSRs with a good amplification pattern were retained for mapping in the *L. perenne* population.

The number of STS primers that has been turned into polymorphic markers was small (five, seven and seven out of sixteen STS markers in respectively the *L. perenne*, *L. x boucheanum* and *L. multiflorum* population). This was mostly due to lack of polymorphism using PAA-gel electrophoresis. The resolution

attained in this kind of gels lies between 10 and 50 bp. The number of polymorphisms would certainly be higher if techniques with a higher resolution were used; techniques like SSCP, CAPS or HD analysis, that can detect single nucleotide polymorphisms.

In the *L. perenne* population, an isolated RGA fragment was turned into a CAPS marker. This demonstrates once more the validity of this approach for the isolation of R-related sequences in different species. However, nothing is known yet about the potential function of this locus in crown rust resistance.

The polymorphic markers identified in this chapter, will be used for linkage map construction. In Chapter 5, the map construction in the *L. perenne* population will be discussed; in Chapter 7, the map construction in the two other populations. A number of SSR and STS markers are polymorphic in the three populations used in the present study. These markers will serve as a basis to align the different maps. In the *L. perenne* population, the heterologous RFLP probes will be used to align the *Lolium* map with other *Gramineae* maps.

Chapter 5

Linkage map construction in the *L. perenne* population and alignment with related linkage maps

5.1 Introduction

Determination of the number and distribution of the genetic loci controlling a trait of agronomic importance is facilitated when knowledge on the genome organization of the species is available. Locating these loci in the genome can easily be achieved for simple heritable monogenic traits, but is also possible for polygenic traits based on more loci. In the latter case, large segregating populations are required to construct a genetic linkage map and to unravel the number of loci involved in the trait (Hayward et al., 1998; Jeuken et al., 2001). A genetic linkage map graphically represents the arrangement of the numerous loci, which may include morphological, isozyme and DNA markers, along the chromosome. The distance between these loci is expressed in centimorgans (cM), representing the recombination rates between the loci (1 cM = 1% recombination). Genetic linkage maps, thus, report the linear order of markers and the recombination frequency between linked markers. They do not contain information on physical distance, neither cytological distance nor number of DNA base pairs between markers, because the recombination frequencies vary along the length of the chromosome. For example, the regions near the centromeres show suppressed recombination, which is reflected in the clustering of markers (Kumar, 1999).

There are four well-defined steps in the construction of a genetic linkage map: 1) development of markers and identification of polymorphic ones; 2) establishment of a segregating population (the parents should be genetically divergent enough to exhibit sufficient polymorphisms and at the same time should not be so far apart so as to cause sterility of the progeny); 3) fingerprinting of the parents and the progeny with markers displaying polymorphism in this population; 4) ordering of markers (recombination frequencies and their standard errors for all pair-wise comparisons between loci are estimated using the maximum likelihood method and map units are calculated

using a map function) (Ritter and Salamini, 1996; Kumar, 1999; Van Ooijen & Voorrips, 2001).

In Table 5.1, three different mapping strategies are compared. In the majority of cases, the published genetic maps are of inbreeding species and mapping populations are derived from inbred lines. In this case, knowledge of the linkage phase between heterozygous loci can be deduced from the parental plants (Maliepaard et al., 1997). In heterozygous outbreeding species such as ryegrasses, potato, sugar beet, apple … difficulties in mapping have been encountered in cases where alternative linkage phases have to be considered (Hayward et al., 1998). One way to circumvent these difficulties is to mate a homozygous (e.g. doubled haploid) or a near-homozygous plant (inbred line) with a heterozygous plant, the so called one-way pseudo-testcross. However, self-incompatibility may block the possibility to construct near- homozygous plants. This is the case in ryegrass, as it is an obligate outbreeder with a gametophytic self-incompatibility system controlled by the loci S and Z (Cornish et al., 1979). In addition, the products of double haploidization and forced selfing are often plants displaying severe inbreeding depression. To overcome such a problem, a statistical procedure has been developed for the estimation of recombination frequencies from populations obtained by crossing two heterozygous parents, the so-called two-way pseudo-testcross. In this strategy, linkage analysis is carried out for each parent separately. The parental maps are integrated based on the availability of markers heterozygous in both parents. They function as allelic bridges (Grattapaglia and Sederoff, 1994; Ritter and Salamini, 1996; Maliepaard et al., 1998).

The differences for linkage analysis within a F_2 strategy and a two-way pseudo-testcross, are due to the number of segregating alleles per locus and the linkage phase of the alleles at different loci (Ritter and Salamini, 1996; Maliepaard et al., 1997). At one locus one may find up to four alleles, and this may vary between loci, while the linkage phases are usually unknown what complicates the detection of recombination events. The presence of null-alleles in the parents of a two-way pseudo-testcross leads to dominance, i.e. two particular genotypes cannot be distinguished by phenotype. In this way, there are seven distinct segregation types providing recombination information in a two-way pseudo-testcross (Maliepaard et al., 1997).

Table 5.1 : Overview of three different mapping strategies. The population usually used for map construction is given in bold.

	Mapping strategy		
	F_2	One-way pseudo-testcross (or BC_1 type)	Two-way pseudo-testcross
Parent plants	2 homozygotes AA x BB	1 heterozygote and 1double haploid (or homozygote) AB x CC	2 heterozygotes AB x CD
F_1 population	AB	**AC:BC**	**AC:AD:BC:BD**
F_2 population	**AA:AB:BB**		

However, these difficulties do not hamper the construction of genetic maps for outcrossing species by the two-way pseudo-testcross. This method is becoming fairly commonly used (apple, Maliepaard et al., 1998; roses, Debener and Mattiesch, 1999; onion, van Heusden et al., 2000; poplar, Wu et al., 2000; chestnut, Casasoli et al., 2001; kiwifruit, Testolin et al., 2001). The increasing availability of genetic maps for outbreeders is also due to the development of software, like JoinMap version 3.0 (Van Ooijen and Voorrips, 2001), able to handle this kind of linkage data.

Several linkage maps for *Lolium* spp. are being developed at different institutes. A (probably) incomplete overview of published and unpublished maps is given in Table 5.2. To date, published linkage maps for *Lolium* spp. have been based on one of three segregating populations (Hayward et al., 1994 and 1998; Bert et al., 1999; Jones et al., 2002a, 2002b; Armstead et al., 2002). Two populations were developed by crossing a double haploid plant (DH) with a heterozygous plant, named the one-way pseudo-testcross strategy. By using a DH parent, the construction of two parental maps and their integration is avoided. The population described by Hayward et al. (1998) showed short longevity, due to presence of genes from the short-lived predominantly biennial species *L. multiflorum.* The maps of Bert et al. (1999) and Jones et al. (2002a, 2002b) were based on a more recently constructed segregating population (p150/112), derived from a cross between a DH and a heterozygous *L. perenne* plant. The map constructed using this population serves nowadays as the reference map of *Lolium*, and is referred to as the ILGI map (International

Table 5.2: Existing genetic maps of *Lolium* spp. The construction of the segregating populations, the marker types and map length is given. (DH = double haploid)

Reference	Plant species	Construction segregating population	# of plants	Marker types	Map length
Hayward et al., 1994 and 1998.	*interspecific*	One-way pseudo-testcross DH (*L. perenne*) x F$_1$ (*L. perenne* x *L. multiflorum*)	89 plants	RAPD RFLP Isozyme	692 cM
Bert et al., 1999. Jones et al., 2002 (a & b)	*L. perenne*	One-way pseudo-testcross (DH x F$_1$)	165 plants	AFLP RFLP EST SSR isozyme	814 cM
Dolstra et al. PRI, the Netherlands (personal communication)	*L. perenne*	One-way pseudo-testcross (DH x F$_1$)	Unknown	AFLP STS SSR	708 cM
Maiko et al., 2002.	*L. multiflorum*	Two-way pseudo-testcross	82 plants	AFLP EST RFLP SSR	1154 cM
Van de Walle et al. CLO-DvP, Belgium (personal communication)	*L. multiflorum*	Two-way pseudo-testcross	110 plants	AFLP STS SSR	925 cM
Armstead et al., 2002	*L. perenne*	F$_2$ population (Selfing of F$_1$ plant derived from a cross between two highly distinct inbred lines)	180 plants	AFLP RFLP STS SSR	515 cM

Lolium Genome Initiative). The third map published by Armstead et al. (2002) is based on a F_2 population obtained by selfing a F_1 plant, which was in turn selected out of a cross between highly distinct inbred lines. More genetic maps are currently under development in different laboratories. In the present study we opted to use a two-way pseudo-testcrosses in order to avoid inbreeding depression or self-incompatibility problems and to be able to choose parent plants with contrasting phenotypes for the trait studied.

In this project, we aim to compare our map to ongoing mapping projects in *Lolium* and to identify syntenic relationships between *Lolium* and other *Gramineae* species. Many *Poaceae* (rice, maize, pearl millet, foxtail millet, maize, sorghum, wheat, barley and rye) show a high level of conserved synteny and colinearity (Gale and Devos, 1998). Specially the genetic structure of maps of wheat, barley and oat is similar (Namut et al., 1994; Van Deynze et al., 1995a, 1995b; Dubcovsky et al., 1996). These three species belong to the *Pooideae* as *Lolium* does. The establishment of syntenic relationships between species enables the transfer of genetic information from well-studied species such as rice to less studied species as *L. perenne*. Jones et al. (2002a) reported the alignment of a *L. perenne* map with the *Triticeae* consensus map. They observed synteny between the genetic maps of *L. perenne*, oat, rice and the *Triticeae*. As a number of heterologous RLFP probes are included in the genetic map presented in this Chapter, a comparison will be made with the genetic maps of the *Triticeae*, oat and rice.

5.2 Objectives and rationale

We aimed to construct a genetic linkage map for *Lolium perenne* based on RFLP, AFLP, SSR and STS markers using the two-way pseudo-testcross approach. The F_1 population was genotyped using the polymorphic markers described in Chapter 4 and using the AFLP markers generated in the BSA analysis (Chapter 3). Our objective was to have a skeletal map with good genome coverage. This map will be used for several purposes. First, the map will be aligned with publicly available linkage maps of *Lolium*. The common RFLP, SSR and STS markers will be useful tools for alignment. Secondly, the RFLP data using heterologous probes derived from wheat, barley, oat, maize and rice, allow the alignment with the *Triticeae* consensus map and the search for

syntenic relationships between *Gramineae*. Thirdly, the location of the markers selected in the BSA analysis will be determined, identifying genomic regions involved in crown rust resistance. Finally, the availability of a map for a population segregating for crown rust resistance, enables the acceleration of the identification of the number and location of the genetic factors involved in this trait. In Chapter 6, this linkage map will be combined with crown rust resistance data in a QTL-approach in order to identify genomic regions, other than those identified in the BSA approach, involved in the determination of the trait.

5.3 Material and methods

5.3.1 Generation of marker data

Marker data were generated on the *L. perenne* mapping population of 252 plants as described in Chapter 2. Genetic data were obtained for an average of 206 F_1 genotypes (with a range from 35 to 252). AFLP, SSR, STS and RFLP fingerprints were generated as described in Chapters 3 and 4. All segregating DNA-fragments were scored dominantly, i.e. for presence or absence of the detected band, and the parental origin of the markers was also recorded. Even for those marker-systems generating co-dominant markers (RFLP, SSR and STS), all DNA-bands were first scored separately as dominant markers. DNA-fragments were classified into three different groups on the basis of their segregation model: (1) a-x--: informative for the TC1 gametes only (SB2 genotype homozygous), (2) --xa-: informative for the SB2 gametes only (TC1 genotype homozygous) and (3) a-xa-: heterozygous in both parents. A first linkage analysis using JoinMap 3.0 (Van Ooijen and Voorrips, 2001) was based on this information.

After this first linkage analysis, RFLP, SSR and STS markers were scored co-dominantly if the bands detected with the same RFLP probe or generated with the same STS or SSR primer combination mapped in the same linkage group. Using this information, six marker classes were then defined: (1) a-x--, (2) --xa-, (3) a-xa-, (4) abxab, (5) abxac, (6) abxcd. Class 3 markers were not included in the linkage analysis, as such markers contribute little information to the map. Recombination frequency estimates obtained with such markers are typically inaccurate (Maliepaard et al., 1998).

5.3.2 Segregation analysis

The χ^2 test integrated in JOINMAP3.0 software (Van Ooijen and Voorrips, 2001) was used to estimate departures from expected segregation ratios: 1:1 segregation (markers in classes 1 and 2), 1:2:1 if only two alleles were involved (marker class 4), or 1:1:1:1 if three or four alleles were involved (marker classes 5 and 6).

5.3.3 Linkage analysis and map construction

In the two-way pseudo-testcross, the total data set is divided into two separate data sets, one for each parent. In the first round, distorted markers (P<0.001) were omitted from the analysis. Markers were grouped into linkage groups at LOD equal or higher than 4 using JoinMap version 3.0 (Van Ooijen and Voorrips, 2001). Markers of classes 4, 5 and 6 were used to identify homologies and to integrate the TC1 and SB2 parental linkage groups. Marker order was calculated at LOD = 1.00 and recombination threshold value (REC) of 0.40. Map distances were calculated using the Kosambi function. In a second step, distorted markers and markers unmapped during the first step, were included in the map only if the map order was not drastically affected. In some cases a LOD = 0.01 and REC = 0.499 had to be used to integrate these markers.

5.3.4 Comparative mapping

Comparative mapping was done as described by Jones et al. (2002a). The comparative location of probes in the *Triticeae* and rice genomes were as reported by Jones et al. (2002a) and/or ascertained using the Graingenes (http://grain.jouy.inra.fr/ggpages/) and Gramene (http://www.gramene.org/) databases.

5.4 Results and discussion

5.4.1 Marker analysis

During the BSA analysis, the *L. perenne* mapping population was fingerprinted using four primer combinations (Chapter 3). These AFLP primer

combinations (PC) generated, next to the putatively R-linked markers, a high number of AFLP markers useful for mapping studies. A total of eight PC (5 *Eco*RI-*Mse*I PC and 3 *Hind*III-*Mse*I PC) were run and resulted in 270 polymorphic AFLP markers (Table 5.3). Although all polymorphic fragments between 75 and 450 bp were assessed for segregation, approximately 50% of the markers selected for mapping were concentrated in the range between 75 and 175 bp (Fig. 5.1). Next to AFLP markers, 46 SSRs, 5 STS and 29 RFLPs, polymorphic in the *L. perenne* mapping population (Chapter 4) were tested on the whole population.

Table 5.3 : Number of polymorphic markers generated with the different AFLP primer combinations (PC). For each PC, selective nucleotides of the AFLP primers and number of putatively linked R markers selected during BSA is given.

Primer Combination		**polymorphic markers**	**R markers**
1	*Eco*RI-ACG-*Mse*I-CAA	29	-
8	*Eco*RI-AAC-*Mse*I-CAC	36	1
26	*Eco*RI-ACA-*Mse*I-CAT	39	3
78	*Eco*RI-AAG-*Mse*I-GCC	25	-
106	*Eco*RI-ACG-*Mse*I-GGC	28	1
168	*Hind*III-TGG-*Mse*I-CAT	25	1
400	*Hind*III-TAC-*Mse*I-GAT	46	-
407	*Hind*III-TGC-*Mse*I-GTT	42	-
	Total number	**270**	**6**
	Mean number/PC	**34**	

5.4.2 Segregation analysis

The law of segregation, which is the most fundamental law in Mendelian genetics, relies on a predictable transmission of alleles from a parent to its offspring, and on a predictable formation of genotypes from the transmitted alleles. Segregation distortion is defined as a deviation of the observed genotypic frequencies from their expected values.

A χ^2 test was performed to test the null hypothesis of Mendelian segregation on all scored markers. In Fig. 5.2, an overview is given of the proportion of distorted markers for each of the marker techniques employed. Overall, 68% of the markers did not show distorted segregation (P>0.05). Segregation distortion
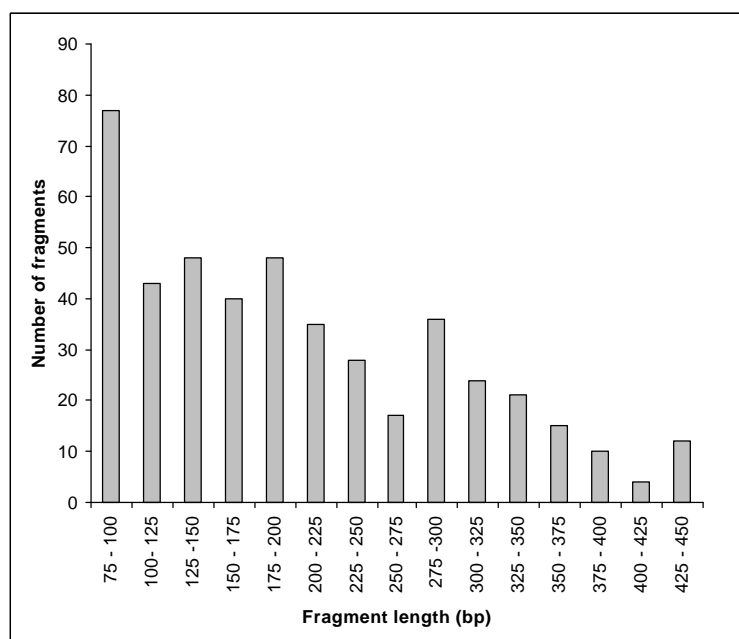
Fig. 5.1 : Frequency distribution of the size of polymorphic AFLP markers generated with eight AFLP primer combinations run on the *L. perenne* mapping population.

has been reported in a wide range of plant species (Casasoli et al., 2001; Testolin et al., 2001) including *Lolium* (Hayward et al., 1998; Bert et al., 1999; Jones et al., 2002a&b; Armstead et al., 2002; Thorogood et al., 2002). Hayward et al. (1998) reported that 20% of the markers displayed distorted segregation at $P<0.01$; in Jones et al. (2002a) 32% of the markers displayed distorted segregation at $P<0.05$ (excluding the AFLPs); Armstead et al. (2002) reported 36% distorted markers at $P<0.05$. These figures are in the same range as the percentage obtained in the present study at $P<0.05$ using the whole data set (32%). However, at $P<0.01$ 15% of the markers mapped by Jones et al. (2002a) were distorted while at this significance level 22% distorted markers is found in the present study. This high percentage can be related to the inclusion of ALFP markers in our study. AFLP markers have the highest proportion of distorted markers among the different marker techniques (Fig. 5.2).

Segregation distortion can be due to different reasons; statistical error, genotyping and scoring errors and biological reasons can lie at the basis. In our

study, genotyping and scoring errors can be one of the reasons for skewed markers. Forty-four markers were skewed towards excess of the marker. This can be due to the superimposition on the gels of non-allelic amplified products corresponding to different loci (fragment homoplasy, as has been demonstrated by Vekemans et al., 2002 for AFLP markers). Thirty-four markers deviated towards absence of the marker. These AFLP markers tended to be fragments with a faint amplification. Biological reasons for segregation distortion can be found in pollen tube competition, pollen lethals, preferential fertilization and selective elimination of zygotes (Lu et al., 2002). These biological reasons for segregation distortion will be discussed in 5.4.4.
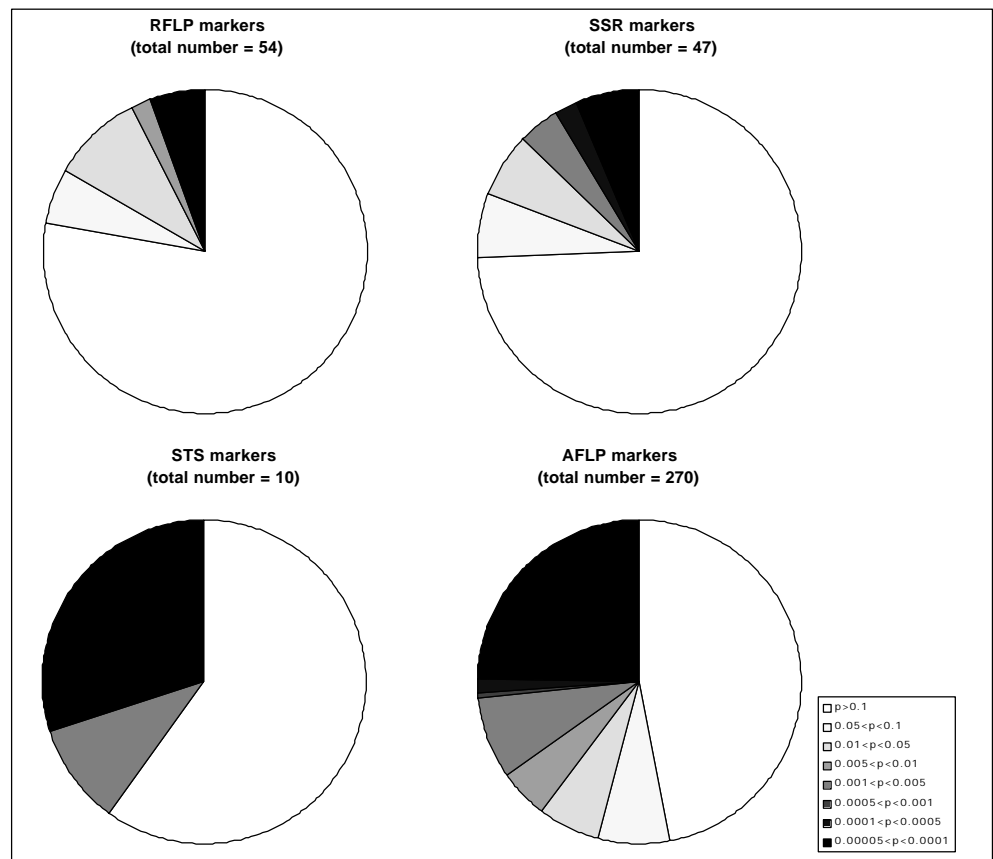
Fig. 5.2: Segregation distortion in the AFLP, SSR, STS and RFLP markers generated in the *L. perenne* mapping population.

5.4.3 Linkage analysis and map construction

Map construction was carried out according to the two-way pseudo-testcross procedure. During preliminary map construction, distorted markers (P<0.001) and class 3 markers (a-xa- see 5.3.1) were omitted. Merging of the TC1 and SB2 map using class 3 markers as allelic bridges can not be performed accurately as simulation experiments have shown that these markers are very limited in producing precise and unbiased estimates of recombination frequencies (Wu et al., 2000).

A total of 237 markers were included in this preliminary analysis: 36% derived from the susceptible parent, 44% from the resistant parent and 20% were heterozygous in both parents. The TC1 map contained 7 linkage groups, including 92 markers. The SB2 map contained 7 linkage groups including 90 markers.

Using less stringent conditions (LOD=0.01 and REC=0.499), markers that were not mapped in the first round were added without changing the marker order drastically. Twenty-three distorted markers (P<0.01) were added without a major change in marker order. The integrated map, obtained by aligning the parental maps on the basis of allelic bridges is presented in Fig. 5.3. It was not possible to integrate linkage group 7S (from the SB2 parental map) with linkage group 7T (from the TC1 parental map) as just one allelic bridge is present (BCD147). After inspection of the ILGI map and the map published by Armstead et al. (2002), 9 SSR markers and 17 RFLP markers situated on LG 7 are potential markers suitable for integration. This represents a priority of future developments with this linkage map.

5.4.4 Genome coverage and marker distribution

The *L. perenne* genetic map is generated from 252 individuals, spans 833 cM and consists of 230 loci. The mean distance between two consecutive loci is 3.62 cM. Assuming that linkage group 7S and 7T represent the same chromosome, we can say that the integrated map represents the *Lolium* genome with its seven chromosomes. The length of the LGs vary from 83 till 159 cM (neglecting LG 7S, which spans 40 cM). Gaps between two adjacent markers

LG1

0	PC400-014*
6	PC407-045
19	LPSSRK03A02_a*
21	PC106-028
22	PC008-051
24	LPSSRK10F08_a
26	PC026-03
27	rye023_a*
30	PC400-149*
32	LPSSRK03A02_b
33	LPSSRK10F08_b
	LPSSRK10F08_c
	PC078-023
34	rye023_b
	LPSSRK10F08_d
	LPSSRK03A02_c
	LPSSRK15H05
35	rye023_c
36	Xrz244
37	LPSSRK12D11
38	PC400-099
	PC026-60
39	PC026-34*
40	PC008-056
41	PC400-151
43	PC106-027
	Xrgc488_a
	LPSSRK07F07
45	PC407-052
	PC400-032
	PC400-091
47	PC008-007
48	PC168-023
	PC008-076
49	**OSE_a**
53	PC026-32
54	**OSE_b***
55	PC008OD-085
56	PC407OD-026*
57	LPSSRK10G04
63	PC026-R2
68	Xcdo89
70	PC400-059
79	PC106-R2
81	PC168-R1
85	PC106-016
102	**OSE_c***
	NBS_a

LG2

0	PC400-137
7	LPSSRK12E06_a
8	PC400-077*
9	LPSSRK12E06_b
14	Xbcd135
17	PC078-075
20	PC407-006
21	LPSSRK08F05_a
	LPSSRK08F05_b
	PC400-022*
25	PC008-014*
28	PC008-088
35	PC078-099*
40	rye022
43	PC008-050*
45	PC400-075
47	PC106-106
49	PC078-121
50	Xcsu6
51	PC168-051
53	PC026-R4
	LPSSRK09G12_a
55	Xbcd855_a
	PC106-022
56	LPSSRK09G12_b
	PC400-036
57	PC026-R3
	Xbcd855_b
58	Xbcd855_c
	PC078-057
59	PC001-079
60	PC400-140
61	Xbcd855_d
64	PC400-113
66	PC001-032
68	PC407-055
69	Xcdo385
71	LPSSRK14B06
74	PC026-39
76	PC008-028
80	rye024_a
102	rye024_b*
106	Xcdo456
133	

LG3

0	PC001-059
11	PC001-065
21	uni001
28	PC168-067
33	PC407-031
38	Xrgc742
39	PC001-037
42	PC106-050
44	PC168-068
47	PC106-056
51	PC078-089
52	PC078-097
59	PC400-136
61	PC407-153
	PC026-41
	PC407-014
64	PC078-067
65	Xrgc390
	PC078-061
66	PC026-41
67	PC400-061
69	PC078-007
70	LPSSRK12H08
72	PC407-007
74	PC026-84
76	PC026-27
78	PC168-038
79	PC008-079
83	Xbcd127
94	PC008-075
105	PC026-43

LG4

0	PC026-26
8	Xc1286_a
9	LPSSRK07C11
11	Xpsr104
17	PC026-28
20	PC168-007
23	PC026-94
28	PC168-066
	PC168-002
34	PC168-065
42	PC008-034
44	PC026-29
47	PC026-63
48	PC026-79
49	PC026-71
51	PC008-080
52	rye012
53	Xcsu25
54	PC008-025
55	PC168-118*
58	PC026-20
60	Xcdo87_a
	PC407-036
64	Xcsu70
65	Xibf64_a
	Xibf64_b
67	LPSSRK04D01
68	LPSSRK08A09
69	Xcdo87_b
76	PC078-053
92	PC407-181
94	rye035

Fig. 5.3: Genetic linkage map for the *L. perenne* cross based on SSR, RFLP, STS and AFLP markers. AFLPs have prefix PC; RFLPs have prefix X; ESTs are indicated in bold; and SSRs have a prefix lp, rye or uni; AFLP markers selected during the BSA analysis as being significantly associated with crown rust resistance start with PC and end with –Rx (with x = number). Distorted markers (P<0.001) are marked with *. Linkage groups are numbered according to the ILGI linkage map.

LG5 **LG6** **LG7S** **LG7T**
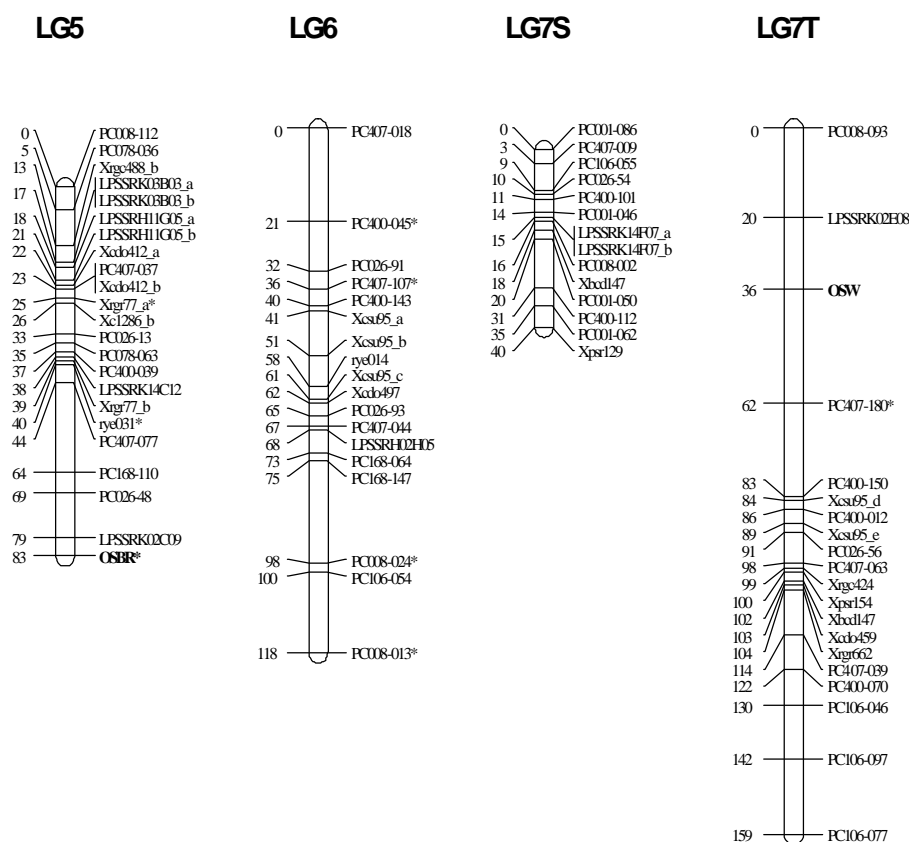
Fig. 5.3 : *continued*

exceeding 20 cM were found on LG 2, LG 6 and LG 7T. The genetic map consists of 28 markers per linkage group, considering LG 7T an dLG 7S as separate linkage groups. A maximum of 48 markers was found on LG 1 and a minimum of 18 on LG 6 (neglecting LG7S, with 14 markers).

Twenty-three of the mapped loci deviated significantly from the expected Mendelian segregation ratios. Segregation distortion in *Lolium* has been reported by several authors : Jones et al. (2002a) found distortion mainly in LG3, LG4 and LG5; Bert et al. (1999) observed for the same mapping population segregation distortion in LG 3 near the GOT/3 locus; Hayward et al. (1998) reported segregation distortion in LG1 and LG3; Thorogood et al. (2002) reported

segregation distortion in LG3 and Armstead et al. (2002) in LG5 and LG7. In the present study a high number of distorted markers was found in LG1 (8 markers) and LG2 (6 markers), but the highest percentage of distorted markers is found in LG6: 4 out of 18 markers are distorted. LG1 and LG2 represent the chromosomes where S and Z have been mapped by Thorogood et al. (2002). In LG2, all distorted loci (except rye024_b) map in the proximity of the Z locus, situated near to BCD135. The presence of the high number of distorted loci in this region can be explained by the SI system present in *Lolium* spp. However, just three out of the 8 distorted loci on LG 1 map close to the S locus, situated in the proximity of CDO89 and OSE. In LG 1 and in LG6, distortion can be more attributed to the presence of a viability gene, as also suggested by Thorogood et al. (2002) on LG3. In the available published maps, segregation distortion appears in different LGs. This indicates that at different regions of the genome, genes are located having influence in generation and survival of the progeny.

The theoretical genome length can be estimated from the observed mean chiasma frequency. Naylor et al. (1960) observed in an interspecific cross, *L. perenne* x *L. multiflorum*, a mean chiasma frequency per chromosome of 1.7. This indicates that the expected size of a genetic map of *Lolium* is 1190 cM. The presented map spans 833 cM covering in this way 70% of the whole genome. The partial coverage is also indicated by the substantial number of markers (152 out of 382) that were not grouped into a linkage group. Additional, preferentially co-dominant markers are needed to integrate these ungrouped markers into the current linkage map.

5.4.5 Mapping of different marker types

The marker types, used to construct the map, are distributed over the different linkage groups. A total of 140 AFLP markers were incorporated in the map. No significant clustering is observed of *Hind*III-generated or *Eco*RI-generated AFLPs. Although the AFLP markers are not very informative in a two-way pseudo-testcross, they fulfill an important role in expanding the linkage groups (for example in LG6 and LG7S/T). They also fill in large gaps between co-dominant markers (f.e. LG4).

Three RFLP markers (C1286, CSU95 and RGC488) detected multiple loci, situated on different LGs. Five RFLP and three SSR markers could not be

scored co-dominantly due to the specifications of the JOINMAP3.0 software. For example, two alleles of marker CDO412 were visualized. CDO412 segregated as a abxcc marker, with c a null allele. This class of marker could not be entered in JOINMAP3.0 and alleles had to be entered as separate markers. Both alleles map 1 cM apart in LG5, confirming that they represent the same locus and not multiple loci.

5.4.6 Mapping of markers selected during the BSA

The clusters of putatively R-linked markers as already discussed in Chapter 3, were mapped. PC026-R3 and PC026-R4 map together on LG2; PC106-R2 and PC168-R1 on LG1. The cluster explaining the highest percentage of variation in the crown rust data (PC106-R2 and PC168-R1), maps close to the resistance gene analogue, NBS_a. QTL analysis will help us to confirm if in these genomic regions, QTLs for crown rust resistance are situated.

5.4.7 Clustering

To study the phenomenon of clustering, the observed and the expected frequency distributions of marker number/10cM interval (Fig. 5.4) were constructed and compared according to Young et al. (1999). Assuming random marker distribution, the number of 10 cM intervals with x markers is expected to follow the Poisson distribution function ($P(x)=e^{-\mu}\mu^{x}/x!$ with $\mu=2.76$ markers/10cM interval). We constructed the observed distribution of 10cM intervals with x markers by sliding a 10 cM interval over the linkage groups, with a step size of 1 cM. The observed distribution shown in Fig. 5.4 deviated significantly from the expected Poisson distribution ($\chi^{2}=1265$, 8 df, P<0.001). This is mainly due to the big differences between observed and expected frequencies in the 10 cM interval with zero markers or the 10cM intervals with nine or more markers. The location of clusters is represented in Fig. 5.5.
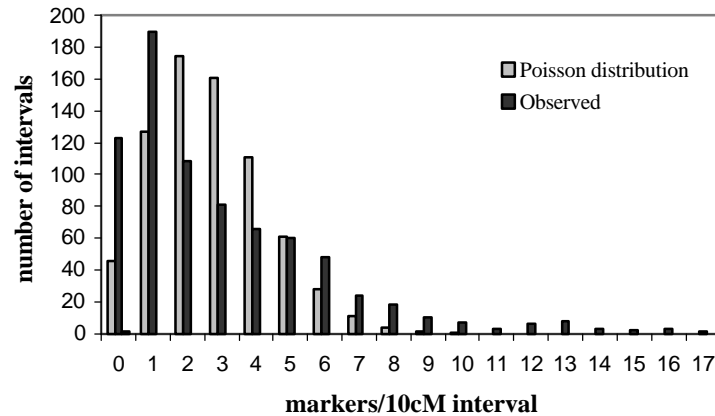
Fig. 5.4 : Distribution of marker number/10 cM interval in the *L. perenne* linkage map. The observed distribution is compared with the expected Poisson distribution ($P(x)=e^{-\mu}\mu^{x}/x!$ with $\mu=2.76$ markers/10cM interval), assuming random marker distribution.

Clusters (10 cM intervals with more than 9 markers) were absent in LG4, LG6, LG7T and LG7S; major gaps (0 markers/10cM) were present in all LG, except on LG3 and LG7S. On LG7T, there were 6 gaps of more than 10 cM without a marker, indicating that these regions do not have good marker coverage. Addition of more markers will help the saturation of these regions.

5.4.8 Alignment with publicly available *L. perenne* maps

The integrated map has been easily aligned with the ILGI map described in different publications (Bert et al., 1999; Jones et al., 2002a&b). Alignment was based on 32 SSRs, 10 RFLPs and 5 STSs that were in common between the presented map and the ILGI map (Table 5.4). Four inconsistencies (3 SSRs and 1 RFLP) with the ILGI map were observed. These inconsistencies can be due to the detection of multiple loci by the respective SSRs or RFLP. This might be the case for markers LPSSRK14B06, LPSSRK08A09 and Xcdo459 as they all detected a fragment that was monomorphic in our mapping population, in addition to the polymorphic mapped fragments.

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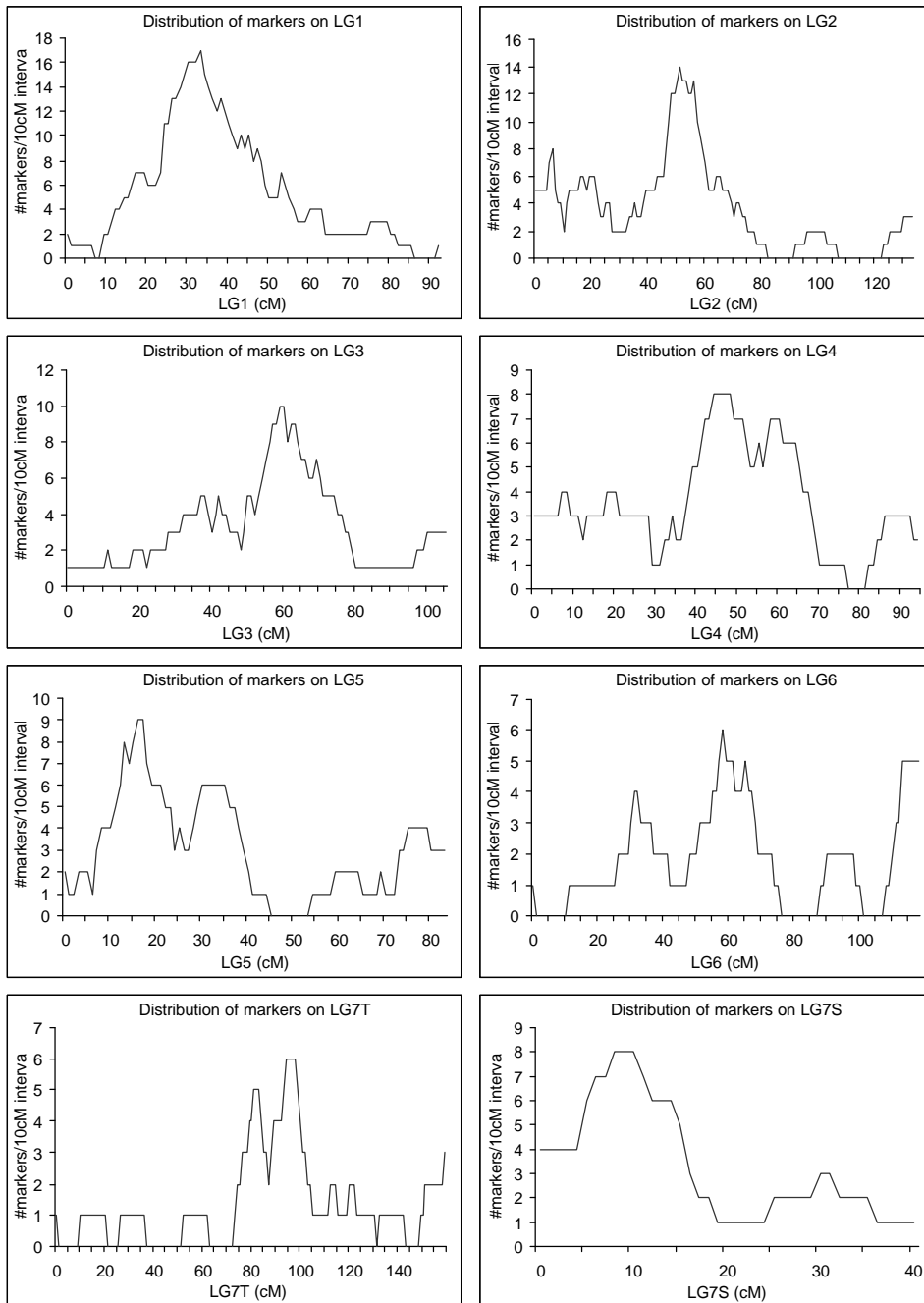
Fig. 5.5 : Marker distribution over the seven linkage groups (LG). Number of markers within 10 cM intervals is presented.

Table 5.4 : Alignment of the presented map and the ILGI reference map (Jones et al., 2002a&b) based on common RFLP, SSR and STS markers. Inconsistencies are indicated in bold.

Common marker	Marker type			Position	
	SSR	STS	RFLP	Presented map	ILGI map
LPSSRK03A02_a	x			1	1
LPSSRK10F08_a	x			1	1
LPSSRK03A02_b	x			1	1
LPSSRK10F08_b	x			1	1
LPSSRK10F08_c	x			1	1
LPSSRK10F08_d	x			1	1
LPSSRK03A02_c	x			1	1
LPSSRK15H05	x			1	1
LPSSRK12D11	x			1	1
LPSSRK07F07	x			1	1
OSE_a		x		1	1
OSE_b		x		1	1
LPSSRK10G04	x			1	1
OSE_c		x		1	1
LPSSRK12E06_a	x			2	2
LPSSRK12E06_b	x			2	2
Xbcd135			x	2	2
LPSSRK08F05_a	x			2	2
LPSSRK08F05_b	x			2	2
LPSSRK09G12_a	x			2	0
LPSSRK09G12_b	x			2	0
Xcdo385			x	2	2/7
LPSSRK14B06	x			2	**1**
Xcdo456			x	2	2
Xrgc390			x	3	3
LPSSRK12H08	x			3	3
LPSSRK07C11	x			4	4
LPSSRK04D01	x			4	4
LPSSRK08A09	x			4	**3**
LPSSRK03B03_a	x			5	5
LPSSRK03B03_b	x			5	5
LPSSRH11G05_a	x			5	5
LPSSRH11G05_b	x			5	5
Xcdo412_a			x	5	5
Xcdo412_b			x	5	5
LPSSRK14C12	x			5	5
LPSSRK02C09	x			5	**4**
OSBR		x		5	5

Table 5.4 : *continued*

Common marker	Marker type			Position	
	SSR	STS	RFLP	presented map	ILGI map
Xcdo497			x	6	6
LPSSRH02H05	x			6	6
LPSSRK02E08	x			7	7
OSW		x		7	7
Xpsr154			x	7	7
Xbcd147			x	7	7
Xcdo459			x	7	**5**
LPSSRK14F07_a	x			7	7
LPSSRK14F07_b	x			7	7

Although the presented map was constructed using the two-way pseudo-testcross approach, a genetic map was obtained which has been aligned with the ILGI map (constructed using the one-way pseudo-testcross approach). The marker order is highly conserved and the map length is in the same order. This demonstrates once more the validity of the two-way pseudo-testcross approach for linkage map construction in outcrossing species. The main advantage of this approach is that the mapping population can be constructed using plants out of the breeding pool and showing extreme phenotypes of the trait studied. In this way, the segregation of the trait studied is ensured.

5.4.9 Comparative mapping

Thirty-one loci were detected by heterologous RFLP probes. These probes were derived from the EGRAM anchor probe set (Chapter 4) and had been previously mapped in rice by Stephenson (1997). They were thus suitable for comparative mapping. In Table 5.5, an overview is given of the map positions of these probes on the rice map (Stephenson, 1997), oat map (Van Deynze et al., 1995a), *Triticeae* consensus map (Gale et al., 1995; Nelson et al., 1995a & b; Van Deynze et al., 1995b; Marino et al., 1996) and the ILGI map (Jones et al., 2002a).

Jones et al. (2002a) carried out a thorough comparative study between the ILGI map and three other *Poaceae* maps (*Triticeae*, rice and oat) on the basis of 109 heterologous probes. A simplified overview of their results is given in Table 5.6.

We can add some additional information to this study based on our data (summarized in Table 5.5).

- For LG1 we can confirm synteny with LG1 of the *Triticeae*, LG5 of rice, and LGA of oat.

- For LG2, we can add one more probe (BCD855) confirming synteny with LG2 of the *Triticeae* and two probes confirming synteny with LG4 and LG7 of rice (CSU6 and BCD855 resp.).

- In LG3, we found 2 additional probes (RGC742 and BCD127) identifying synteny with rice LG1.

- In LG4, we found 2 probes (PSR104 and CDO87) confirming synteny with LG3 of rice, however, the other 3 probes do not. Synteny with LGE of oat and LG4 of the *Triticeae* is identified, but 1 probe (CDO87) maps in the *Triticeae* on LG3.

- In LG5 and LG6 we found additional synteny with rice. In LG5, 2 probes map on LG10 and 11 of rice; in LG6, 1 probe maps on LG6 of rice.

- In LG7, we found 3 additional probes showing synteny with rice LG6 and 8. Two of those probes confirm synteny with LG7 of the *Triticeae*.

Although our data set was very small for comparative mapping, it provided additional information to the study made on the basis of the ILGI map. The gained information includes some confirmation but also some inconsistencies to the results obtained by Jones et al. (2002a). Integration of available *Lolium* maps will improve the knowledge about the existence of syntenic relationships between *Lolium* and other *Gramineae*.

Table 5.5 : Overview of map positions of heterologous RFLP probes on the rice map (Stepenson, 1997), oat map (Van Deynze et al., 1995a), the *Triticeae* consensus map (Marino et al., 1995; Nelson et al., 1995a&b; Van Deynze et al., 1995b; Gale et al., 1995) and the ILGI map (Jones et al., 2002a).

LG presented map	Probe	LG rice	LG oat	LG Triticeae	LG ILGI
1	RZ244	5	A/C	1	-
	RGC488	10	-	-	-
	CDO89	5	D	1	-
2	BCD135	4	B	-	2
	CSU6	4	-	-	-
	BCD855	7	-	2	-
	CDO385	7	B	-	2
	CDO456	4	B	2	2
3	RGC742	1	-	-	-
	RGC390	8	-	-	3
	BCD127	1	-	-	-
4	C1286	1	-	-	-
	PSR104	3	-	4	-
	CSU25	11	E	-	-
	CDO87	3	E	3	-
	CSU70	12	-	-	-
	IBF64	-	-	-	-
5	RGC488	10	-	-	-
	CDO412	9	E	-	5
	RGR77	11	-	-	-
	C1286	-	-	-	-
6	CSU95	6	-	-	-
	RGC424	7	G	7	6
7T	CSU95	6	-	-	-
	RGC424	2	-	-	-
	PSR154	8	-	6	7
	BCD147	8	D	3	7
	CDO459	12	F	3	5
	RGR662	8	-	1/7	-
7S	BCD147	8	D	3	7
	PSR129	6	-	7	-

Table 5.6 : Overview of the results found in the comparative study made by Jones et al. (2002a) between the *Lolium* map (ILGI reference map) and the maps of the *Triticeae*, oat and rice.

LG ILGI	LG Rice map	LG Oat map	LG Triticeae map
1	5	A	Complete synteny with LG1
2	4/7	B	Small non-syntenic regions with LG2
3	1/5	C/G	Complete synteny with LG3
4	3	E/F	Small non-syntenic regions with LG4
5	2/9	E	Complete synteny with LG5
6	2	D/G	Large non-syntenic regions with LG6
7	6/8	None	Small non-syntenic regions with LG7

5.5 Conclusions

Here we represent the first genetic map of *L. perenne*, based on the two-way pseudo-testcross approach. The mapping population consists of 252 F_1 individuals. The parents were highly heterozygous and showed extreme contrasting phenotypes for crown rust resistance. A genetic map of 833 cM included 230 loci (RFLP, STS, AFLP and SSR), grouping into seven linkage groups. These seven linkage groups represent the genome of *Lolium* with seven chromosomes. Six linkage groups were integrated; it was not possible to integrate the parental linkage groups of the seventh chromosome. After inspection of the ILGI map and the map published by Armstead et al. (2002), potential markers suitable for integration of LG 7T and LG 7S were identified. This represents a priority of future developments based on these linkage maps.

The map size (833 cM) was in the same range of other published linkage maps of *Lolium*. The genome coverage is expected to be 70% of the theoretical expected linkage map length. The coverage is far from complete as was seen by a high number of markers, that could not be mapped in this study. Additional preferentially co-dominant and gene-specific markers will help the full coverage of the genome in future research.

Thirty two percent of the markers showed segregation distortion (P<0.05), which was in the same range as reported for other *Lolium* mapping studies. A high number of distorted markers mapped in LG1, LG2 and LG6. LG1 and LG2 are the LG to which the S and Z loci map. The presence of these self-incompatibility genes may explain the segregation distortion of loci mapping close to S and Z. However, segregation distortion has been reported to be

present in other LGs than reported in this study, indicating the presence of different loci linked with viability over the *Lolium* genome.

Markers generated with different marker techniques map evenly on the genetic map. AFLP markers served as a dense backbone and RFLP, SSR and STS markers were useful markers for map integration. This enabled the alignment with other *Lolium* maps and maps of closely related species. The markers identified in the BSA have been mapped and are placed on LG1 and LG2. The RGA fragment maps on LG 1 close to the markers selected in the BSA.

The alignment with the ILGI map was straightforward and was mainly based on the SSR markers in common. Just a few inconsistencies were observed, but this might be explained by markers detecting more than one locus. Other *Lolium* maps are under construction at this moment. It is beyond any doubt that the integration of the different maps will result into an integrated map containing a balanced set of anchor probes. This integrated map will permit further investigation focusing on the structure, evolution and function of the *Lolium* genome.

As reported in previous studies, a high level of synteny between *Lolium* and the *Triticeae* was found. Using the presented map, some additional information to the comparative mapping study made by Jones et al. (2002a) between *Lolium* and related species (oat, rice and *Triticeae*) was obtained. Expanding the knowledge of synteny between these species will allow the transfer of information obtained in model-species as rice to less studied species, with larger genomes, like *Lolium*. Mapping of genes or loci with known function are of special interest.

As Wu et al. (2000) suggest that a genetic map with a wide marker spacing of 20 or even 50 cM may be optimal to initially scan the genome for QTL mapping, this genetic map will be used for identifying QTLs for crown rust resistance.

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Chapter 6

Detection of QTLs for crown rust resistance in the *L. perenne* population

6.1 Introduction

6.1.1 Disease resistance

Qualitative resistance is usually referred to as resistance which complies with a gene-for-gene interaction; for each major resistance gene (R genes) in the plant host there exists an avirulence gene in the pathogen (Flor, 1971). R genes are often clustered in the genome and molecular analysis has shown that the gene-for-gene model holds true for several plant pathogen systems (reviewed by Hammond-Kosack and Jones, 1997). In these systems, the R-genes are thought to encode receptor molecules, which perceive a pathogen signal, whereupon a resistance response is triggered (Baker et al., 1997). This type of resistance is often discovered when an interaction between a specific plant and a specific pathogen strain is studied. On the other hand, when studying resistance, intermediate reaction types are often found indicating that minor genes also contribute to resistance. In this case, resistance is more quantitative than qualitative. Quantitative resistance is defined by a complex, polygenic inheritance pattern with multiple genes having small additive effects on the resistant phenotypes. These minor genes may be equally effective to different pathogen strains (pathotypes) and the resistance may be relatively durable in the field.

6.1.2 Quantitative trait loci and disease resistance

Many traits of economic importance in plants such as yield, quality, maturity, resistance to several biotic and abiotic stresses are of quantitative nature; the observed phenotypes follow a continuous distribution and reflect the action of many Quantitative Trait Loci (QTLs), together with environmental effects. The availability of genetic markers has aided to unravel the nature of these QTLs. It is possible to assign chromosomal positions to these QTLs, to determine the types and magnitude of gene effects of individual QTLs, and also to determine

which parent possesses the positive allele at each QTL. The attractiveness of using QTL analysis over qualitative studies is that it allows for the simultaneous detection of all loci contributing to the expression of the trait. This simultaneous detection permits to study the interaction between loci and to determine the variation explained by each locus separately (Kumar et al., 2000; Kover & Caicedo, 2001).

In the present study, the aim was to identify loci involved in crown rust resistance. The inheritance pattern of resistance in the *L. perenne* population was explained in Chapter 2 by the action of major genes of which the action was influenced by minor genes. A similar pattern of semi-quantitative disease resistance, has been found in several other QTL mapping studies (Young, 1996). Although some examples exist where quantitative resistance is explained by more than five QTLs, it is common to find two to five loci underlying complex disease resistance (summarized in Young, 1996). In these cases, resistance is polygenic in the sense that a few genes have large effects on the phenotype.

Evidence is accumulating that a wide variety of molecular and genetic mechanisms may underlie quantitative resistance. Genes previously identified to confer qualitative resistance have been found to contribute to quantitative resistance and some genes previously considered to be quantitative can vary their expression according to the genetic background. For example, a major R-gene which triggers a specific HR (hypersensitive reaction) response upon recognition of the appropriate avirulence gene product, may have reduced effect when a mutated version of this avirulence gene product is encountered. Thus there is support for the idea that resistance in plants is ultimately determined by the combination of the products of many genes encompassing all sorts of functions, including secondary compound production, phenology, physiology and other structural features that might affect pathogen accessibility and growth in the host (Rouppe van der Voort et al., 2000; Kover & Caicedo, 2001).

An overview of QTL analyses of disease resistance in different host-pathogen systems was given by Kover & Caicedo (2001). They observed in the different studies between 0 and 18 QTLs contributing to host resistance. On average, each mapping population contained 4.6 QTLs, but the distribution was skewed towards a smaller number of QTLs, with the majority of studies identifying two or more loci. This can be due to limitations inherent to the statistical methods for QTL-detection, as it is difficult to locate more than 12 QTLs in any given

population at one time or to demonstrate the existence of more than three QTLs per chromosome because of the wide confidence intervals (Asins, 2002). On average, each QTL explained 20% of the phenotypic variance, but the distribution was also skewed, with 67% of the QTLs explaining less than 20% of the variance. It was also common to find on the same chromosome QTLs for resistance to different pathogens (Kover & Caicedo, 2001).

6.1.3 QTL detection methods

Different approaches have been used to date to detect associations between a quantitative trait and the marker alleles segregating in a population (Mauricio, 2001).

- The earliest approach consists of looking at all individual associations between marker alleles and phenotype using either a χ^2 test or simple linear regression. In this case, no linkage map is required. Despite being more robust to violations of normality than more elaborated approaches (such as IM or MQM), these methods cannot extract all the information in the data (Liu, 1998). In general, the drawbacks of these methods include that the phenotypic effects of QTLs are systematically underestimated, the genetic locations of QTLs are not well resolved because distant linkage cannot be distinguished from small phenotypic effects, and the size of progeny required for detecting QTLs is very large. These methods of testing many genetic markers also involve the multiple testing problem. This is the increased risk that false positives will be detected (Lander & Botstein, 1989; Kearsey & Farquhar, 1998).

- These drawbacks can be overcome by the application of interval mapping (IM). This approach of QTL mapping requires prior construction of a genetic linkage map (Lander and Botstein, 1989; Knapp et al., 1990; Haley & Knott, 1992; Jansen, 1992; Carbonell et al., 1992; Mauricio, 2001). Intervals between adjacent markers along a chromosome are scanned and the likelihood profile of a QTL being located at any particular point in each interval is determined; or to be more precise, the LOD (a measure for the likelihood of the odds) of there being one versus no QTL at a particular point is estimated. Those maxima in the LOD-profile which exceed a specified significance level, indicate the likely sites of a QTL.

Significance levels have to be adjusted to avoid false positives resulting from multiple tests. The confidence intervals of the QTL position are set as the map interval corresponding to a 1.5 unit LOD decline either side of the peak (Kearsey & Farquhar, 1998; Kumar, 1999). Interval mapping has the disadvantage that during the selection of intervals linked with the trait, the effect of other QTLs present in the genome is neglected. This leads to the selection of ghost QTLs.

- This problem is solved in the third approach, composite interval mapping (CIM) or multiple QTL mapping (MQM). In this case, markers located nearby putative QTLs identified by e.g. interval mapping, are used as co-factors in an approximate multiple-QTL model with additive and dominant gene actions only. At each testing point the effect of one or more co-factors is included (Manly & Olson, 1999).

  Currently, the usual way to detect QTLs is mostly based on IM, or on methods employing an approximate MQM mapping (Lander and Botstein, 1989; Haley & Knott, 1992; Jansen 1992, 1993, 1994; Zeng, 1993, 1994; Jansen & Stam 1994; Xu & Atchley, 1995; Kao & Zeng 1997; Maliepaard et al., 2001). The MQM step is used to absorb the effects of previously identified QTLs and to remove 'ghost' QTLs, increasing the power to identify additional QTLs (Kearsey & Farquhar, 1998; Mauricio, 2001). The χ^2 test and regression analysis are still broadly used for initial data exploration and for the verification of results obtained with other methodologies (Kearsy and Farquhar, 1998).

- Permutation-based methods have been described by Churchill & Doerge (1994) to determine appropriate empirical thresholds (critical values) against which to compare test statistics (LOD scores or likelihood ratios). These methods involve repeated shuffling of the quantitative trait values over the progeny and the generation of a random sample of the test statistic from an appropriate null distribution. These methods are statistically valid when likelihood or regression based test statistics are used and for any distribution of the quantitative trait. Thus, they are useful for QTL analysis of non-normal distributed data and take into account the multiple testing problem by considering the permutation distribution of the maximum test statistic over all markers.

Doerge and Churchill (1996) designed two methods yielding threshold values that can be used to construct tests for the presence of minor QTL effects while accounting for effects of known major QTLs : conditional empirical threshold (CET) and residual empirical threshold (RET).

In CET, the data set is divided into subsets depending on the presence/absence of a marker allele significantly associated with a first QTL. In this way the effect of the first identified QTL is removed from the subsets. Within the subsets, permutation-based tests yield new empirical threshold values suitable for identifying subsequent marker alleles associated with other QTLs additional to the first QTL. Next, the newly identified marker allele associated with a second QTL can be used to divide the subsets in new subsets, and so on.

In RET, the first identified QTL and its associated marker allele is fixed. The effect of this QTL is substracted from the trait value of each plant. These new trait values are then used in a new round of permutation testing. Subsequent rounds of RET are done until no new significant associations are found.

In this study, both permutation-based methods are used to verify the results obtained with the IM and MQM method, especially as the crown rust data used for QTL analysis are not normally distributed.

6.1.4 QTL mapping in an outcrossing species

QTL mapping studies commonly make use of F_2 or backcross progenies derived from inbred lines. In a two-way pseudo-testcross, as used in the present thesis, a QTL or a marker can segregate for four distinct alleles; the only detectable QTLs are those for which one or both parents are heterozygous with alleles of strong alternative effect. These effects should not be masked either by dominance or the environment in which phenotyping is conducted (Marques et al., 1999). The use of a two-way pseudotestcross has several advantages as breeding material can be used in the mapping studies. Both parents contribute to the variation, which involves the sampling of more variation (up to 4 alleles in one locus) than in inbred lines (Grattapaglia et al., 1996; Asins, 2002). A severe disadvantage in a two-way pseudo-testcross is the low information content of dominant marker types. Therefore, tracking the inheritance of multiple alleles at

QTLs in an outbred pedigree necessitates the use of co-dominant multi-allelic markers.

Ryegrass QTL-mapping studies published to date were based on the use of one-way pseudo-testcrosses or F_2 populations. QTLs for water-soluble carbohydrate content (Turner et al., 1998), for flowering and chlorophyll breakdown (Thorogood et al., 1999a) and for (temperature dependent) crown rust resistance (Thorogood et al., 1999b) have been identified. In this chapter, the *L. perenne* map, presented in Chapter 5, will be used to identify QTLs linked with crown rust resistance.

6.2 Objectives and rationale

In the present study, the first aim was to identify the number and the chromosomal location of loci affecting crown rust resistance. The second goal was to determine the parental source of beneficial QTL alleles, and the magnitude of their effect on the phenotype. Using the genetic map presented in Chapter 5, different approaches to identify QTLs were used : interval mapping and multiple QTL mapping. As the phenotypic data are not normally distributed, permutation tests will be used to increase the power to detect significant associations. In the permutation testing, also the unmapped markers are included, providing information on the eventual presence of significant associations between crown rust resistance and alleles of unmapped markers.

QTL analysis will be performed on the parental maps as well as on the integrated map. Identified QTLs and their associated marker alleles are further evaluated for their potential use in marker assisted selection programs.

6.3 Material and methods

6.3.1 Linkage map

A slightly modified map compared with the map represented in Chapter 5 was used for QTL detection: markers added in the second mapping round (forced addition of unmapped markers) were removed, as their addition to the map often caused small rearrangements in marker order. The TC1 linkage map used for

QTL detection consisted of 138 framework markers in 7 LGs and covered 729 cM, while the SB2 map used for QTL detection contained 132 markers in 7 LGs covering 454 cM. The integrated map consisted of 228 loci in 7 LGs (the integration of the two parental LGs 7 into one LG was not possible) covering 779 cM.

6.3.2 Phenotypic data

F_1 plants had been analyzed for crown rust resistance in the greenhouse as described in Chapter 2.

6.3.3 Map-based QTL analysis

QTL analyses were performed using the software MapQTLv.4.0. (Van Ooijen et al., 2002). This version of the program enables QTL analysis of a two-way pseudo-testcross with up to four alleles per segregating locus. Three parametric approaches based on the maximum likelihood method were applied : IM (Lander & Botstein, 1989), automatic co-factor selection and MQM mapping (Jansen, 1993; Jansen & Stam, 1994). These procedures are implemented in the MapQTLv.4.0. program.

In IM, intervals contained 5 neighboring markers. Markers with a LOD-score > 3 were selected and used as co-factors in the next procedures. This threshold corresponds to an error rate of 5% per linkage group, for the average linkage group length (Van Ooijen, 1999).

Next, the automatic co-factor selection procedure implemented in MapQTLv4.0 was used to test different combinations of co-factors selected in IM. In this procedure, the non-significantly linked co-factors in a particular set are rejected from the set. The aim was to identify co-factors that were consistently retained when different sets of co-factors were tested.

In the MQM analysis, markers consistently retained in the automatic co-factor selection were fixed as co-factors. In subsequent rounds of MQM analysis, co-factors were added or dropped according to the LOD > 3.0 threshold, until a stable result was obtained.

6.3.4 Permutation-based QTL analysis

As the crown rust data used for QTL analysis do not follow the normal distribution, permutation-based methods as described by Doerge and Churchill (1996) were used to verify the results obtained in IM and MQM. In these tests, also unmapped markers, which were not included in the IM and MQM analysis, were tested for significant linkage with crown rust resistance. Permutation-based methods were developed in S-Plus 6.0 professional release 2 (Insightful Corp.) by Moerkerke et al. (University of Gent, personal communication).

In these methods, an original test statistic (T_0) is computed for each marker (the standardized regression coefficient, equivalent to a t-test). This T_0 will take its maximum at markers linked with QTLs. To set the critical threshold values for significant linkage, trait values are randomly permuted among the progeny, destroying the relationship between the trait values and the genotypes of the marker loci in the observed data. A new test statistic is estimated for each marker in the permuted data set and the maximum test statistic obtained in each permutation is recorded. This procedure is repeated numerous times, giving a distribution of maximum test statistic values expected if there was no QTL linked to any of the marker loci. Values at appropriate percentile points of the empirical distribution can be used as test statistic threshold values to establish significance of the observed T_0. We tested two-sided at $\alpha=0.05$. In this case the 2.5 and 97.5 percentile value are the empirical threshold values for significance. We just used 200 permutations; in this case we had to construct 95% confidence intervals for the 2.5 and 97.5 percentile values.

To identify multiple QTLs, two approaches were followed. In a first approach (CET), the marker with the highest significant linkage was used to stratify plants in two subpopulations: plants with and without the marker. Next, for each marker, a new stratified T_0 value was calculated. Permutation testing (200 permutations) within the strata yielded new empirical threshold values (2.5 and 97.5 percentile values) and the accompanying 95% confidence intervals. The marker with the highest significant linkage was subsequently used to stratify within the subpopulations. This was done until no more significant linkages were found.

In a second approach (RET), the marker with the highest significant linkage was fixed as a QTL. The effect of this locus was subtracted from the crown rust

score in each plant. This new crown rust score was then used for a subsequent round of permutation testing (200 permutations). Subsequent rounds were done until no new significant linked markers were found.

In cases for which T_0 values were located within the confidence intervals of the empirical threshold values, narrower confidence intervals were constructed by performing up to 3200 permutations.

6.3.5 Phenotypic variance explained

The percentage of variance explained by a given marker was determined by 1) linear regression, 2) by IM and 3) by MQM analysis. In addition, the power of the identified QTL alleles was estimated by looking at the differences between the population mean crown rust score and the mean crown rust score for each QTL genotype. Mean crown rust scores for each QTL genotype were determined using MQM in MapQTLv.4.0.

6.4 Results and discussion

6.4.1 Map-based QTL analysis

As demonstrated in Chapter 2, we can use the average of the crown rust score for QTL analysis. However, the distribution of these data deviates from the normal distribution. A log-transformation or arcsin-transformation of the crown rust data did not significantly improve normality, mainly due to their categorical nature. Untransformed phenotypic data were used for QTL analysis.

QTL analysis was performed on both parental linkage maps (TC1 and SB2) and on the integrated map. In a preliminary analysis, IM was used to identify QTLs linked with crown rust resistance. No marker with LOD score > 3.0 has been found on the SB2 parental map. On the TC1 parental map, 12 markers with LOD > 3.0 were identified : 4 markers on LG1, 5 on LG2, 2 on LG5 and 1 on LG6 (Fig. 6.1). On the integrated map, 20 markers showed a LOD > 3.0 : 5 on LG1, 14 on LG2 and 1 on LG5 (Fig. 6.2). Some LOD scores > 3.0 were found at positions between markers. These positions were not retained for further analysis as these intervals represent 'ghost' QTLs. This phenomenon is

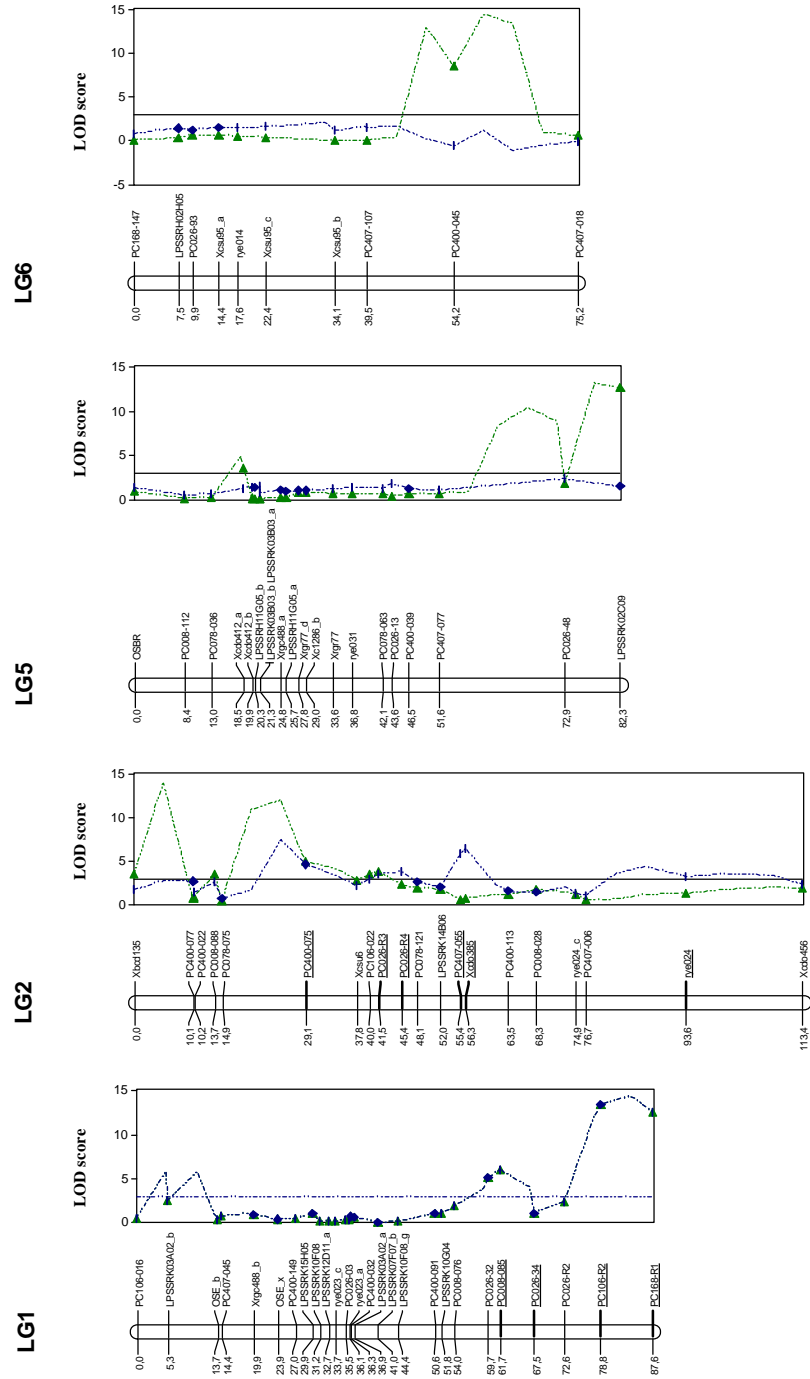Fig. 6.1 : IM (━▲━) and MQM (┅◆┅) results obtained using the TC1 parental map. Markers with LOD score higher than 3 were retained for MQM analysis; markers selected in the MQM analysis are underlined.

Fig. 6.2 : IM (—▲—) and MQM (—◆—) results obtained using the integrated map. Markers with LOD score higher than 3 were retained for MQM analysis; markers which were selected after MQM are underlined.

often seen when the phenotypic data deviates from the normal distribution, as in this study (J. Van Ooijen, PRI, The Netherlands, personal communication). The SB2 parental map was not analyzed further as no QTL positions were identified using the IM algorithm.

Automatic co-factor selection available in MapQTL v.4.0, was used to evaluate the markers displaying significant effects in IM. In the TC1 map, the automatic co-factor selection procedure retained PC008-085 (LG1), PC106-R2 (LG1), PC168-R1 (LG1), PC026-32 (LG1) and PC400-75 (LG2) when different sets of co-factors were tested. In the integrated map, PC106-R2 (LG1), PC168-R1 (LG1) and PC001-79 (LG2) were consistently retained as significant co-factors. PC168-R1 and PC106-R2 were alternatives to each other, as often just one of both markers was chosen as a significant co-factor.

Next, in each consecutive round of MQM mapping, the marker with the highest LOD-score (>3.0) was added to the list of fixed co-factors. For the other markers it was tested whether they explained additional variance of crown rust resistance to the variance explained by the fixed co-factors. In the TC1 parental map, three additional regions with a LOD-score >3 were identified in LG2. Markers representing these regions are : PC026-R4, Xcdo385 and rye024 (Fig. 6.1). In the integrated map, three additional regions were found (one in LG1 and two in LG2). Marker PC008-085 represents the additional region in LG1 and markers Xbcd135 and PC106-022 represent the two additional regions in LG2. The markers previously selected on LG5 and LG6 using IM did not display a LOD score > 3 in MQM mapping.

The results obtained in the TC1 parental map and the integrated map using MQM are compared in Table 6.1. Seven different QTLs were identified. The QTLs detected in both maps, show a high degree of similarity. Four QTLs are in common: QTL1, QTL2, QTL3 and QTL4. QTL5 and QTL6 were detected just in the TC1 parental map and QTL7 just in the integrated map. QTL5 has a high LOD-score (6.46), but this should be taken with care as the marker Xcdo385 is located in an unsaturated region and the number of plants fingerprinted with this marker was very low (40 F_1 individuals). QTL5 will not be taken into consideration in the continuation of the QTL analysis. QTL6 and QTL7 will also be dropped for further analysis as they were not consistently detected and their LOD-scores were not high (<4.0). For further discussion, we consider just QTL1, QTL2, QTL3 and QTL4.

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Table 6.1: Comparison of QTLs identified using MQM in the TC1 parental map and the integrated map. The QTLs are described by the marker with the highest LOD score in the corresponding QTL region.

QTL number		TC1 map		Integrated map	
		Marker	LOD score	Marker	LOD-score
LG1	QTL1	PC008-085	6.07	PC008-085	7.37
	QTL2	PC106-R2	13.46	PC168-R1	30.76
LG2	QTL3	PC400-075	4.66	PC400-075	6.35
	QTL4	PC026-R4	3.82	PC106-022	5.81
	QTL5	Xcdo385	6.46	Not significant	-
	QTL6	Rye024	3.28	Not significant	-
	QTL7	Not significant	-	Xbcd135	3.88

Dupuis and Siegmund (1999) showed with simulations that for dense maps (markers at every 1cM) 1-LOD and 1.5-LOD support intervals provided a QTL coverage probability of approximately 90% and 95%, respectively, and an even greater percentage for sparse maps. The 1.5-LOD support intervals of the QTLs detected in both maps using MQM are given in Table 6.2 and represented in Fig. 6.3. The 1.5-LOD support interval of QTL3 and QTL4 in the TC1 parental map do overlap. This is probably due to the lower marker density in the TC1 parental map. In the integrated map, the 1.5-LOD support intervals of QTL3 and QTL4 are distinct. The number and size of the QTLs identified in the present study are in line with those identified in other QTL studies for disease resistance in plants (Kover and Caicedo, 2001).

6.4.2 Permutation-based QTL analysis

Permutation testing was used to confirm the results obtained in the MQM analysis and for analyzing the unmapped markers. Unmapped markers were not integrated in the IM and MQM analysis. In Fig. 6.4, the results of the first round of permutation-based QTL analysis and of the first round of CET are given.

In the first round of permutation testing, the T_0 values of two markers were more extreme than the 95% CI of the 2.5 percentile threshold value (PC106-R2 and PC168-R1 situated in LG1). This indicates significant linkage of PC106-R2

and PC168-R1 with crown rust resistance. The T_0 statistic of seven other markers (PC026-032 and PC008-085 located in LG1; PC106-22, PC008-088, PC400-075 and PC026-R3 located in LG2; PC008-044 unmapped) fell within the 95% CI of the 2.5 or 97.5 percentile threshold values. The markers selected in this analysis (except the unmapped marker) are the markers representing the four QTLs identified in the MQM analysis.

Table 6.2: 1.5 LOD support intervals giving a 95% probability of QTL location in the TC1 and integrated map (I) using MQM.

	MARKER	LG	Position (cM)	LOD score	Left border (cM)	Right border (cM)	Size of 1.5 LOD-interval (cM)
TC1 map							
QTL1	PC008-085	1	61.7	6.07	58.4	65.4	7.0
QTL2	PC106-R2	1	78.8	13.46	78.0	94.3	16.4
QTL3	PC400-075	2	29.1	4.66	23.6	34.5	10.9
QTL4	PC026-R4	2	45.4	3.82	18.8	48.9	30.1
I map							
QTL1	PC008-085	1	72.5	7.37	70.2	73.4	3.2
QTL2	PC168-R1	1	96.1	30.76	95.2	-	0.9*
QTL3	PC400-075	2	42.2	6.35	24.6	43.0	18.4
QTL4	PC106-22	2	58.4	5.81	55.4	65.6	10.2

*Just the left border was calculated as PC168-R1 is the last marker in LG1.

In a second step, the most significantly linked marker (PC106-R2) was used to divide the plant population into two subpopulations : with and without the marker. CET permutation testing identified four residual markers significantly linked with crown rust resistance (PC400-075, PC026-R3, PC008-088 and PC106-022). These four markers had T_0 statistics that were more extreme than the boundaries of the 95% CI of the 2.5 or 97.5 percentile threshold values. All four markers are located on LG2 (Fig. 6.4). Three markers were situated within the boundaries of the 95% CI of the 2.5 or 97.5 percentile threshold values (PC078-121 on LG2; PC106-028 on LG1 and the unmapped marker PC001-026). PC400-075 was the most significant linked marker and was used to stratify the data again. No additional markers were identified in a subsequent round of CET. Even when the confidence intervals were narrowed by performing up till 3200 permutations.
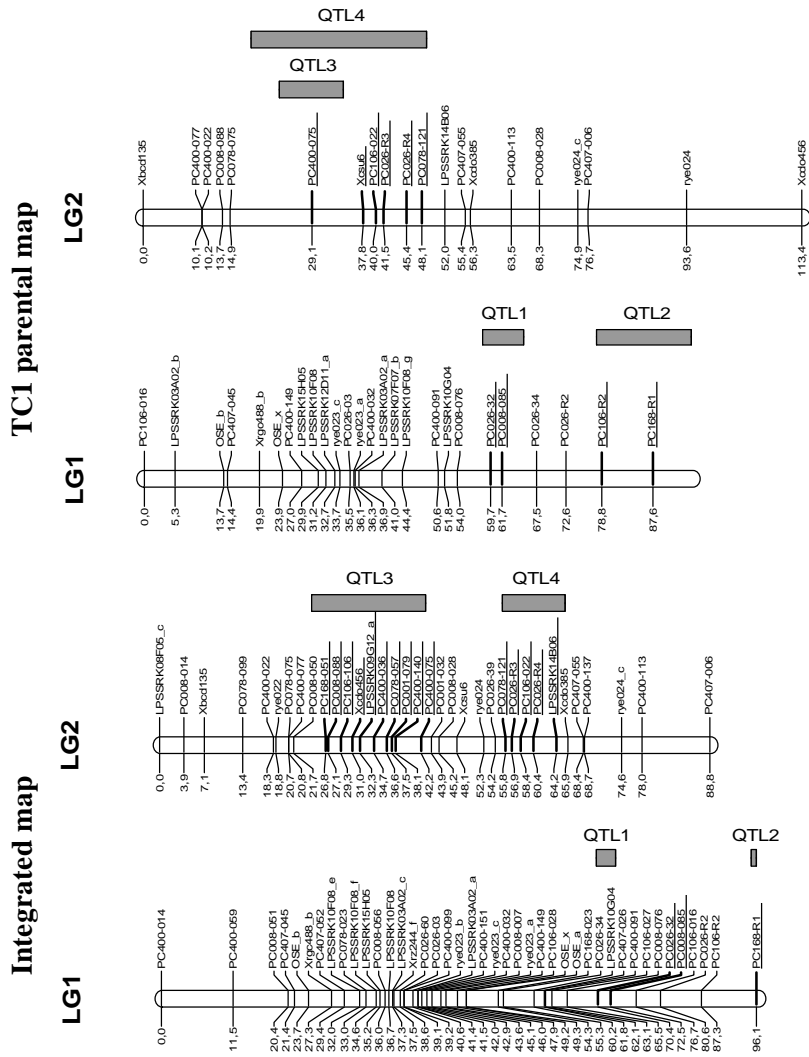
Fig. 6.3 : 1.5-LOD support intervals of QTL1, QTL2, QTL3 and QTL4 detected in the integrated map and the TC1 parental map. Markers included in the interval are underlined.
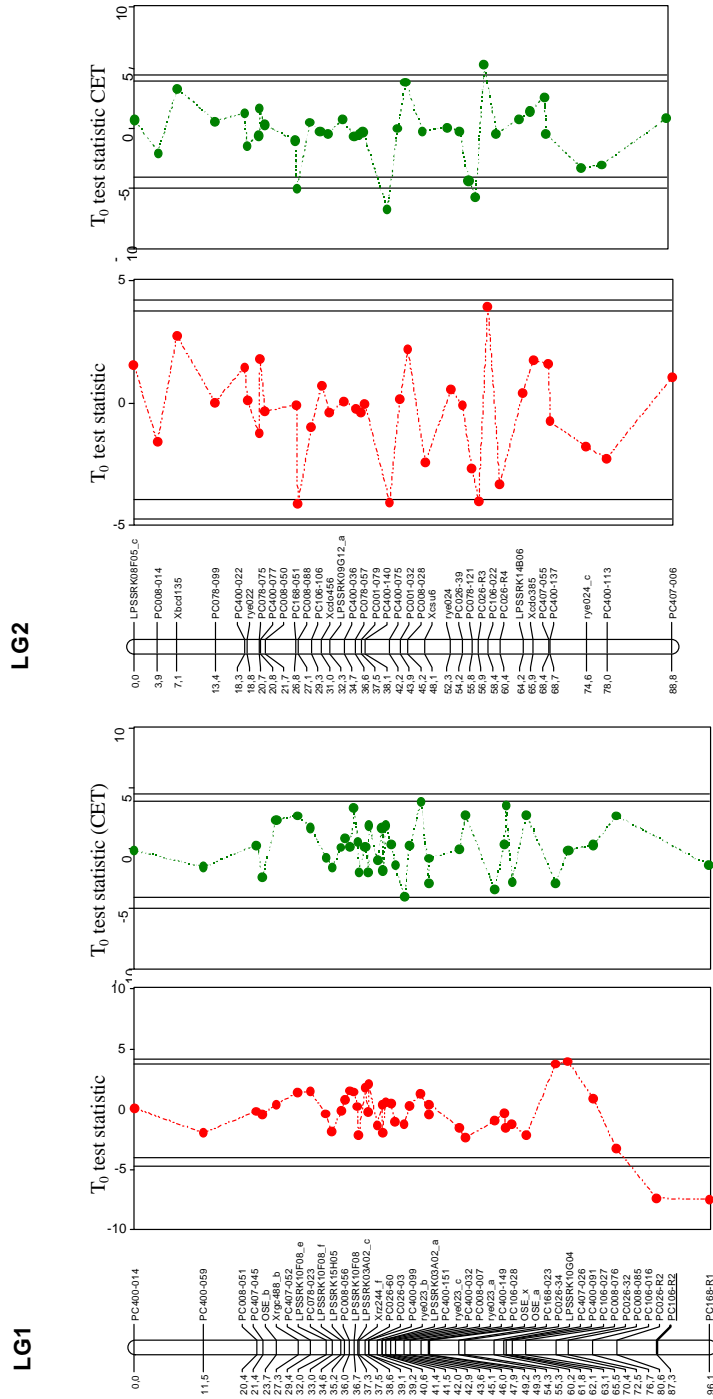
Fig. 6.4: T_0 test statistics obtained in the first round of permutation testing (red) and subsequently in the CET approach (green) after fixing the effect of the QTL linked with PC106-R2 (underlined). The 95% CI of 2.5 and 97.5 percentile threshold value are indicated (black lines). Markers with a T_0 test statistic extremer than the boundaries of the 95% CI of the 2.5 or 97.5 percentile threshold value are markers displaying significant linkage with crown rust resistance. Results of unmapped markers or markers mapped on other LG than LG1 and LG2 are not shown.

Using the RET approach, similar results were obtained: PC106-R2 and PC400-075 were selected as the most significant markers. No additional markers to these two markers displayed significant linkage (results not shown).

The permutation-testing confirmed partly the results obtained in MQM, but as expected QTL analysis using map information is able to reveal more QTLs than permutation tests do. Inclusion of map data into the permutation test may increase the power of the tests. Therefore, testing for linkage between intervals and crown rust resistance instead of single marker-trait linkage represents a priority of future developments of this research.

6.4.3 Variance explained

Most commonly, QTLs are described by four aspects: chromosomal location, the magnitude of their phenotypic effect, the effect of gene dosage at the locus, and their interactions with other QTLs or unlinked genetic loci (Paterson, 1996). In this study we detected four QTLs involved in crown rust resistance. The proportion of variance explained by these QTLs was estimated using three alternative methods: simple linear regression, IM and MQM (Table 6.3). All three methods provided similar results, however, the proportion of phenotypic variance explained by a given QTL is estimated most accurately using MQM as it takes into account map information and the action of the other QTLs. Using this method, QTL1, QTL2, QTL3 and QTL4 explained respectively 12.5%, 24.9%, 5.5% and 2.6 % of phenotypic variance (Table 6.3). The QTLs explaining over 20% of the phenotypic variance are strong QTLs, such as QTL2. The traits controlled by such QTLs can be considered almost Mendelian and are of extreme interest for breeders to be used in the breeding scheme (Manly and Olson, 1999).

However, as the plants used in this study were evaluated just under one set of environmental conditions, it is impossible to predict at this stage the importance of the genomic regions, identified in this PhD, if the infection takes place in a different environment. Furthermore, as only one spore mixture was used for the infection tests, it is impossible to predict whether the genomic regions identified, represent genetic factors activated by the attack of specific strains of the pathogen. They might even represent genomic regions which are activated by different pathogens or even by different biotic and abiotic stresses.

Table 6.3 : Percentage of phenotypic variance explained by the identified QTLs using three methods of estimation : simple linear regression, IM and MQM on the integrated map.

QTL	Linear regression	IM	MQM
QTL1	7.4%	13.4%	12.5%
QTL2	20.7%	23.7%	24.9%
QTL3	9.0%	10.6%	5.5%
QTL4	3.7%	6.4%	2.6%

6.4.4 Potential use of the identified QTLs for MAS

To evaluate the potential use of a marker (or QTL) in MAS, the trait mean of different QTL genotype classes (Table 6.4) can be used. This provides information on the effect of QTL alleles on trait performance and may provide a more useful estimate of the potential gain to be realized by selecting a favorable QTL allele or allele combination. As in this study, a two-way pseudo-testcross design is used, we can just test the difference between the average trait value of (Q1Q3+Q1Q4) vs. (Q2Q3+Q2Q4) for parent Q1Q2 and (Q3Q1+Q3Q2) vs. (Q4Q1+Q4Q2) for the parent Q3Q4. Thus just the effect of an allele substitution can be tested, which is much less powerful than testing the difference between the trait value of Q1Q1 vs. Q2Q2. If dominant markers are used the phase and power limitation clearly increase (Asins, 2002). However, an attempt to estimate the effect of the different QTL alleles has been made and is represented in Table 6.4.

For QTL1, the genotype bc is the most resistant genotype; genotype ad is to be avoided as the mean crown rust score increases with 0.87 units. Therefore, absence of marker PC008-085 is favorable. Presence of marker PC008-085 involves an increase in the population crown rust score of 0.28 units or 0.87 units depending on the accompanying allele (c or d).

For QTL2, presence of the marker PC168-R1 results in a decrease of 0.18 units of mean crown rust score and absence results in an increase of 0.75 units. Presence of this marker is favorable.

Table 6.4: Mean crown rust score for each genotype of the four identified QTLs calculated using MapQTLv.4.0. QTLs are defined by the marker with the highest LOD-score in the QTL region. The allele visualized by each marker in the TC1*SB2 cross (abxcd) is given. Genotypes containing the visualized allele are indicated in bold.

		Visualized allele (abxcd)	Mean rust score of each genotype				Population Mean	Difference to population mean			
			ac	ad	bc	bd		ac	ad	bc	bd
QTL1	PC008-085	a	**2.12**	**2.71**	1.75	1.87	1.84	**0.28**	**0.87**	-0.09	0.03
QTL2	PC168-R1	b	2.59	2.59	**1.66**	**1.66**	1.84	0.75	0.75	**-0.18**	**-0.18**
QTL3	PC400-075	b	2.05	1.93	**1.76**	**1.59**	1.84	0.21	0.09	**-0.08**	**-0.25**
QTL4	PC106-022	a	**2.06**	**1.77**	1.70	1.69	1.84	**0.22**	**-0.07**	-0.14	-0.15

Genotype bd is the most favorable genotype for QTL3 and genotype ac is to be avoided. Allele b can be visualized by PC400-075 and allele d by the presence of PC001-032, a marker mapping 1.7 cM apart from PC400-075.

QTL4 is influenced by allele b. This allele causes a decrease in mean crown rust score of 0.14 or 0.15 units depending on the accompanying allele (c or d). Allele b cannot be visualized by PC106-022. The closest marker which can describe allele b is PC026-R3 which is 1.5 cM away from PC106-022. Presence of PC026-R3 is linked with a decrease of the crown rust score.

In summary, the ideal QTL conformation to obtain resistant plants is as summarized in Table 6.5. Overall, the positive alleles (linked with resistance) were derived mainly from the resistant parent (TC1). Just for QTL3, allele d, found in the susceptible parent (SB2) is favorable to resistance.

Table 6.5: Ideal genotype per QTL for selecting resistant plants, based on the results described in Table 6.4.

QTL	Ideal genotype	Markers
QTL1	bc or bd	Allele b = presence of PC008-085
QTL2	bc or bd	Allele b = presence of PC168-R1
QTL3	bd	Allele b = presence of PC400-075
		Allele d = presence of PC001-032
QTL4	bc or bd	Allele b = presence of PC026-R3

Gene action at QTLs is determined by the same principles as are employed for monogenic traits: additivity, dominance, recessivity and epistasis. Epistasis in this context means that the resistant phenotype conferred by one locus depends on the alleles present in another locus in the same individual (Kover and Caicedo, 2001). Loci pairs were thus tested for epistatic interactions using GLM analysis (Kover and Caicedo, 2001) but no epistasis has been proven.

6.4.5 Synteny at QTL level

R genes have also been identified and mapped in other monocots. Several agreements exist between the results found in the present study and those of other QTL-mapping studies:

- Van Deynze et al. (1995a) made a thorough study of group 1 chromosomes of *Triticeae* species and their relation to chromosomes in rice and oat. This group of chromosomes contains the leaf rust genes identified in wheat. The homologous chromosome in oat, the group A chromosomes contain resistance genes against *Puccinia coronata* Cda f.sp. *avenae*. The group 1 chromosomes of the *Triticeae* and the group A chromosomes of oat are homologous to the LG1 of *Lolium*; on this LG, we found QTLs linked with crown rust resistance.

- Yu et al. (1996) identified homoeologous regions for resistance to obligate biotrophs in *Avena, Hordeum* and *Zea mays*. These regions were located in the homoeologous group 1. This group corresponds to LG1 in *Lolium* and in which we found QTLs for crown rust resistance.

- Yu & Wise (2000) mapped a cluster of crown rust resistance loci (*Pca* cluster) to LG B of diploid oat. This LG is homologous to the LG 2 of the *Triticeae* and LG2 of *Lolium*, on which we found QTLs for crown rust resistance.

These findings indicate the potential presence of homologous regions for resistance genes in the *Gramineae*. If group 1 and 2 chromosomes of *Triticeae* descended from the same chromosomes of a common ancestor, the disease resistance loci on them might be orthologous. However, we just have very fragmented data on the synteny between LG1 and LG2 of *Lolium* and other monocots to make a detailed synteny analysis for QTLs linked with resistance.

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6.5 Conclusions

Within the *L. perenne* mapping population, four QTLs involved in crown rust resistance have been identified and localized. Two QTLs are located on LG1 and two on LG2. They explain respectively 12.5%, 24.9%, 5.5% and 2.6 % of phenotypic variance. No epistatic interaction was found between these four QTLs.

Different techniques were used for QTL analysis. Consistent results were obtained with IM and MQM on the parental and integrated maps. Using, permutation-based methods, the presence of QTL2 and QTL3 were confirmed. However, QTL1 and QTL4 were not detected using these methods. Probably, addition of map information in CET and RET, may improve the power of the permutation tests.

The map used for QTL analysis was of good quality as the 1.5-LOD intervals for the four QTLs were between 3 cM and 19 cM broad (except for QTL4 in the TC1 parental map, which is 30 cM). This indicates a good saturation around the QTL position. However, there were also indications for three additional QTLs in LG2 (QTL5, QTL6 and QTL7). Saturation of the linkage map in these particular regions, will help clarify the presence of additional QTLs in LG2. Also saturation of the region around QTL2 is useful as QTL2 maps in a telomeric region of LG1. Saturation of this region with co-dominant markers from which linkage phase can be easily reconstructed, is preferable.

Indication was found for the presence of synteny at the QTL level between homologous groups of chromosomes within the *Gramineae*. LG1 and LG2 show homology with group A and B chromosomes of oat on which crown rust resistance genes were identified. They show also homology with the group 1 chromosomes of the *Triticeae* on which leaf rust resistance genes were identified.

Although the confidence intervals of the four identified QTLs are still large, plant breeders may not need to know the QTL locations with great accuracy if they intend to introgress them by marker assisted backcrossing. They will be mainly interested in those QTLs which have a large effect, to incorporate them in elite plants. Marker information can be used to increase the frequency of positive QTLs (and to decrease the frequency of negative QTLs) in these plants. Probably the greatest value of markers in this context is the reduction of linkage

drag during introduction of QTLs by backcrossing. In this context, marker information can help to break the unfavorable correlations between quantitative characters of interest. Furthermore, marker information around and within a QTL can be used to develop selection indexes (Kearsey & Farquhar, 1998).

As in this study the dissection of a complex trait was carried out using parents with high breeding values, a first delivery of this study to the breeding program, are resistant genotypes with specific (favourable) QTL configurations. However, the identified markers shown to be linked to QTLs with big effects can not yet be exploited on a broad scale as there are still some restrictions which need to be solved. It is likely that QTL action varies across environments, across different genetic backgrounds and across different spore mixtures. Therefore these QTLs should be tested in different environments, with different spore mixtures and an assessment of the diversity of QTL alleles and their action present in a broad genetic population should be made (Marques et al., 1999; King et al., 2000; Miflin, 2000). But given that we find synteny at QTL level with other species, the QTLs identified might represent genomic regions in general resistance or genomic regions in which R genes are clustered (gene-for-gene resistance). The latter case is likely as the mapped RGA fragment coincides with QTL2 on LG1. It is interesting to analyze this population for resistance to other diseases affecting *Lolium* like bacterial wilt, leaf rust and stem rust and to know the positions of QTLs for resistance to these diseases. This will give an indication of genomic regions involved in general resistance.

In a first attempt to characterize the QTL alleles identified in this cross, the selfings of F_1 individuals (as described in Chapter 2) are currently being genotyped. The results of this analysis are however, out of the scope of the present thesis, and will be reported in the future.

Chapter 7

Map construction and QTL detection in the *L. x boucheanum* and *L. multiflorum* populations

7.1 Introduction

   L. perenne and *L. multiflorum* are genetically highly related. They are interfertile, resulting in hybrid ryegrass *L. x boucheanum*. These three ryegrass types are morphologically nearly indistinguishable (Jahuar, 1993), and the close relationship between them has been proven in studies based on morphology (Terrell, 1968), allozymes (Loos, 1993), RAPD data (Stammers et al., 1995) and SSR data (Kubik et al., 1999; Jones et al., 2001). In most cases, markers developed in one species can readily be used in the other closely related species. For example, Kubik et al. (1999) and Jones et al. (2001) developed SSR markers in *L. perenne*, which can be used in *L. multiflorum* and *L. x boucheanum*. Yu and Wise (2000) cited that co-migrating AFLPs are usually common fragments among mapping populations and closely related species. Therefore, we assumed that co-migrating AFLP fragments in *L. perenne*, *L. x boucheanum* and in *L. multiflorum* represent the same genetic locus.

   As *Lolium* spp. are interfertile, characteristics of one species can be introduced into the other species by means of interspecific crosses. Therefore, it was of interest to study crown rust resistance in *L. multiflorum* and in the interspecific hybrid. This was in parallel to the study of crown rust resistance in *L. perenne*. The alignment of *L. perenne*, *L. multiflorum* and *L. x boucheanum* maps enables the comparisons of QTLs and their positions. One can expect to find QTLs which are conserved between species or one can identify species-specific QTLs. Conserved QTLs can contain species-specific alleles that are absent in related species. Such alleles are of particular interest for the introduction of novel resources of resistance in a related species (Asins, 2002).

7.2 Objectives and rationale

In this chapter, it was the aim to construct genetic linkage maps for the *L. x boucheanum* and *L. multiflorum* populations described in Chapter 2. Maps were constructed using mainly AFLP markers and the co-dominant markers proven to be polymorphic in these populations (discussed in Chapter 4). Using these maps for interval mapping and multiple QTL mapping, the objective was to identify genomic regions involved in crown rust resistance.

To compare QTLs, identified in the three populations, the alignment of the genetic maps or their integration is necessary. Therefore, a common subset of AFLP primer combinations was run on the three populations.

7.3 Material and methods

7.4.1 Plant material and phenotypic data

The *L. x boucheanum* mapping population (2A2*1B12) consisted of 70 plants; the *L. multiflorum* mapping population (Axis3*B-90) consisted of 227 plants. Phenotyping of these populations was described and discussed in Chapter 2.

7.4.2 Marker data and map construction

AFLP, SSR and STS markers were generated as described in Chapter 4. Map construction was done as described in Chapter 5, but in this case, markers from class a-xa- were also included in the linkage analysis.

7.4.3 QTL analysis

Three methods were used for QTL analysis : IM, MQM and permutation-based methods (RET and CET). Methods are described in Chapter 6.

7.4 Results and discussion

7.4.1 *L. x boucheanum* genetic map

The genetic map of the *L. x boucheanum* population is given in Fig. 7.1. A total of 541 markers were included in this analysis: 16% were heterozygous in the susceptible parent (1B12), 19% were heterozygous in the resistant parent (2A2) and 65% were heterozygous in both parents. Distorted segregation at P<0.05 was observed for 38% of the markers (AFLP, SSR and STS together), which is slightly higher than in the *L. perenne* population discussed in Chapter 5.

The 1B12 map consisted of 13 LGs; the 2A2 map consisted of 12 LGs. The integrated map had 15 LGs, consisted of 189 loci and spanned 1881 cM. In the integrated map, four LGs could not be integrated: LG12T was just identified in the 2A2 parental map and LG13S, LG14S and LG15S were just identified in the 1B12 parental map. The mean distance between two consecutive loci is 6.78 cM. The length of the linkage groups varies from 130 cM till 24 cM. The largest gap between two adjacent markers is 28 cM and is situated on LG 1. On average the genetic map consists of 12.6 markers per LG with a maximum of 37 on LG2 and a minimum of 4 on LG12T, LG13S and LG15S. Twenty one percent of the mapped markers deviated significantly from the expected Mendelian segregation ratios (P<0.05). Putatively R-linked markers, identified in the BSA analysis, map to LG2 (PC078-R3, PC157-R1, PC157-R2, PC008R2, PC008R3, PC400-R2 and PC400-R3). PC078-R2, also a marker putatively linked with crown rust resistance, could not be mapped.

7.4.2 QTL analysis in the *L. x boucheanum* population

In the *L. x boucheanum* mapping population, IM revealed 10 loci spread over seven LG of the integrated map to be involved in crown rust resistance. Using MQM mapping, a QTL was identified on LG1. The marker with the highest LOD-score within this QTL is PC008-R2, one of the markers selected during BSA (Chapter 3).

LG1

Position	Marker
0	PC400-007
2	PC407-063
11	PC026-066
20	PC407-072
21	PC407-021
22	PC400-035
28	PC178-058
31	PC407-032
33	**rye021**
36	PC178-146
40	PC400-086
45	**ose**
48	PC026-033
60	PC026-108
69	PC178-068
88	PC400-081
93	PC008-036
101	PC178-151
129	PC400-093
130	PC078-054

LG2

Position	Marker
0	PC178-045*
6	PC178-018*
7	PC178-078
15	PC078-069
	PC026-086
18	PC178-046*
24	PC078-070
	PC026-048
30	PC026-101
35	PC178-044
38	**uni001**
49	PC157-100
50	PC178-084*
66	PC178-019
67	PC078-090
72	PC078-081
74	PC078-106
76	PC078-R3
78	PC157-019*
81	PC157-105
82	PC078-088
	PC407-056
84	PC157-R1
	PC078-086
86	PC178-064
88	PC008-105
91	PC157-R2
92	PC008-R2
	PC078-105
94	PC008-034
97	PC008-048*
100	PC400-072
103	PC178-021
105	PC400-R2
107	PC178-077
112	PC008-R3
116	PC400-R3

LG3

Position	Marker
0	**rye024**
16	PC407-002*
42	PC157-098*
48	PC178-035
56	PC400-041*
57	PC026-052*
63	PC400-015
64	PC407-064*
66	PC178-060*
	PC008-007*
67	PC008-090*
76	PC008-029
78	PC008-065
80	PC008-047
82	PC026-028
88	PC407-031
97	PC026-073

LG4

Position	Marker
0	PC178-081
16	PC157-067
22	PC178-083
34	PC026-075
37	PC008-084
41	PC008-022
42	PC078-066
43	PC400-089
44	PC008-061
	PC407-007
45	**ADH**
	PC008-010
46	PC026-062
51	PC178-154
55	PC178-066
66	PC407-045
75	PC026-090

LG5

Position	Marker
0	PC400-039
19	PC157-036
23	PC178-112
	PC407-004
25	PC008-087
26	PC407-025
30	PC157-001
36	PC157-059
37	PC078-063
42	PC407-048
44	PC008-018
49	PC400-005
51	PC400-037
66	PC157-031

LG6

Position	Marker
0	PC178-043*
8	PC178-009*
15	PC178-110*
	PC178-109
27	PC178-108
30	PC157-111
32	PC178-132
36	PC178-131*
37	PC157-128
38	PC157-085*
39	PC178-104*
40	PC157-102
41	PC178-105*
42	PC178-120
43	PC178-106*
46	PC178-107*
48	PC178-092*
53	PC178-101*
57	PC178-103*
63	PC178-102*

LG7

Position	Marker
0	PC400-040
	PC407-055
7	PC078-057
11	PC178-016
15	**ADP**
17	PC008-003
18	PC178-085
22	PC026-029
23	PC026-095
	PC026-082
27	PC008-006
	PC008-095
30	PC157-026
36	PC157-017
41	PC026-044
47	
50	**OSW**

LG8

Position	Marker
0	PC178-155
8	PC400-028
22	PC407-085
24	PC157-025
28	PC008-042
47	PC407-071

LG9

Position	Marker
0	PC400-107*
20	PC078-098
22	PC407-089
	PC407-059
23	PC078-058*
	PC078-101*
24	PC178-039
	PC400-109
26	PC026-080*
27	PC178-006
31	PC407-040
45	PC400-087

LG10

Position	Marker
0	PC407-082
15	PC157-087
16	PC157-086
24	PC400-046
35	PC157-124

LG11

Position	Marker
0	PC157-126
12	PC400-079
14	PC400-078*
	PC008-011*
17	PC400-084*
24	PC178-067*
25	PC157-096*

LG12T

Position	Marker
0	PC157-023
20	PC008-020
36	PC400-017
49	PC157-127

LG13S

Position	Marker
0	PC407-054*
15	PC157-065*
16	PC400-025
28	PC400-061*

LG14S

Position	Marker
0	PC157-125
26	PC157-099
33	PC078-015
34	PC026-050*
39	PC400-001

LG15S

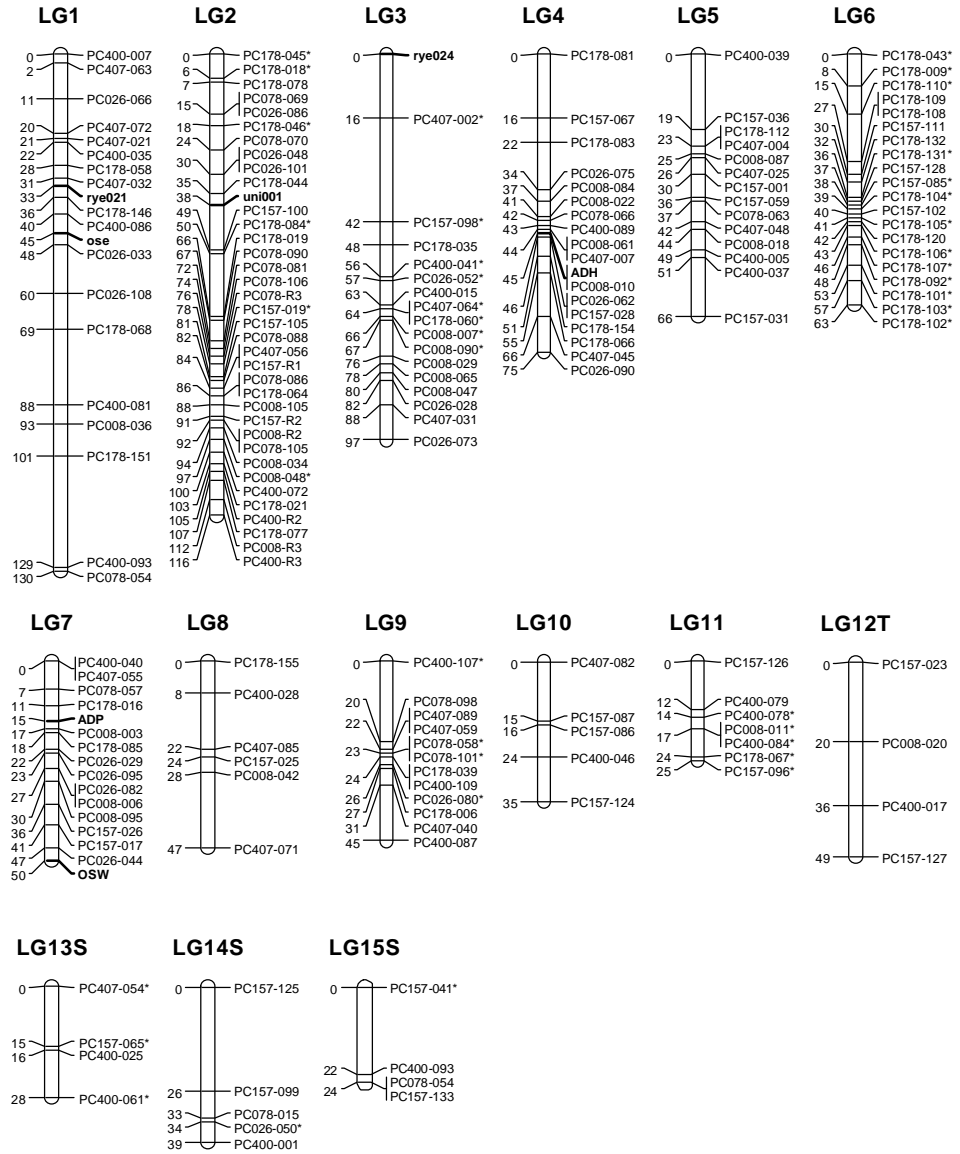| Position	Marker
0	PC157-041*
22	PC400-093
24	PC078-054
	PC157-133

Fig. 7.1 : Linkage map of the *L. x boucheanum* cross based on SSR, STS and AFLP markers. AFLP markers have prefix PC; STS and SSR markers are indicated in bold; AFLP markers selected in the BSA analysis start with PC and end with –Rx (with x=number). Distorted markers (P>0.001) are marked with *. LGs with an extension S or T are LGs just found in the 2A2 (T) parental map or in the 1B12 (S) parental map. LGs are not numbered according to the ILGI map.

The permutation-based approach revealed different results than the MQM analysis (Fig. 7.2). In the first cycle of permutation testing, PC008-031 turned out to be the most significant marker. The T_0 value of another marker, PC026-091, fell within the 95% CI of the 2.5 percentile threshold value. In the second cycle of permutation testing (CET), after stratification on marker PC008-031, a second marker linked with crown rust resistance was identified: PC400-053. None of these markers have been mapped on the *L. x boucheanum* linkage map.

The markers identified in the BSA analysis, were detected in the MQM analysis. This demonstrates once more the usefulness of BSA to identify QTLs with big effects. However, BSA failed to identify the other markers detected by the permutation-based testing. As they are both a-xa- markers, they were not included in the BSA analysis (just a-x-- or --xa- markers were selected in the BSA analysis).

This permutation-method seemed to detect other effects than BSA did. These are represented by the markers PC008-031 and PC400-053. PC008-031 seems to be a promising marker as linear regression analysis revealed that it explains 43% of the variance ($R^2=0.43$), while PC400-053 does not explain any variance ($R^2=0.000$).

The phenotypic analysis of this population discussed in Chapter 2, revealed the presence of two major genes involved in crown rust resistance. If the results in the QTL analysis are summarized, one QTL, explaining 85% of the variance is identified in LG1 using MQM, and one unmapped marker (PC008-031) linked with crown rust resistance was detected using permutation-based methods, explaining 43% of variance. This results in the same conclusion as in the phenotypic analysis : two major loci are involved in resistance. However, the percentage of variation explained by the QTL identified in MQM should be taken with care. The quality of the genetic map was too low to perform a thorough QTL analysis due to large gaps in the genetic map, the low degree of map integration and the high amount of a-xa- markers. It is not possible to discuss the position and allelic effect of the different QTLs. A better map saturation and the incorporation of co-dominant markers are needed before conclusions can be made in this population. The mapping of PC008-031 is of special interest.

a) first round of permutation-based QTL analysis
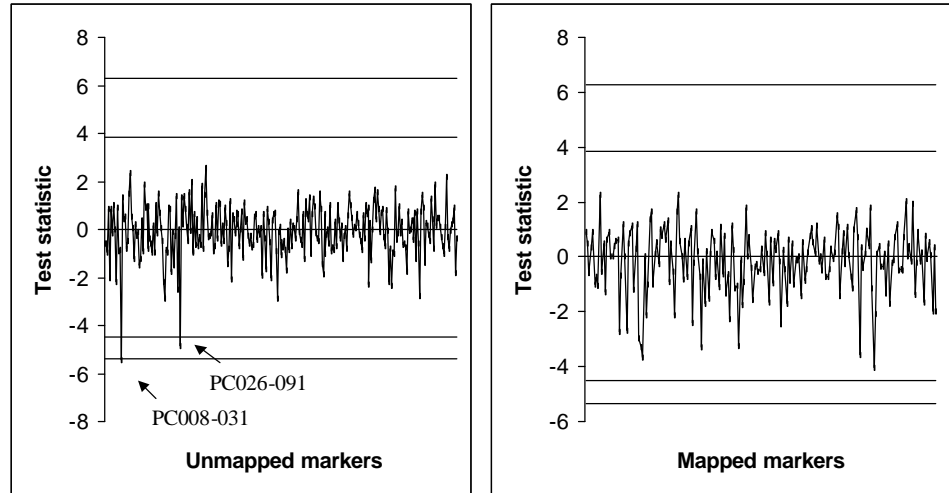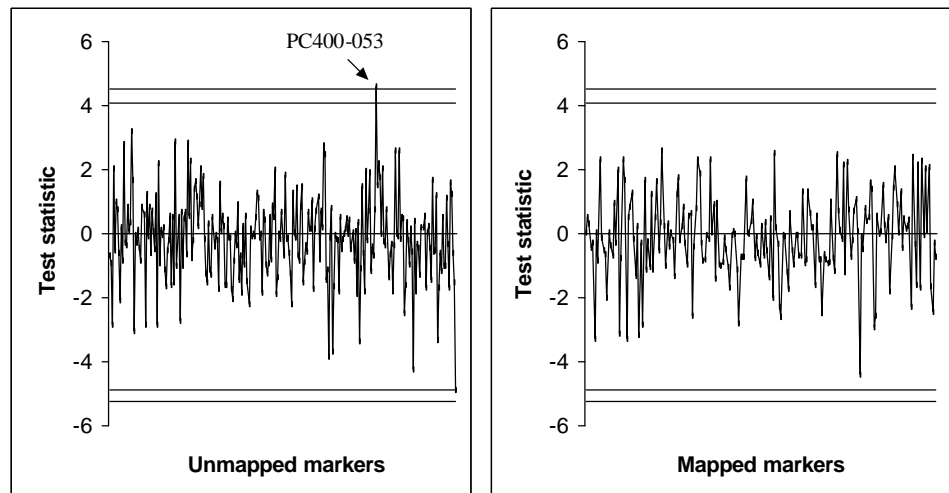

b) CET testing after stratification on PC008-031

Fig. 7.2: Permutation-based QTL analysis : a) test statistics for the mapped and unmapped markers in the *L. x boucheanum* mapping population obtained after the first round of permutation testing; b) CET test statistics obtained after stratification on PC008-031. 95% CI of the 2.5 and 97.5 percentile threshold values are indicated.

7.4.3 *L. multiflorum* genetic map

The genetic map of the *L. multiflorum* population is shown in Fig. 7.3. A total of 553 markers were included in this analysis: 22% were heterozygous in the susceptible parent (B-90), 30% were heterozygous in the resistant parent (Axis3) and 48% were heterozygous in both parents.

The Axis3 map consists of 10 LGs; the B-90 map consists of 15 LGs. The integrated map includes 133 loci, consists of 18 LGs and spans 1372 cM. It was difficult to integrate both parental maps. Eleven LGs of the integrated map are LGs identified in just one of the parental maps. LG8T, LG9T and LG10T were just found back in the Axis3 parental map; LG11S, LG12S, LG13S, LG14S, LG15S, LG16S, LG17S and LG18S were just found in the B-90 map. The mean distance between two consecutive loci is 14.35 cM. The length of the linkage groups varies from 153 cM till 5 cM. The largest gap between two adjacent markers is 50 cM and is situated on LG12S. On average the genetic map consists of 7 markers per chromosome with a maximum of 18 on LG2 and a minimum of 3 on LG10T. Of the mapped markers, 56% are markers deviating significantly from the expected Mendelian segregation ratios. Some LGs include just deviating loci such as LG6, LG7 and LG18S. Potentially R-linked markers, identified in the BSA analysis were PC065-R1, PC065-R2 and PC175-R1. PC065-R1 maps on LG9T; the other two markers could not be mapped.

7.4.4 QTL analysis in the *L. multiflorum* population

Interval mapping revealed 12 markers spread over eight LG of the integrated *L. multiflorum* map to be involved in crown rust resistance. MQM mapping identified two QTLs : one in LG12S and one in LG13S, two linkage groups identified in the susceptible parent. The QTL located in LG12S explains 76% of variance and the QTL located in LG13S explains 3% of variation. The QTLs identified by MQM should be taken with care as they appear in very small linkage groups, (containing four and seven markers), with large gaps between the markers.
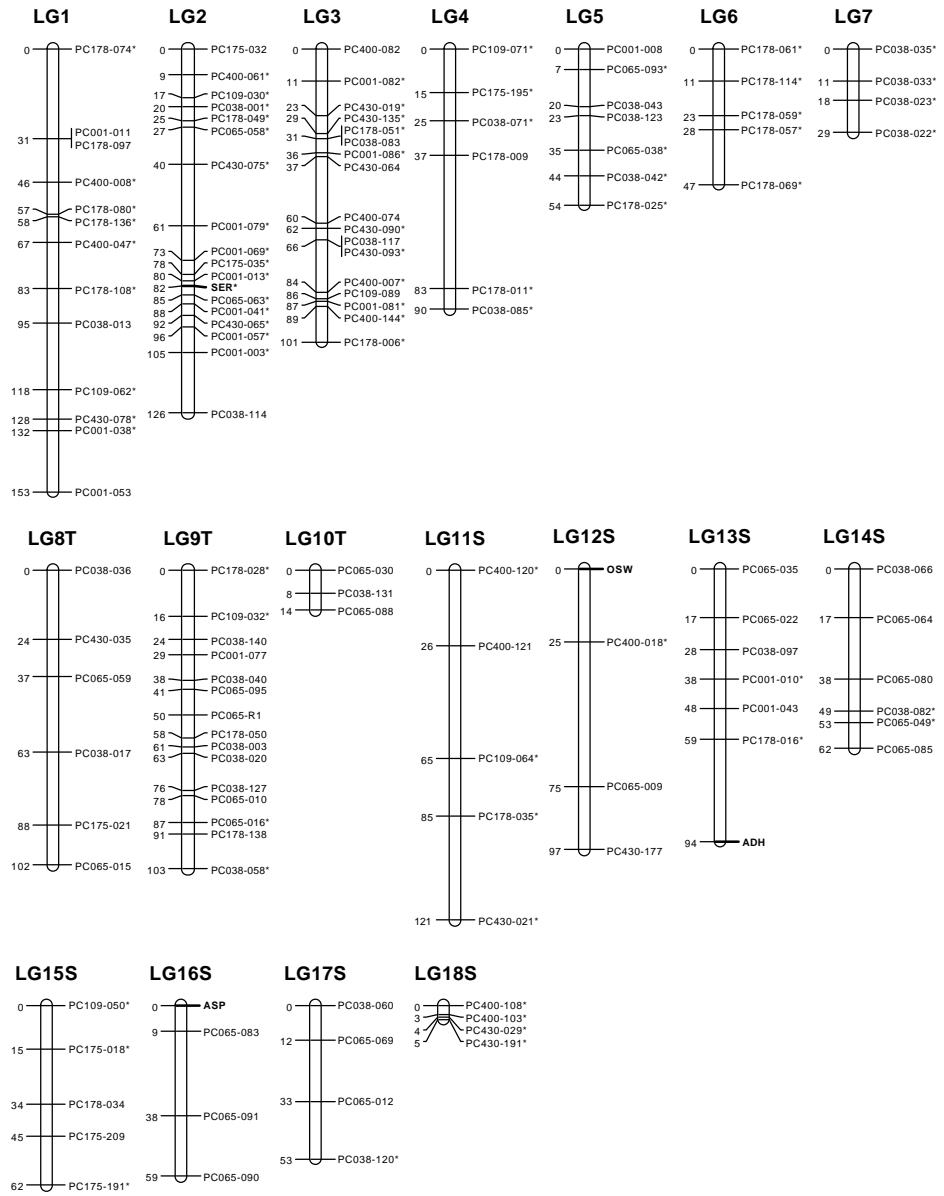
Fig. 7.3 : Linkage map of the *L. multiflorum* cross based on SSR, STS and AFLP markers. AFLP markers have prefix PC; STS and SSR markers are indicated in bold. Distorted markers (P<0.001) are marked with *. LGs with an extension S or T are LGs just found in the Axis3 (T) parental map or in the b-90 (S) parental map. LGs are not numbered according to the ILGI map.

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The results obtained with MapQTLv.4.0. were not consistent with the results obtained in the permutation-based method. The permutation-based approach identified in a first step PC038-118 (Fig. 7.4). After stratification on PC038-118, CET identified PC430-97 as the most significant marker by CET. No other significantly linked markers have been identified in the *L. multiflorum* population. PC038-118 and PC430-97 have not been mapped in this *L. multiflorum* population.

The markers identified in the permutation-based method are more promising. Linear regression revealed R^2 values of 0.157 and 0.112 for PC038-118 and PC430-97 respectively. Linkage analysis showed that the recombination frequency between these markers is 0.40, indicating that they are probably not on the same chromosome. Although they were both a-x-- markers, they were not selected in the BSA analysis as they appeared as well in the resistant as in the susceptible bulk.

The phenotypic analysis revealed that in the *L. multiflorum* population, crown rust resistance was inherited in a quantitative way, no major genes were suspected to be present (Chapter 2). None of the three markers selected during BSA analysis was significantly linked with crown rust resistance when tested on the whole population (Chapter 3). This was confirmed by the MQM analysis as PC065-R1 in LG9T (the only mapped marker of the 3 markers identified in the BSA), did not show up as a significant QTL. The markers found in the permutation-based methods are in agreement with the phenotypic analysis in a way that they explain a small amount of variation. This indicates that they are linked with minor genes.

Saturation of the *L. multiflorum* genetic map is necessary before a thorough QTL analysis can be carried out. Especially, mapping of the two markers identified in the permutation-based method should be aimed for.

a) first round of permutation-based QTL analysis
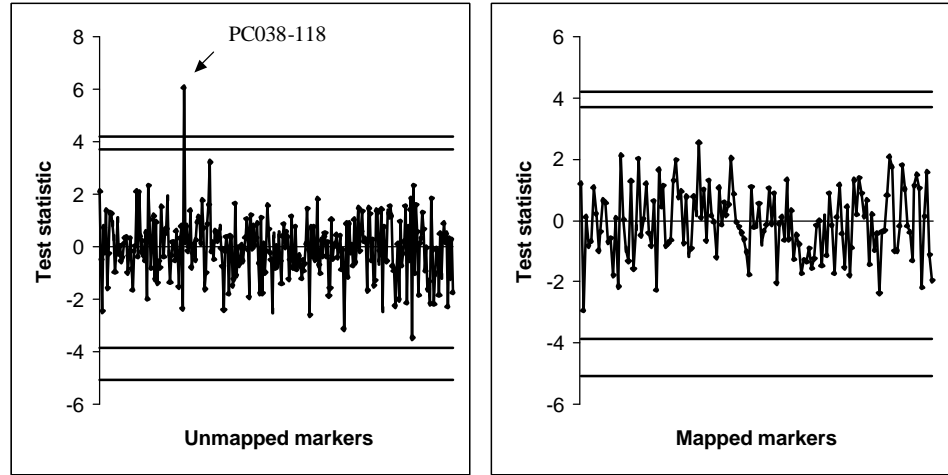
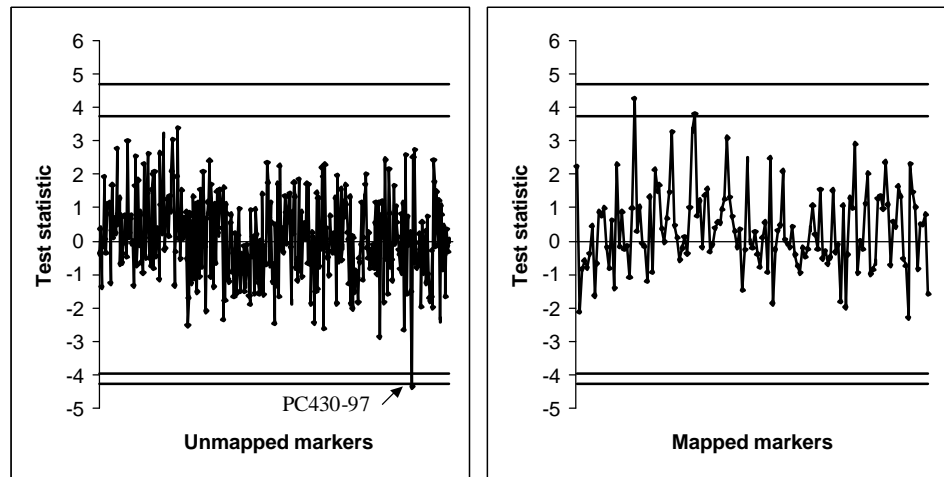b) CET testing after stratification on PC038-118

Fig. 7.4: Permutation-based QTL analysis : a) test statistics for the mapped and unmapped markers in the *L. multiflorum* mapping population obtained after the first round of permutation testing; b) CET test statistic obtained after stratification on PC038-118. 95% CI of the 2.5 and 97.5 percentile threshold values are indicated.

7.4.5 Integration of *L. perenne*, *L. x boucheanum* and *L. multiflorum* maps

To have a general view on QTLs for crown rust resistance in the three populations, the integration of the different maps was planned. Therefore, AFLP primer combinations (PC) revealing a high number of AFLP markers in common between the three populations were run on all three populations. An overview of the AFLP PC and the number of fragments in common are given in Table 7.1. Between the *L. perenne* and *L. multiflorum* map, just two markers were in common; between the *L. perenne* and *L. x boucheanum* map, eleven markers and between the *L. x boucheanum* and *L. multiflorum* map, six markers. This low number of markers in common did not permit the integration of the maps.

Table 7.1 : Number of polymorphic and mapped markers in the three populations and number of mapped, polymorphic markers in common between the populations. (- = PC was not run on this population, NC = PC was not in common).

	# polymorphic markers			# mapped markers			# commonly mapped		
AFLP PC	lp	lh	lm	lp	lh	lm	lp-lh	lp-lm	lm-lh
1	86	-	88	9	-	16	NC	2	NC
8	61	62	-	20	23	-	2	NC	NC
26	44	69	-	26	18	-	2	NC	NC
78	47	53	-	13	18	-	1	NC	NC
178	-	102	76	-	43	22	NC	NC	4
400	67	86	101	24	28	12	3	0	2
407	49	87	-	20	21	-	3	NC	NC
Total	354	459	265	112	151	50	11	2	6

7.5 Conclusion

Using mainly AFLPs and a few co-dominant markers, we were not able to construct genetic maps of high quality in the *L. x boucheanum* and *L. multiflorum* populations. Several linkage groups were just identified in one parental map and could not be integrated with a linkage group of the other parental map. Large gaps between markers were also present in the maps,

indicating a low level of saturation. The low information content of AFLP markers and in the *L. x boucheanum* population the small population size were the main reasons for the low quality of the maps. This had its consequences for QTL analysis. QTL analysis using MQM and the permutation-based method did not reveal the same QTLs. Given the characteristics of the maps, the results obtained in the MQM analysis should be taken with care because of the low quality of the genetic maps. Many markers were not mapped, and as consequence they were not included in the MQM analysis. Under these circumstances, the permutation-based methods provide probably more reliable results.

MQM in the *L. x boucheanum* population identified one QTL at a genomic region containing markers selected in the BSA analysis. This result confirms the conclusions obtained in the BSA, that those markers identify loci involved in crown rust resistance. Whether this QTL coincides with one of the QTLs identified in the *L. perenne* map remains to be tested. The markers identified in the permutation-based method were promising as one marker identified in the *L. x boucheanum* population had a R^2 value of 0.43. Unfortunately we were unable to map this marker. In summary, we identified two QTLs (one mapped and one unmapped) involved in crown rust resistance. This is in line with the hypothesis obtained after phenotypic analysis in which we assumed that crown rust resistance in the *L. x boucheanum* is conferred by two major genes.

In the *L. multiflorum* population, MQM identified QTLs on small LGs. These results should be taken with care as on these LGs large gaps were present. On the other hand, the permutation-based approach revealed two interesting markers : PC038-118 and PC430-97 with R^2 values of 0.15 and 0.11. These markers were unmapped and tended to be unlinked. This assumes that the markers map to different chromosomes. These markers, if tightly linked with QTLs, respresent genomic regions with minor effects. This is in accordance with the phenotypic analysis in which we hypothesised that resistance in the *L. multiflorum* population was conferred by minor genes with additive effect.

We were unable to integrate the three maps, due to both the low quality of the *L. x boucheanum* and *L. multiflorum* maps, and the low number of co-dominant markers suitable for map-integration. We can therefore conclude that the high amount of co-dominant markers and the big population size used to construct the *L. perenne* map are necessary conditions to establish a genetic

linkage map which can be aligned with other maps and on which a thorough QTL analysis can be performed. Co-dominant marker techniques are indispensable and needed next to a high-throughput marker system generating a high number of markers like AFLP. The lack of freely available, high-throughput co-dominant marker systems like SSRs and STS hampers the establishment and alignments of linkage maps in the outcrossing species *Lolium*. For example, the high number of SSRs included in the *L. perenne* map could not be tested in the *L. x boucheanum* or the *L. multiflorum* population as they are not freely available.

To improve the quality of the study, the *L. x boucheanum* and the *L. multiflorum* maps will be saturated with dominant and co-dominant markers. This will enable the alignment of the different maps and mapping of promising markers identified in the permutation-based QTL analysis.

Chapter 8

General discussion, conclusions and future perspectives

8.1 General discussion and conclusions

8.1.1 Inheritance of crown rust resistance

In this PhD thesis, crown rust resistance in *Lolium* was studied on the basis of F_1 populations segregating for crown rust resistance. Phenotypic analysis revealed no maternal effects on crown rust resistance in the three selected populations. In the *L. perenne* and *L. x boucheanum* population, crown rust resistance was found to be oligogenic; one or two major dominant genes are involved, of which the action is modified by minor genes. In the *L. multiflorum* populations, quantitative resistance conferred by several minor genes with additive action was hypothesized.

As *Puccinia* spp. are biotrophic fungi, genes that display characteristic gene-for-gene specificity were expected to be active in these populations. Wise et al. (1996) stated that this kind of genes are often found in the resistance of monocotyledonous species to obligate fungal biotrophs, such as *Zea mays* to *Puccinia sorghi*, *Triticum aestivum* to *Puccinia* spp. and *Hordeum vulgare* to *Erysiphe graminis*. The major genes identified in the *L. perenne* and *L. x boucheanum* population resemble this kind of genes. Qualitative resistance against *Puccinia coronata* in *Lolium* had already been reported (McVeigh, 1975; Wilkins, 1975; Schmidt, 1980; Lellbach, 2000). McVeigh (1975) reported on qualitative resistance controlled by recessive alleles, but in the present study, dominant alleles were hypothesised. Dominant resistance alleles were also found by Wilkins (1975) and Schmidt (1980). Schmidt (1980) observed that the action of the major genes was modified by several complementary minor genes with additive interaction, which is also the case in the *L. perenne* and *L. x boucheanum* population presented in this study.

An extreme case of action of minor genes was found in the *L. multiflorum* population, in which no major R genes were found and in which resistance

seemed to be conferred by the combined action of multiple genes with small individual effect on the phenotype.

8.1.2 Detection of trait linked markers

To dissect the genetic background of the resistance mechanisms found, DNA markers were used to identify genomic regions linked with the trait. Three approaches were employed to identify significant linkages between marker and trait : Bulk Segregant Analysis, map-based QTL analysis and permutation-based QTL analysis.

In the *L. perenne* population with oligogenic resistance, BSA revealed two groups of markers tagging major genes involved in resistance. The same markers were identified by the map-based and permutation-based QTL analysis. However, the map-based QTL analysis revealed two additional groups of markers significantly linked with crown rust resistance.

In the *L. x boucheanum* population, also displaying oligogenic resistance, BSA revealed markers significantly linked with crown rust resistance. Map-based QTL analysis identified the same markers to be linked with the trait. The permutation-based QTL analysis was able to identify additional markers significantly linked with crown rust resistance, but which had not been mapped, and were therefore not detected during the map-based QTL analysis.

In the *L. multiflorum* population with polygenic resistance, BSA failed to identify markers significantly linked with crown rust resistance. Due to the low quality of the genetic linkage map, a map-based QTL analysis was not appropriate for this population. The permutation-based method showed to be the most successful, as two unlinked markers were identified to be significantly linked with crown rust resistance.

Therefore, we have demonstrated that when oligogenic traits are studied, BSA is an interesting approach to identify markers linked with loci having a big effect on the trait (e.g. in *L. perenne* and *L. x boucheanum* population). However when studying oligogenic resistance, map-based or permutation-based QTL analysis are able to identify minor genes influencing the action of the major genes (f.e. in the *L. perenne* population) while BSA is not. If polygenic resistance is studied, BSA is not suitable; at least in the way BSA was performed

in this study (e.g. *L. multiflorum* population). In the case of polygenic resistance, map-based or permutation-based QTL analysis are both suited. If the quality of the map is acceptable, map-based QTL analysis is the most appropriate choice when dealing with polygenic resistance. Permutation-based methods are especially useful when no linkage map is available or when the available map is unsaturated and fragmented (as in the *L. multiflorum* and *L. x boucheanum* population). As it does not use map information, permutation-based QTL analysis is helpful to screen unmapped markers for significant associations with the trait.

The incorporation of map data in the permutation-based methods used in this PhD thesis should be aimed for, as in contrast to available map-based methods they can handle phenotypic data deviating from the normal distribution, while the described permutation-based method can. Another important advantage is that they take into account the multiple testing problem.

A demonstration that the two-way pseudo-testcross is suited to linkage mapping in *Lolium* opens new perspectives for the identification of markers linked to other traits, as the most extreme phenotypes can be used as crossing parents or the construction of segregating populations.

8.1.2.1 Markers identified in the L. perenne *mapping population*

The highest resolution of genetic dissection of the trait studied in the *L. perenne* population was achieved with the map-based QTL analysis. Four QTLs were identified explaining 45.5% of the variation. Two QTLs are situated in LG1 and two in LG2. These results can explain the phenotypic segregation data. Out of the phenotypic segregation data, two major genes showing no interaction were expected to be present in this population. It was probably not possible to distinguish the QTLs situated in the same linkage group on the basis of the phenotypic data and QTLs on the same linkage group were phenotypically identified as one major gene.

BSA, initially proposed for mapping major genes, was very effective in targeting markers linked with loci with a big effect on crown rust resistance. Two clusters of markers linked with resistance were identified. Cluster 1 (PC106-R2 and PC168-R1) explains 25% and cluster 2 (PC026-R3 and PC026-

R4) 5% of the phenotypic variance. These markers coincide all with QTLs identified in the QTL analysis. BSA cluster 1 coincides with QTL2 on LG1 and cluster 2 coincides to QTL4 on LG2. Therefore, BSA identified the two major genomic regions involved in crown rust resistance in this population. But as in the phenotypic analysis, the QTLs localised on the same LG could not be differentiated from each other using BSA. This indicates that BSA is a suitable method for the rapid identification of markers linked with major genes. A map-based QTL analysis enables more precise localization and identification of closely linked QTLs with minor effects.

8.1.2.2 Markers identified in the L. x boucheanum mapping population

In the *L. x boucheanum* population, a group of linked markers which explained 15% of variation was identified in the BSA. One unmapped marker explaining 43% of variation was identified in the permutation-based method. This complies with the phenotypic segregation data suggesting that two major genes are involved in crown rust resistance.

The unmapped marker explaining the highest variation was not identified in the BSA as it was an a-xa- marker. These markers were not of interest in the BSA as just a-x-- and --xa- are analysed. This is a disadvantage of BSA, as this method can just detect regions which the parents are polymorphic for.

8.1.2.3 Markers identified in the L. multiflorum mapping population

In the *L. multiflorum* population, two unlinked, unmapped markers were identified using the permutation-based method, explaining 15% and 11% of the phenotypic variation. These markers, if tightly linked with QTLs, represent genomic regions with minor effects. This is in accordance with the phenotypic segregation data, from which minor genes were expected to be involved in crown rust resistance. BSA was not effective as it did not detect any marker linked with the polygenic trait, probably due to the lower phenotypic effect of each individual QTL, in comparison to the *L. perenne* population.

In the *L. x boucheanum* and *L. multiflorum* populations, the constructed linkage map was unsaturated. If the quality of the map is improved, a map-

based QTL analysis would be the most appropriate choice; especially in the case of the *L. multiflorum* population as in this population just quantitative resistance was observed.

8.1.3 Map quality, marker quality

The high resolution QTL mapping achieved in the *L. perenne* population was mainly due to the high quality of the genetic map. The map was highly saturated with co-dominant markers generated with different marker techniques. These markers were highly informative for linkage phase determination.

High quality maps could not be obtained in the *L. multiflorum* and *L. x boucheanum* population, proving that the two-way pseudo-testcross procedure with mainly dominant markers is not optimal for map construction and QTL analysis in this kind of segregating populations. The incorporation of the a-xa-markers into the linkage analysis was necessary for the integration of the parental maps, but these markers are not informative for linkage phase determination (Maliepaard et al., 1998) and contributed to the low quality of the maps. In the *L. multiflorum* and *L. x boucheanum* population, it was difficult to integrate the parental linkage maps and linkage groups were fragmented resulting in numerous, small groups. In contrast to the permutation-based technique, which was successful in identifying AFLPs linked with crown rust resistance in *L. x boucheanum* and *L. multiflorum* populations, the map-based method was not suitable for QTL analysis due to the quality of the maps. Improving the quality of both maps by using co-dominant markers, will give a more detailed view on the genetic organization of crown rust resistance in these populations and will allow the alignment of the three genetic maps generated in this study. This could not be done now solely on the basis of AFLPs and a few co-dominant markers.

In the *L. perenne* population, the RFLP markers and the SSRs developed by Jones et al. (2002b) enabled the alignment of the integrated map with the ILGI map. The RFLP markers, included in the presented *L. perenne* map, revealed additional information and mostly confirmation of the synteny study made by Jones et al. (2002a) was observed. However, inconsistencies with the results presented by Jones et al. (2002a) were found. RFLP probes not mapped by Jones et al. (2002a) but mapped in the present linkage map revealed evidence for different syntenic relationships than those proposed by Jones et al. (2002a).

Alignment of all available *Lolium* maps containing markers suitable for comparative mapping will help to improve and generalize the knowledge on syntenic relationships between *Lolium* and other *Gramineae.*

Some speculations have been made on syntenic relationships at the QTL level. The presence of homologous regions containing resistance genes in different monocot species (*Triticeae*, oats, rice) had been reported by Van Deynze et al. (1995a), Bush and Wise (1998) and Yu and Wise (2000). The QTLs identified in the *L. perenne* population map onto LG1 and LG2 of *Lolium*, which are syntenic with LGA and LGB of oat respectively and with LG1 and LG2 of the *Triticeae* respectively. These homologous groups of chromosomes are chromosomes on which resistance genes have been identified; leaf rust genes in wheat and crown rust genes in oat. This outcome indicates that a detailed comparative mapping study of these two LGs is certainly challenging.

A number of markers with sequence information was used. The STS markers and RGA markers amplified sequences with homology to expressed genes. Promising was that the RGA fragment map on LG1; a LG on which QTLs for crown rust resistance in the *L. perenne* population were identified. These STS markers were often co-dominant markers, with a high information content for linkage phase determination. The importance of employment of STS markers derived from expressed sequences is increasing, especially when the identification of candidate genes for QTLs linked with specific traits are aimed for. This is the main aim of a currently ongoing EU-project (GRASP) at the DvP

8.2 Future perspectives

The conclusions of the present thesis, especially for the *L. perenne* population open nice perspectives both for further research on crown rust resistance and for the development of MAS schemes. We should bear in mind that the results of this study are based on phenotypic analysis on seedlings using one spore mixture in an artificial inoculation method. This is a good starting point to identify genomic regions involved in crown rust resistance in *Lolium* populations, as the environmental conditions are controlled and an uniform spore mixture is used. However, it is very interesting to compare these results with phenotypic data obtained on adult plants, using different spore mixtures or single spore isolates

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and in different environments. This constitutes one of the future aims in the framework of this research. But given that we find synteny at QTL level with other species, the QTLs identified might represent genomic regions in general resistance or genomic regions in which R genes are clustered (gene-for-gene resistance). The latter case is likely as the mapped RGA fragment coincides with QTL2 on LG1. It is interesting to analyze this population for resistance to other diseases affecting *Lolium* like bacterial wilt, leaf rust and stem rust and to know the positions of QTLs for resistance to these diseases. This will give an indication of genomic regions involved in general resistance.

The QTLs identified in this study will be further explored. In the first place, these QTLs can be accurately described and followed in crosses. A start has been made to study the different alleles present in the four QTLs. The selfings described in Chapter 2 are being genotypically analysed and the QTL configurations will be determined. This will allow to study the effects of alleles in different configurations (homozygous, specific allele combinations, QTL combinations) on the phenotype.

In practical plant breeding, these QTLs can be employed for genotype-building, which means the use of markers to design new genotypes combining favourable alleles previously detected at a number of loci (Hospital et al., 2001). The QTLs can be studied in different configurations, and the best performing configuration (homozygous, heterozygous, epistatic effects) can be selected. Elite plants can then be used in polycrosses serving as a donor for resistance or plants can be exploited in an introgression program. Interesting alleles can be introgressed into the breeding pool and used to build up a resistant variety.

A second direction, that will be taken with the obtained results, is divergent selection on the basis of marker presence. The efficiency of divergent selection on the basis of marker presence/absence will be compared with the efficiency of divergent selection based on the phenotype (resistant/susceptible). The *L. perenne* F_1 population will be divided into subpopulations based on the presence/absence of markers positively linked with crown rust resistance. The same will be done on the basis of the phenotypic value of the F_1 plants. These sub-populations, based on phenotypic selection and on marker selection will be multiplied and the progress of crown rust resistance within these multiplied populations will be compared. This will allow us to evaluate the efficiency of divergent selection on the basis of phenotype and marker configuration.

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In these two applications, we have to bear in mind that the efficiency of marker-based selection is restricted by the recombinations taking place between the markers and the QTL. Hence, one has to accelerate the response to selection to fix favourable QTL alleles before marker-QTL linkage disequilibrium vanishes (Hospital et al., 2001).

A third direction, in which this research will proceed, is in testing the marker allele-QTL association in a genetically broader population. Often it is stated that results obtained in one cross only apply in this analysed cross and that certain risks are taken in extrapolating them to other genotypes (Miflin, 2000). The marker alleles linked with identified QTLs will be tested for their association with crown rust resistance in collections of plants with broad genetic basis. The breeding pool of DvP has been sampled for this purpose. The validity of the markers in other mapping populations is being tested in collaboration with R. Kolliker (FAL, Switzerland).

Finally, the linkage map constructed for *L. perenne* represents an important tool for further developments in ryegrass-research. The map will be further saturated with molecular and phenotypic information (such as heading date and seed characteristics).

The development of more co-dominant markers and map saturation is aimed for. A collection of 137 cDNA sequences, derived from a cDNA library constructed from leaf tissue of a *L. perenne* plant, is available at the DvP. This database is used to develop co-dominant STS markers. These markers are of high interest as they detect expressed sequences. Interesting homologies with sequences with known function were found. Incorporating this type of markers will increase the chance to discover potential candidate genes within QTLs (Walsh, 2001). A set of co-dominant markers, evenly distributed through the genome and with sequence information is of extreme interest. This set can help to identify QTLs, to characterize them and to perform association studies in a broad germplasm.

Furthermore, by aligning different genetic maps (constructed at DvP or at other institutes) with QTL information for different traits (e.g. nitrogen use efficiency, water soluble carbohydrate content, plant habitus, biotic and abiotic stress resistance, …), a general view will be obtained of the genomic organization of the *Lolium* genome. This will give a better understanding of the basis for genetic correlations between economically important traits (linkage and/or

pleiotropic relationships between gene blocks controlling associated traits; e.g. flowering time and biomass, inflorescence size and inflorescence number, productivity and crown rust resistance). This can facilitate more efficient incremental improvement of specific target traits without the risk of linkage drag of undesirable characters that are linked to a desirable QTL (Asins, 2002).

Summary

Crown rust, causes severe yield and quality losses in ryegrasses. The causal agent is the biotrophic fungus *Puccinia coronata*. The aim of this present study was to identify genomic regions in *Lolium* spp. involved in crown rust resistance. F_1 populations segregating for crown rust resistance were created for each species included in this study (*L. perenne*, *L. x boucheanum* and *L. multiflorum*). Phenotypic analysis revealed oligogenic resistance in the *L. perenne* and *L. x boucheanum* populations. The presence of major genes, of which the action was modified by minor genes was put forward. In the *L. multiflorum* population, polygenic resistance conferred by minor genes with additive action was observed. Markers linked with the resistance genes detected in the three populations were obtained and were in accordance with the inheritance pattern observed in the phenotypic analysis. Three approaches were employed for the identification of resistance linked markers : bulk segregant analysis (BSA), map-based QTL analysis and permutation-based QTL analysis. BSA identified markers linked with loci having a big effect on the trait. Map-based QTL analysis was appropriate when a linkage map with good genome coverage was available. Permutation-based methods were employed when map data was of insufficient quality. In the *L. perenne* population, four QTLs were identified explaining 45% of variation. Two QTLs with major effects were detected by BSA and by permutation-based methods, however the other two QTLs with minor effects were just detected using the map-based approach. In the *L. x boucheanum* population, two genomic regions were identified explaining 15% and 43%. In the *L. multiflorum* population, two genomic loci with minor effect (16% and 11%) were identified. Due to low genome coverage of the *L. x boucheanum* and *L. multiflorum* genetic maps, just one of the two resistance linked loci identified in the *L. x boucheanum* population and none of the two resistance linked loci identified in the *L. multiflorum* population have been mapped. This indicates that the high-resolution QTL mapping in the *L. perenne* population was mainly due to the big population size and to the high number of co-dominant markers (SSR, RFLP and STS) used in the linkage map construction. These co-dominant markers enabled the alignment of the presented map with other linkage maps of *Lolium* and *Gramineae* species. Indications of syntenic relationships between *Lolium* and *Gramineae* (oat,

wheat, barley and others) were found at the genomic level and at the QTL level. QTLs identified in the *L. perenne* population mapped onto LG1 and LG2 which are syntenic with LGA and LGB of oat on which crown rust resistance genes have been identified. LG1 is also syntenic with the group 1 homologous chromosomes of the *Triticeae*. On these *Triticeae* chromosomes leaf rust resistance genes were identified in wheat and barley. This study made available a genetic linkage map suitable for further research and markers linked with crown rust resistance in different *Lolium* spp. These markers are now available for the plant breeder to evaluate their use for MAS strategies.

Samenvatting

Kroonroest veroorzaakt aanzienlijke opbrengst- en kwaliteitsverliezen in raaigrassen. Deze ziekte is het gevolg van een infectie met de biotrofe schimmel *Puccinia coronata*. Het doel van deze studie was genomische regio's, die betrokken zijn bij kroonroestresistentie in *Lolium* spp. op te sporen. Hiertoe werden F_1 populaties, die segregeerden voor kroonroestresistentie gecreëerd en dit voor de twee species opgenomen in deze studie (*L. perenne* en *L. multiflorum*) en hun interspecifieke hybride *L. x boucheanum*. Fenotypische analyse toonde aan dat in de *L. perenne* en de *L. x boucheanum* populaties kroonroestresistentie oligogeen was. De aanwezigheid van enkele major genen, van dewelke de actie gewijzigd wordt door minor genen, werd als hypothese vooropgesteld. In de *L. multiflorum* populatie werd vooropgesteld dat kroonroestresistentie polygeen was, waarbij meerdere genen met additieve actie betrokken zijn. Drie benaderingen werden gebruikt om merkers gekoppeld met kroonroestresistentie te identificeren : Bulk Segregant Analysis (BSA), kaartgebaseerde QTL analyse en permutatiegebaseerde QTL analyse. BSA identificeerde loci die een groot effect hadden op de bestudeerde eigenschap. Kaartgebaseerde QTL analyse was geschikt wanneer een koppelingskaart met een goede genoombezetting beschikbaar was. Permutatiegebaseerde QTL analyse werd gebruikt wanneer de kaartgegevens van onvoldoende kwaliteit waren. In de drie populaties werden groepen van merkers gevonden die gekoppeld waren met kroonroestresistentie. In alle populaties was er een overeenkomst tussen de hypotheses vooropgesteld na de fenotypische analyse en de geïdentificeerde DNA-merkers gekoppeld met kroonroestresistentie. In de *L. perenne* populatie werden vier QTLs gekarakteriseerd die samen 45% van de fenotypische variatie in de populatie verklaarden. Twee QTLs waren gekoppeld met een groot effect en werden gedetecteerd met behulp van de kaartgebaseerde en de permutatiegebaseerde QTL analyse. De merkers gekoppeld met de kleine effecten werden slechts gedetecteerd wanneer de kaartgebaseerde methode gebruikt werd. In de *L. x boucheanum* populatie werden twee genomische regio's geïdentificeerd die respectievelijk 15% en 43% van de fenotypische variatie verklaarden. In de *L. multiflorum* populatie werden twee genomische loci met een klein effect geïdentificeerd (16% en 11%). Doordat de *L. x boucheanum* en *L. multiflorum* koppelingskaarten geen goede genoombezetting

hadden, kon slechts één van de twee resistentie loci geïdentificeerd in *L. x boucheanum* populatie en geen enkel van de twee loci in de *L. multiflorum* populatie gekarteerd worden. Dit geeft aan dat de hoge resolutie bekomen bij de QTL analyse in de *L. perenne* populatie hoofdzakelijk te wijten is aan het groot aantal planten in de karteringspopulatie en het groot aantal co-dominante merkers (SSR, RFLP and STS) gebruikt tijdens de constructie van de koppelingskaart. Deze co-dominante merkers maakten het tevens mogelijk om de bekomen kaart te vergelijken met andere beschikbare koppelingskaarten van *Lolium* en andere *Gramineae* species. Er werden, zowel op het genomisch als op QTL niveau, indicaties gevonden van synteny tussen *Lolium* en *Gramineae* (haver, tarwe, gerst en andere). De QTLs opgespoord in de *L. perenne* populatie karteren op koppelingsgroep 1 en 2. Deze koppelingsgroepen vertonen synteny met koppelingsgroep A en B van haver. Op deze groepen zijn genen gelocaliseerd die gekoppeld zijn met kroonroestresistentie in haver. Er is ook synteny tussen koppelingsgroep 1 van *Lolium* en koppelingsgroep 1 van de Triticeae. In gerst en tarwe werden op deze groep genen gevonden die gekoppeld zijn met roestresistentie. Dit doctoraatsonderzoek leidde tot koppelingskaarten die beschikbaar zijn voor verder onderzoek en tot de identificatie van merkers gekoppeld met kroonroestresistentie. Deze merkers zijn nu beschikbaar voor de plantenveredelaar om ze verder te evalueren naar bruikbaarheid in merker gestuurde veredeling.

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Wilkins, P.W., Armstead, I., Deakin, C., Dhanoa, M.S. and Davies, R.W. (2002). Marker-assisted selection for herbage yield and quality in an advanced breeding population of perennial ryegrass. In: Proceedings Eucarpia meeting Fodder crops and amenity grasses section : Grass for Food and Grass for leisure, 24th meeting, 22-25 September 2002, FAL, Braunschweig, Germany, Vorträge für Pflanzenzüchtung, 74-79.

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Wu, R.L., Han, Y.F., Hu, J.J., Fang, J.J., Li, L., Li, M.L. and Zeng, Z.B. (2000). An integrated genetic map of *Populus deltoids* based on amplified fragment length polymorphisms. Theoretical and applied genetics, 100, 1249-1256.

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Young, N.D. (1996). QTL mapping and quantitative disease resistance in plants. Annual review in phytopathology, 34, 479-501.

Young, W.P., Schupp, J.M. and Keim, P. (1999). DNA methylation and AFLP marker distribution in the soybean genome. Theoretical and applied genetics, 99, 785-790.

Young, N. (2000). The genetic architecture of resistance. Current opinion in plant biology, 3, 285-290.

Yu, Y.G., Buss, G.R. and Maroof, M.A.S. (1996). Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proceedings of the national academy of sciences of the USA, 93, 11751-11756.

Yu, G.X., Bush, A.L. and Wise, R.P. (1996). Comparative mapping of homoeologous group 1 regions and genes for resistance to obligate biotrophs in *Avena*, *Hordeum*, and *Zea mays*. Genome, 39, 155-164.

Yu, G.X. and Wise, R.P. (2000). An anchored AFLP- and retrotransposon-based map of diploid *Avena*. Genome, 43, 736-749.

Zeng, Z.B. (1993). Theoretical basis for separation of multiple linked gene effects in mapping quantitative trait loci. Proceedings of the national academy of sciences USA, 90, 10972-10976.

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Curriculum vitae

1. Personalia

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Nationaliteit : Belg

Geboren :   te Menen, op 30 oktober 1972

Civiele status : gehuwd met Mik Van Gaever

2. Studies

Middelbaar onderwijs :

  tot 1990: Humaniora ASO optie Wiskunde (8 uur), Sint Alloysiuscollege, Menen

Universitair onderwijs :

  1990-1991: eerste kandidatuur bio-ir., RUG, onderscheiding

  1991-1992: tweede kandidatuur bio-ir., RUG, grote onderscheiding

  1992-1993: eerste jaar bio-ir. optie cel- en genbiotechnologie, RUG, onderscheiding

  1993-1994: tweede jaar bio-ir. optie cel- en genbiotechnologie, RUG, onderscheiding

  1994-1995: derde jaar bio-ir. optie cel- en genbiotechnologie, RUG, onderscheiding

    Scriptie: Conversie van somatische embryo's bij *Rosa* spp.

      Promotor : Prof. dr. Ir. P. Debergh

  1993-1995: geaggregeerde voor het onderwijs in de toegepaste biologische wetenschappen, RUG, onderscheiding

1995-1996 : aanvullende opleiding landbouwontwikkeling

Deze opleiding werd gevolgd in een Erasmus-uitwisseling aan de University of London (Wye College) waar ik een MSc Plant Biotechnology volgde,

Scriptie: PCR-based strategies for DNA-typing and detection of *Colletotrichum capsici.*

Promotor : Dr. S. Mantell

3. Beroepservaring

Sept. 1996–Juni 1998: Wetenschappelijk medewerkster aan de vakgroep plantenziekten van de Faculté Universitaire de Sciences Agronomiques, Gembloux op het IPGRI poject : 'Development of techniques for elimination of virus diseases from *Musa*' onder begeleiding van Prof. Dr. ir. P. Lepoivre.

Sept. 1998–Dec. 1998: Junior Associate Software Engineer, CTG Diegem. Uitgestuurd als consultant naar Jansen Pharmaceutica, Beerse.

Jan. 1999 tot heden: Wetenschappelijk medewerkster aan het CLO–DvP, labo biotechnologie, als doctoraatstudent met als thesisonderwerp: 'Genetic dissection of crown rust resistance in ryegrasses (*Lolium* spp.) using molecular markers' onder de begeleiding van Dr. I. Roldán-Ruiz en. Prof. Dr. Ir. E. Van Bockstaele.

4. Publicaties :

Publicaties in wetenschappelijke tijdschriften zonder referee :

– MUYLLE, H.; ADAMS, E.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (1999). Mapping crown rust resistance genes in *Lolium*. Med. Fac. Landb. en Toegepaste Biol. Wet. Ugent, 64/5, 179-182.

– MUYLLE, H.; ADAMS, E.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (1999).   Mapping of crown rust resistance genes in *Lolium* using BSA and comparative genetics with other monocots. 13 th Forum for Applied Biotechnology. Med. Fac. Landb. en Toegepaste Biol. Wet. Ugent, 64/5b, 381-387.

– HELIOT, B.; PANIS, B.; LOCICERO, A.; REYNIERS, K.; MUYLLE, H.; VANDEWALLE, M.; MICHEL, C.; SWENNEN, R. ; LEPOIVRE, P. (2000). Development of in vitro techniques for elimination of virus

diseases from *Musa*. 4th International symposium on in vitro culture and horticulture breeding, 2-7 July 2000, Tampere, Finland.

– PANIS, B.; HELIOT, B.; REYNIERS, K.; LOCICERO, A.; VANDEWALLE, M.; MUYLLE, H.; MICHEL, C.; LEPOIVRE, P.; SWENNEN, R. (2000). Assessment of cryopreservation for cucumber mosaic virus (CMV) eradication in banana plantlets. 4th International symposium on in vitro culture and horticulture breeding, 2-7 July 2000, Tampere, Finland.

– MUYLLE, H.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (2000). Identifying resistance gene analogues in *Lolium* spp. 14 th Forum for Applied Biotechnology. Med. Fac. Landb. en Toegepaste Biol. Wet. Ugent, 65/3b: 431-433.

– REHEUL, D.; BAERT, J.; BOLLER, B.; BOURDON, P.; CAGAS, B.; EICKMEYER, F.; FEUERSTEIN, U., GAUE, I.; GHESQUIERE, A.; GRAS, M.C.; HOKS, I.; KATOVA, A.; LELLBACH, H.; MATZK, F.; MUYLLE, H.; OLIVEIRA, J.A.; PRONCZUK, M.; ROLDÁN-RUIZ, I.; THOROGOOD, D.; VAN BELLINGHEN, C.; VAN WIJK, A., VISSCHER, J.; VIJN, R.; WOLTERS, L. (2000). Crown rust, *Puccinia coronata* Corda : recent evolutions. Proceedings 3th Conference on Harmful and Beneficial Microorganisms in Grassland, Pastures and Turf, 25-27 sept. 2000, Paderborn, Germany, 17-28.

– MUYLLE, H.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (2001). Molecular characterization of crown rust resistance in *Lolium* using BSA and comparative genetics. Book of abstracts: second symposium Molecular breeding of Forage Crops 2000, 19-24 nov. 2000, Victoria, Australia, p. 104

– MUYLLE, H.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (2001). Linkage map construction in the outcrossing species *Lolium perenne* and *L. multiflorum*, and analysis of the syntenic relationships with other grass species. 15 th Forum for Applied Biotechnology. Med. Fac. Landb. en Toegepaste Biol. Wet. Ugent, 66/3b, 401-408.

– MUYLLE, H.; VAN BOCKSTAELE, E.; ROLDAN-RUIZ, I.; (2002). Selection of AFLP Markers Linked with crown Rust Resistance in *Lolium*: Efficiency of Bulk Segregant Analysis in an *Allogamous* Species. Proc. 6th Conf. EFPP 2002, Prague (PL). Plant. Protect. Sci, 38 (special issue 1): in press

- MUYLLE, H.; VAN BOCKSTAELE, E.; ROLDAN-RUIZ, I.; (2002). Genetic dissection of crown rust resistance in a *Lolium perenne* mapping population. Proceedings Eucarpia meeting 2002 (In Press).

Interne eindverslagen contractonderzoek :

- Development of techniques for elimination of virus diseases from *Musa*: Interim Progress report, March 1997, INIBAP-IPGRI, 18 pp.

- Development of techniques for elimination of virus diseases from *Musa*: First annual report, September 1997, INIBAP-IPGRI, 30 pp.

- Development of techniques for elimination of virus diseases from *Musa*: Interim Progress report, March 1998, INIBAP-IPGRI, 20 pp.

- The European Gramineae Mapping project (EGRAM). Second year report for the period 1.10.1998 to31.8.1999.

- The European Gramineae Mapping project (EGRAM). Third year report for the period 1.9.1999 to31.8.2000.

- The European Gramineae Mapping project (EGRAM). Final report, April 2001.

6. Wetenschappelijke activiteiten

Bijwonen van internationale congressen:

Met voordracht :

- 13 th Forum Applied Biotechnology, Gent,22-23 September 1999 : MUYLLE, H.; ADAMS, E.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (2000)   Mapping of crown rust resistance genes in *Lolium* using BSA and comparative genetics with other monocots. 13 th Forum for Applied Biotechnology. Med. Fac. Landb. en Toegepaste Biol. Wet. Ugent, 64/5b, 381-387.

- 15 th Forum Applied Biotechnology, Gent,24-25 September 2001 : MUYLLE, H.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (2001). Linkage map construction in the outcrossing species *Lolium perenne* and *L. multiflorum*, and analysis of the syntenic relationships with other grass species. 15 th Forum for Applied Biotechnology. Med. Fac. Landb. en Toegepaste Biol. Wet. Ugent, 66/3b, 401-408.

- MUYLLE, H.; VAN BOCKSTAELE, E.; ROLDAN-RUIZ, I.; (2002).
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  Efficiency of Bulk Segregant Analysis in an *Allogamous* Species. Proc. 6th
  Conf. EFPP 2002, Prague (PL). Plant. Protect. Sci, 38 (special issue 1): in
  press.

- MUYLLE, H.; VAN BOCKSTAELE, E.; ROLDAN-RUIZ, I.; (2002).
  Genetic dissection of crown rust resistance in a *Lolium perenne* mapping
  population. Proceedings Eucarpia meeting 2002 (In Press).

Met poster :

- Symposium Durable Disease Resistance key to sustainable agriculture, 28
  nov.–1 dec. 2000, Wageningen, The Netherlands: MUYLLE, H.; DE
  LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-
  RUIZ, I. (2000). Characterization of resistance gene analogues in *Lolium
  perenne.* Book of abstracts: Symposium Durable Disease Resistance key to
  sustainable agriculture, 28/11–1/12/2000, Wageningen, The Netherlands, p
  85

- 52nd International Symposium on Crop Protection, 9 mei, 2000, Gent,
  België : Identification of resistance genes responsible for crown rust
  resistance in *Lolium* using BSA. 52nd nternational Symposium on Crop
  Protection, 9 mei 2000, Gent, Book of Abstracts, p. 169.

- 14 th Forum for Applied Biotechnology, Brugge, 27-28 september 2000:
  MUYLLE, H.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE,
  E.; ROLDÁN-RUIZ, I. (2000). Identifying resistance gene analogues in
  Lolium spp. 14 th Forum for Applied Biotechnology. Med. Fac. Landb. en
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Begeleiden van scripties :

- De Boelpaep Ann; 1999-2000; Opsporen van AFLP merkers gekoppeld aan
  kroonroestresistentie in *Lolium x hybridum* d.m.v. BSA. Eindwerk CTL,
  Gent, 101 p.

- Gabriels Sofie; 1999-2000; Opsporen en karakterisering van
  resistentiegenanalogen in *Lolium* spp. via de PCR techniek. Eindwerk
  Katholieke Hogeschool Sint Lieven, Gent, 60 p.

- Smet Francis; 2000-2001; Maken van een genetische koppelingskaart in *L.
  perenne* op basis van co-dominante merkers. Eindwerk CTL, Gent, 114 p.

- De Saeger Marijke; 2002-2003; Karakteriseren van QTLs voor kroonroestresistentie in Engels raaigras en hybride raaigras. Eindwerk CTL, Gent.

Vergaderingen met actieve bijdrage bijgewoond in het kader van projecten:

- Meeting 'Banana Streak Virus', Voorstelling van behaalde resultaten in het project 'Development of techniques for the eradication of virus diseases in *Musa* spp.', 19-21 januari 1998, INIBAP, Montpellier, France.

- Joint meeting EGRAM-EUDICOT program, Montpellier, 25-26 February 1999. MUYLLE, H.; ADAMS;E.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (1999). Mapping of crown rust resistance genes in *Lolium* using BSA.

- Meeting European Gramineae Mapping project, Asker , Norway, 12-14 Mei, 2000.MUYLLE, H.; ADAMS;E.; DE LOOSE, M.; PEERBOLTE, R.; VAN BOCKSTAELE, E.; ROLDÁN-RUIZ, I. (2000) Mapping of crown rust resistance genes in *Lolium* using BSA.

- Meeting European project NIMgrass, DvP-CLO, Gent, 22-23 januari 2001.

- Meeting European project NIMgrass, Banbury, UK, 25-26 juni 2002.

- Meeting European project NIMgrass, DvP-CLO, Gent, 29 november 2002.

- Wetenschappelijk comité, O&O project : Ontwikkeling en toetsing van rekenalgoritmes voor merker ondersteunde plantenveredeling voor een duurzame of biologische landbouw, 24 juni 2002, DvP, Melle.

- Gebruikerscomité, O&O project : Ontwikkeling en toetsing van rekenalgoritmes voor merker ondersteunde plantenveredeling voor een duurzame of biologische landbouw, 6 december 2002, DvP, Melle.

- Meeting European project GRASP, PRI, Wageningen, Nederland, 24-25 maart 2003.

7. Externe Dienstverlening

- Organisatie werkwinkel van DvP op de KVLV studiedag: Kennismakingsdag biotechnologie beter begrijpen. Leuven, Aula de Somer, 18 november 2000.

8. Andere activiteiten

Deelname aan internationale congressen met passieve deelname :

- 51st International Symposium on Crop Protection, 4 mei, 1999, Gent, België

- 53rd International Symposium on Crop Protection, 8 mei, 2001, Gent, België

- Eucarpia : 20th international symposium section ornamentals: strategies for new ornamentals. EUCARPIA, 3-6 juli 2001, Melle, België.

Deelname aan studiedagen en opleidingen:

- Workshop: In situ amplificatie methoden: IS-PCR en PCR in situ hybridisatie, 20-22 januari 1997: Leiden (Nl), Leidse hogeschool.

- 48ste PUO-dag : GMO's in de voeding. FLTBW, 1 december 1999, Gent, België.

- Studie- en vervolmakingsdag : Impact van de veredeling op de plantenteelt : Recente evolutie en verwachtingen. Technologisch Instituut, Genootschap Plantenproductie en Ecosfeer, Werkgroep Plantenteelt, 18 mei 2000, CLO Melle, België

- Seminar day, Applied bioinformatics. Technologisch instituut, sectie biotechnologie, 21 november 2000, Antwerpen, België.

- Grondige en toegepaste plantenveredeling, Prof. Dr. Ir. E. Van Bockstaele, RUG, academiejaar 2000-2001.

- Praktijkgerichte statistiek, module 3, L. Boullart, RUG, academiejaar 2000-2001.

- ICES – course in Bio-informatics: Instituut voor Permanente Vorming, RUG, academiejaar 2001-2002

- Colloquium : Grasland in Vlaanderen, CLO-DFE, Gent, 18 september 2002.

- Flow cytometrie, KAHO St. Lieven hogeschool, 13 februari 2003.