Automation of Molecular Markers in Practical Breeding of Spring Barley (*Hordeum vulgare* L.)

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Abstract

Plant breeders constantly need to adapt their research to the ever-changing market needs and agricultural practices. To achieve these goals, they need to competently combine different genetically-governed characters in a genotype. This is a complex, time-consuming and labour intensive task. It is undeniable that the advent of molecular markers and their application inhold tremendous possibilities to increase plant breeding efficiency. However, the requirements specific to practical plant breeding represent also a limitation to their full application. To be attractive it is necessary that molecular technology is able to promptly handle sufficiently large amounts of material at reduced costs. Recent developments in other molecular genetic areas, providing solutions for improved assay-throughputs, are today available to crop development programmes. However, because of the still important investments involved in investigating whole genomes, this trend has been slow and has primarily benefited major crops.

   This thesis is part of the Øresund Food Network collaboration "Efficient use of DNA markers for improved development of healthy plants" and its general aim is to investigate the automation of molecular markers in practical plant breeding programmes. For this purpose, the different uses of molecular markers are presented and their availability discussed. The specific needs of molecular applications in practical plant breeding are investigated and the specific approach of a plant breeding company to automate them, in order to increase their availability to breeding programmes, is detailed. The uses of the developed fully-automated system are exemplified using specific marker-resistance gene associations for important diseases in spring barley (*Hordeum vulgare* L. ssp. v*ulgare*).

Keywords: automation, molecular markers, practical plant breeding, barley, disease-resistance, powdery mildew, leaf rust, nematode.

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A ma famille

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Appendix

Paper I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Dayteg, C., S. Tuvesson, A. Merker, A. Jahoor & A. Kolodinska-Brantestam. 2007. Automation of DNA marker analysis for molecular breeding in crops: practical experience of a plant breeding company. *Plant Breeding 126*, 410-415

II. Dayteg, C., M. Rasmussen, S. Tuvesson, A. Merker & A. Jahoor. 2008. Development of an ISSR-derived PCR marker linked to nematode-resistance (*Ha2*) in spring barley. *Plant Breeding 127, 24-27*

III. Dayteg Christophe, S. Tuvesson, M. Rasmussen, A. Merker & A. Jahoor. 2007. Localization of QTLs for barley leaf rust (*Puccinia hordei* Otth) resistance in a cross between a barley cultivar and a wild barley (*Hordeum vulgare* ssp. *spontaneum*) line. (Submitted)

IV. Dayteg Christophe, S. Tuvesson, M. Rasmussen, A. Merker & A. Jahoor. 2007. Characterization and chromosomal location of a putative exotic barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) resistance in two crosses between a barley cultivar and wild barley (*Hordeum vulgare* ssp. *spontaneum*) derived line. (Manuscript)

Introduction

Over the last 50 years, enormous progress in crop productivity has been achieved largely through the genetic improvement of agriculturally important plants (Fig. 1). These achievements have been mainly realized by conventional breeding targeted toward the selection of observable phenotype, representing the collective effect of all genes and the environment. This is a time consuming effort that is largely dependant on the performance of the selected candidates under certain environmental conditions. It is limited by the necessity that the phenotype has to be observable before the time when selection decisions have to be made or by its effectiveness in resolving negative association between genes. Hence, plant breeders' great interest in technologies that could make this procedure more efficient (Dekkers & Hospital, 2002; Korzun, 2003).
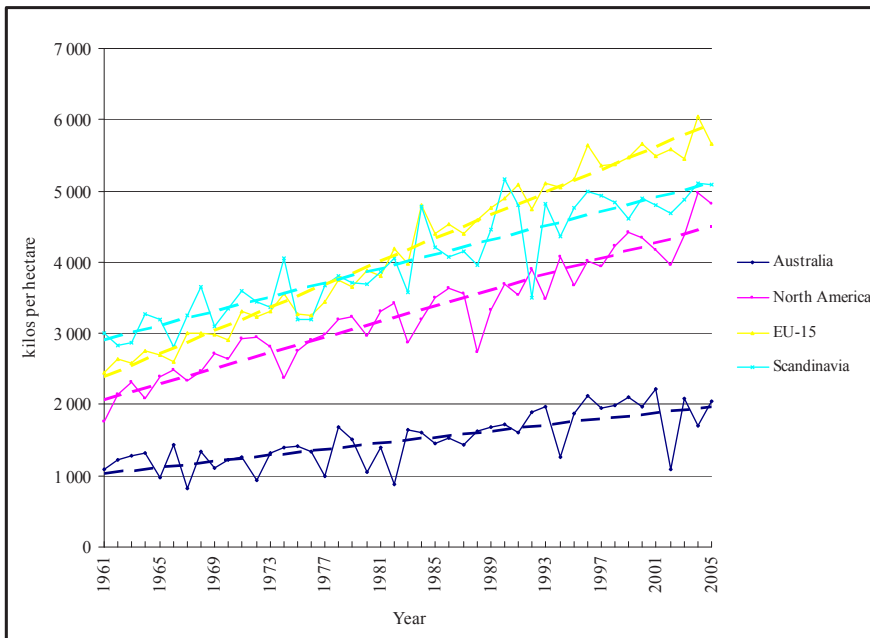
Fig.1. Cereal average yield from 1961 to 2005 in different part of the world. Although the average yield has fluctuated from year to year, primarily due to local weather conditions, there has been a consistently increasing trend as shown by the regression lines (dashed). More than half of this increase is an answer to genetic improvement (Duvick, 1984). Data from FAOSTAT | © FAO Statistics Division 2007.

The exploitation of factors co-segregating with a trait in a simple Mendelian fashion in order to understand its inheritance is an old notion, but these simply inherited morpho-physiological variants are very rare[1] (Bergal & Friedberg,

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[1] In 1875 von Proskowetz used ear selection as a predictor of malting quality. In the 1920's simple colour traits were used to predict seed weight in common bean, and fruit size in tomato. Others were used for varietal discrimination (DUS).

1940). They remained of restricted use for practical breeding purposes until the development of biochemical markers in the 1960's (Koebner, 2003). However, it was not until the introduction of DNA marker technology in the 1980's, that a large enough number of environmentally insensitive genetic markers could be generated. Under the past decades, the molecular marker technology has rapidly evolved into a valuable tool able to dramatically enhance the efficiency of conventional plant breeding (Peleman & van der Voort, 2003).

Application of molecular markers in plant breeding

Molecular tools

Restriction fragment length polymorphisms (RFLPs) were the first DNA markers to be successfully used in plants (Helentjaris *et al.*, 1985). However, as these markers are time-consuming, labour-intensive and require large amounts of DNA, their use was gradually supplanted by more user-friendly techniques (Gupta *et al.*, 1999). Indeed, the development of the polymerase chain reaction (PCR, Saiki *et al.*, 1988) has made DNA marker-techniques quicker and cheaper. PCR is a technically simple and quick method, requiring only small amounts of more or less crudely extracted DNA. Several PCR-based markers such as random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs or microsatellites), inter-SSRs (ISSRs) have been developed and applied to a range of crop species including cereals. The relative pros and cons of these techniques are summarized in Table 1.

Table 1. *Comparison of the most common used marker systems in crops. Adapted from Korzun (2003)*

Feature	RFLPs	RAPDs	AFLPs	ISSRs	SSRs	SNPs
PCR-based	no	yes	yes	yes	yes	yes
Ease of use	low	high	high	high	high	high
Number of polymorphic loci assayed	1-3	1-50	10-100	5-30	1-3	1
Reproducibility	high	low	high	high	high	high
Amenable to automation	low	mod.	mod.	mod.	high	high
Amount DNA required	high	low	mod.	low	low	low
DNA quality	high	high	mod.	low	low	mod.
Cost per analysis	high	low	mod.	low	low	low

mod. : moderate.

Application

Marker technology enables DNA markers to be linked to traits of interest and to direct the selection towards these markers instead of the phenotypic reaction of superior plants (Edwards & Mogg, 2001). Hence, the selection of desirable

genotypes can be done directly at the DNA-level in a non-destructive manner with no interference of the environment and regardless of the plant developmental stages, thus allowing a greater efficiency of field trials (Peleman & van der Voort, 2003). The use of molecular information can enhance breeding strategies based on phenotype-selection, which is broadly referred to as marker-assisted selection (MAS, Dekkers & Hospital, 2002). In practice, markers rather than known genes, are likely to be used (Villanueva *et al.*, 2002). This is especially useful on its own or in combination with phenotypic testing when selecting for:

-traits with small phenotypic effects i.e., when the phenotype is a poor predictor of the breeding value (low heritability).

-traits difficult or expensive to assess (e.g. nematode resistance, Barley Yellow Dwarf Virus-resistance).

-plants heterozygous for recessive traits (e.g. powdery mildew *ml-o* resistance in barley requires one more generation).

-traits expressed in a late development stage or where the individual needs to be sacrificed to score its phenotype (e.g. male sterility in *Brassica napus*, final attenuation in malting barley).

-alleles not expressed in the selection environment.

-combining traits that might mask each other's effects (e.g. pyramiding resistance genes).

Modern plant breeding is not only based on genotype-building but also on manipulating variation within gene-pools of a cross, DNA-fingerprinting of breeding lines using molecular markers as well as detailed genome analysis of plants provide in this aspect a very powerful and efficient tool to characterize, monitor and protect germplasms (Lombard *et al.*, 2000). Multilocus marker-types are usually preferred for their discrimination potential, as they reveal polymorphism information at several loci simultaneously. However, any set of representative DNA-markers is capable to cover the whole genome (Gupta*, et al.*, 1999). The use of molecular markers for genetic studies has been very diverse, the main applications include:

-identification and fingerprinting of genotypes.

-assessment of genetic variability and/or line purity (e.g. conservation or expansion of the gene-pool, pure line or inbreeds-check).

-estimation of genetic relatedness between breeding material and/or populations (e.g. estimate of heterosis, allele frequency).

-foreground (genotyping at target loci) and background (genotyping at loci across the genome) selection for marker assisted backcrosses (MAB) (e.g. introgression of novel traits from unadapted germplasm into elite breeding lines).

-increase of the genetic variability of improved lines (single large-scale marker-assisted selection (SLS-MAS), Ribaut & Bertrán, 1999).

-characterisation and rare allele selection of exotic germplasm.

-linkage analysis.

Simultaneously as biotechnology produces efficient tools to assist plant breeders in their enterprise, it also provides them with new possibilities of gene transfer. To breed and/or distinguish genetically modified (GM) individuals, may not differ

much from other traits however, molecular markers is the only technique available capable of differentiating GM transformation-events.

Approaches

There are to date two main approaches to the use of markers in commercial plant breeding:

MAS, shifting the traditional phenotype-based selection to genotype-based selection (Fig. 2), is routinely used in plant breeding programs mainly for selecting alleles with large effects on traits with relatively simple inheritance (Holland, 2004). The technology is indispensable for GM-quality control of commercial cultivars and empowers breeding programmes. Added value can be created through the introduction of new traits that would have been difficult or required additional steps by classical breeding e.g.: difficulties in phenotypic scoring, selection of rare recombinants or necessity of test crossing (Dayteg *et al.*, 2008; Tuvesson *et al.*, 1998). As several markers can be used for selection, new possibilities to incorporate different genes into the same line are given to the plant breeders, thus attempting to slow down the evolution of pathogen virulence (Hospital, 2003; Werner *et al.*, 2005). Knapp (1998) showed, in his models, that a breeder using phenotypic selection must test 1.0 to 16.7 times more progeny than a breeder using MAS to be assured of selecting one or more superior genotypes. However, the advantages of MAS over phenotypic selection are considerably reduced when conducted in later generation (Liu & Knapp, 1990). Consequently MAS though providing more accurate responses also dramatically increases the frequencies of superior genotypes in early generations.
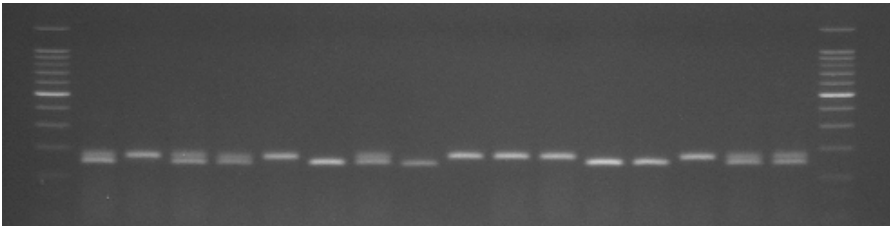

Fig. 2. Example of MAS for BaYMV resistance. Band profile of a segregating barley population using a BaYMV-linked marker, amplification obtained on 1.4% (w/v) agarose and 100-bp ladder. As illustrated the selection of resistant and susceptible genotypes can be directly done at the DNA-level by using linked codominant markers, heterozygous genotypes can also be detected. (Courtesy of Stine Tuvesson).

Fingerprinting enables the characterization of genotypes and the estimation of genetic relatedness between lines (Fig. 3). This information is crucial to allow plant breeders to appropriately choose the parental lines for their crosses especially for hybrid production (Ma *et al.*, 2003), but also for an effective exploitation of the germplasm by monitoring the diversity of their gene-pools. Because of the rapid evolution and occurrence of new and virulent races of pathogens, a broad genetic diversity is paramount in resistance breeding. Unfortunately, most elite cultivars of the small-grain cereals are bred on a quite narrow genetic base, and the limited genetic diversity may impede the deployment

of new sources of resistance for a pathogen or the discovery of new positive alleles for a character. The introduction of novel characteristics from unadapted wild or exotic germplasms into elite breeding lines has been shown to counteract this limitation (Ivandic *et al.*, 1998; Ordon *et al.*, 1996).
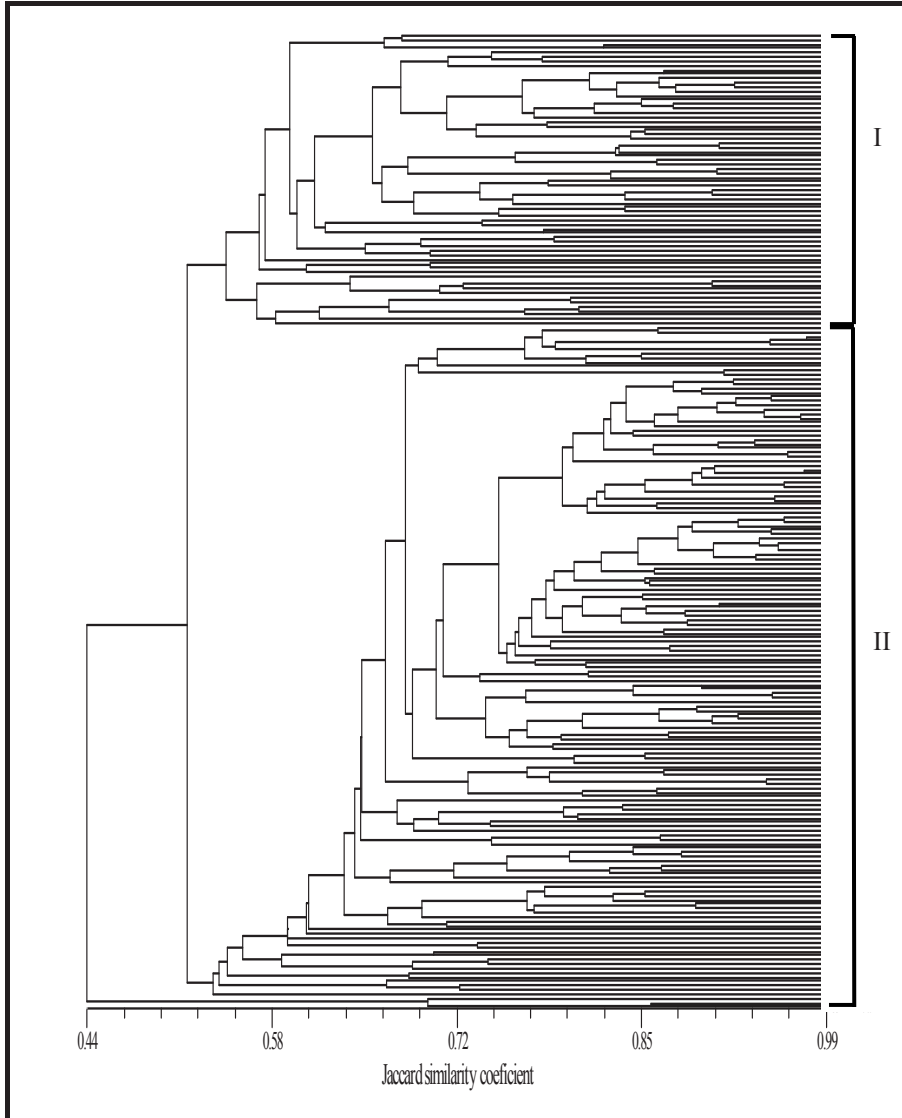
Fig. 3. Example of an UPGMA dendrogram illustrating the genetic relationship between 227 Nordic and Baltic barley accessions. Two main clusters can be distinguished: cluster I includes mainly six-rowed barley while cluster II includes mainly two-rowed barley. Source: Kolodinska-Brantestam *et al.* (2004). Molecular markers enable genotype-discrimination and the estimation of genetic relatedness between lines for an effective exploitation of the germplasm (see text).

H. vulgare ssp. *spontaneum*, a wild relative and a progenitor of barley, is a rich source of useful resistance genes to leaf rust, powdery mildew, barley yellow

dwarf virus, scald, net blotch, *septoria*, etc. (Fetch *et al.*, 2003; Jahoor & Fischbeck, 1987). Breeders, however, are usually reluctant about using wild germplasms in their breeding programs because of complex, long-term and unpredictable outcomes, particularly in crops where quality traits are important criteria (Peleman & van der Voort, 2003). Marker assisted backcrossing (MAB) is an effective aid to selection in backcrossing: first as the target trait can be directly monitored, hence avoiding phenotypic scoring. Then, as markers closely linked to the target gene can limit the surrounding DNA from the donor parent, thus removing possible linkage drag. Finally, as markers dispersed over the genome permit the selection of progeny with higher proportions of the recurrent parent genetic background (Holland, 2004). Any kind of DNA markers can be used, however codominant markers are considered to be the most useful as they allow the selection of heterozygous individuals, as Chen and colleagues (2000) have shown using 128 RFLP loci to MAB of the *Xa21* gene in rice. Melchinger (1990) reviewed the advances of MAB. He compared conventional schemes described by Allard in 1960 to MAB models described by Tanksley and Rick in 1980. They demonstrated that the proportion of the recurrent parent in the first generation of MAB could correspond to that expected after three generations of conventional backcrosses. These results were verified by Frisch and colleagues (1998) who estimated to two the number of generation needed to obtain a genotype with 98% or 99% genetic similarity to the recurrent parent. Considering that Allard estimated the adequate number of generations to six, MAB represents a considerable gain of time. However, they also stressed that the number of markers and material to be screened would be very large.

Gene Mapping - Marker "discovery"

The fact that DNA markers enable indirect selection to be carried out represents by far the most appealing aspect to enhance conventional breeding. The discovery of such marker-traits associations can broadly be classified into the three following groups:

"Text mining":

A considerable amount of molecular markers linked to economically important traits can now be readily found in plant science literature (Cahill & Schmidt, 2004). Example of marker-trait associations for monogenic traits e.g.: fungal resistance (Backes *et al.*, 2003; Graner *et al.*, 2000; Jahoor & Fischbeck, 1993; Kicherer *et al.*, 2000; Shtaya *et al.*, 2006) or virus resistance (Ordon*, et al.*, 1996; Tuvesson*, et al.*, 1998; Werner*, et al.*, 2005) but also for more complex characters e.g.: agronomic and quality traits (Cahill & Schmidt, 2004) are available and this is likely to increase in the coming years. Because of the abundance of the information available, new tools are being developed for an efficient exploitation of the literature, i.e.: www.ojose.com : Online JOurnals Search Engine or privately developed (Maarten Stuiver, personal communication).

Linkage analysis:

The "traditional" establishment of linked markers involves the evidence of empirical association of marker genotypes with trait phenotype in order to identify genetic factors which contribute to resistance and other qualitative traits of interest. Usually, the identification of regions of the chromosome affecting the phenotype is done first, then focus on the polymorphism in a candidate gene can identify particular alleles as having a causative role (Dekkers & Hospital, 2002). This approach to gene mapping, also referred as *linkage analysis*, uses families with a known pedigree structure. Individuals are genotyped at random markers spread across the genome. If a disease-resistance gene is close to one of the markers then, within the pedigree, the inheritance pattern at the marker will mimic the inheritance pattern of the resistance itself. Linkage analysis has been highly successful at finding genes for simple genetic resistances, as demonstrated in most of the publications mentioned above, in which a single major gene is responsible for the disease resistance in a given pedigree, and environmental factors are not very important. This approach in essence requires a good phenotyping as well as access to sufficient DNA markers. Several tools have been developed for the recognition of specific molecular patterns in the sequence files databases available in the public domain, e.g.: HarvEST for ESTs, PlantMarkers (Rudd *et al.*, 2005) for SNPs and SSR, Sputnik or MIcroSAtellite (Varshney *et al.*, 2005) for SSRs, making the DNA marker availability today nearly "unlimited" (Koebner, 2003). This undeniably will enable the establishment of well saturated molecular maps in many crops and should facilitate the genotyping part of "conventional mapping" putting more emphasis on the phenotyping and the comprehension of the processes' underlying genetics.

The increasing insight provided by the genomics era will also present wider possibilities to compare gene structure and function in divergent organisms. *Comparative mapping* allows the transfer of information among orthologous genes or homologous chromosomes. This is not only useful for gene cloning and characterization but also for marker discovery (Sorrells & William, 1997).

Association mapping:

Another approach to gene mapping uses associations at the population level and is referred as *association*, or *linkage disequilibrium (LD) mapping*. The idea is that a resistance mutation arises on a particular haplotype background, and so individuals which inherit the mutation will also inherit the same alleles at nearby marker loci. It involves identifying markers with significant allele-frequency difference between individuals sharing a phenotype in a population of "unrelated" individuals, rather than looking for phenotype given marker-haplotypes in a population with known relationship (Aranzana *et al.*, 2005). In a sense, association mapping is not fundamentally different from linkage analysis, but instead of using a family pedigree, unknown population genealogy is used. Because the population genealogy assumes many generations, recombination will have removed association between a QTL (quantitative trait loci) and any marker not tightly linked to it, and allows much finer-scale mapping than does linkage analysis

(Jannink & Walsh, 2002). The use of multiple validation populations is also considered better, as genetic distances may vary among single population. Thus, providing a powerful tool for future analysis of disease resistance genotype x pathotype x environment interactions (Williams, 2003). However, this approach is currently limited by an elevated rate of false positives correlated to population structures (Aranzana, *et al.*, 2005; Pritchard *et al.*, 2000) or the loss of power when multiple alleles affect the trait studied (genetic heterogeneity, Jannink & Walsh, 2002). Nonetheless, it has also been advocated (Risch & Merikangis, 1996) that in conjunction with new technology for rapid genotyping (i.e. single-nucleotide polymorphisms, SNPs), this method will ultimately be more powerful than linkage analysis for identifying loci involved in the inheritance of complex traits and for isolating genes of small effect (Pritchard, *et al.*, 2000). Furthermore, it presents the double advantage to sample more alleles than bi-parental crosses, thus providing a more representative sample of the existing variation (Williams, 2003) and to re-use existing databases to speed-up and reduce genotyping costs (Pritchard, *et al.*, 2000).

Need for automation

Considering the ever-changing requirements and needs of consumers and agricultural practices, plant breeding is likely to remain a never-ending quest requiring all tools in hands to promptly release its products in a highly competitive market. 1) Quality of the product 2) low production cost 3) prompt release are key factors in which automated molecular markers can play a major role.

As previously described, breeding resources can be efficiently exploited using molecular markers first, by reducing the number of inadequate lines requiring extensive phenotypic evaluation in later generations (Holland, 2004). Then, by optimising the use of the gene-pools and finally, by speeding up the introgression process of new characters. In a practical breeding perspective, however, this requires an adaptation of the methodology to allow plant material to be monitored in realistic high number of individuals in early generations (Dayteg *et al.*, 2007). The high number of individuals and the economic constrains involved in a breeding program compel molecular markers to be technically easy to use, cheap and informative (Hernandez, 2004). While most PCR-based markers fulfil these requirements (Table 1), automating PCR-procedures faces a few problems. First, amongst the PCR-based markers, there is not today a single established or universal marker technology and each type of marker might require its own procedure. Then, marker technology as a whole is in a growing phase and evolves rapidly. Technologies as well as the availability of the appropriated markers may constitute a shortage in the practical approach to marker applications. Finally, DNA-marker being a broad concept, each of its specific application might require its own marker technology or technical challenges (Dayteg, *et al.*, 2007).

The evolution of robotics in biotechnology and the progress of bioinformatics have been significant for the development of high throughput system (Cahill & Schmidt, 2004). However, the spin-off effects of the pharmaceutical industry

remain limited in practical breeding due to their important investment costs. Because of their economical value major crops have essentially been in focus for such investments. Barley breeding in that perceptive has benefited from its agronomical importance and of its role as a model crop in genomic studies for cereals grown in temperate climate.

Crop and pathogens

Barley

Barley (*Hordeum vulgare* L. ssp. *vulgare*) is the world's fourth most important cereal crop after wheat, rice, and maize. It is globally grown on about 70 million ha (Fig. 4) and global production is about 160 million tones annually (FAO, 2006). Barley is certainly a staple grain for many animal feeds or in many countries for human food, although its importance for malt beverages is a cultural factor that contributes to its significance in certain areas. Clear evidence of early domestication and cultivation dates back to approximately 10 000 years ago in the area of the Fertile Crescent (Zohary & Hopf, 1988). It is grown over a broader environmental range than any other cereal, and much of the world's barley is produced in regions with climates unfavourable for production of other major cereals. In Tibet, Nepal, Ethiopia, Eritrea and the Andes, it is cultivated on the
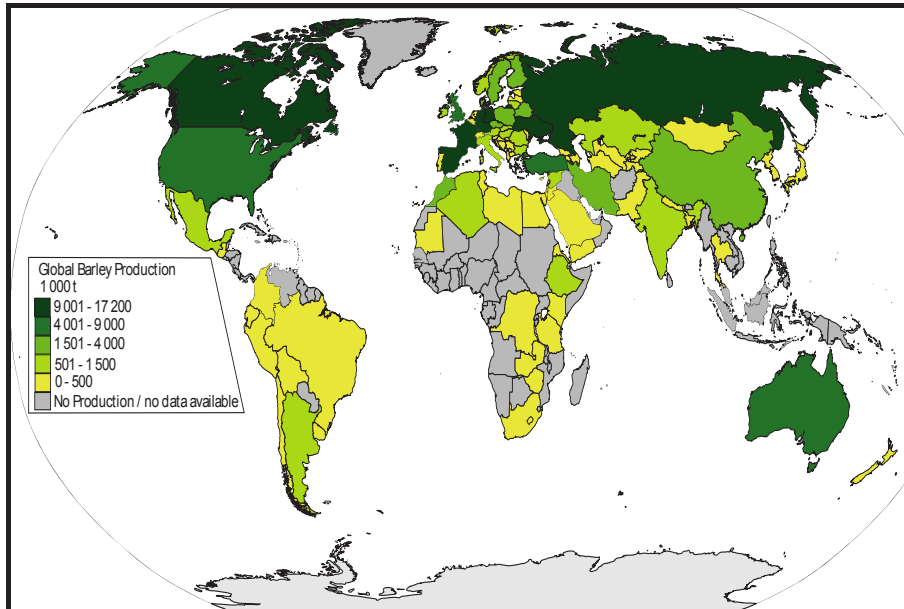

Fig. 4. Average barley production in the world in 2006. Barley is the world's fourth most important cereal crop. It is globally grown on about 70 million ha and global production is about 160 million tones annually. It is grown over a broader environmental range than any other cereal (see text). Data from FAOSTAT | © FAO Statistics Division 2007.

mountain slopes at elevations higher than other cereals. In many areas of North Africa, the Near East, Afghanistan, Pakistan, Eritrea and Yemen, it is often the only possible rain-fed crop. It is the only cereal grown at latitudes above 65°. In most developing countries barley is a typical crop of poor farmers and of hostile, dry and cool environments. Therefore, neither the area nor the production reflect the actual importance of the crop (FAO, 1999).

This wide distribution is the result of an original very wide genetic variation within the species, with specific varieties adapted to specific environments. This is well demonstrated by the extended number of accessions of barley varieties, landraces, breeding material and to some extent, genetic stocks available in genebanks around the world. The European Barley Database for instance registers more than 150 000 accessions for collections held in European genebanks as well as three outside (ICARDA, Australia and Japan). The USDA National small grain collection at Aberdeen holds about 25 000 accessions. In addition, the worldwide availability of significant cDNA, Expressed Sequence Tags (EST)[2] and large insert libraries, i.e.: yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC), greatly promote genomic studies and gene cloning efforts (Scherrer *et al.*, 2005; Yu *et al.*, 2000).

Besides the genetic and genomic resources available, barley presents some significant advantages to work with as a model genome for small grain *Triticeae* crops. It is self-pollinated, true diploid (2n=2x=14) and closely related to the outbreeding diploid rye, the cultivated diploid, tetraploid and hexaploid wheats, and related to the diploid, tetraploid and hexaploid oats and rice (Hori *et al.*, 2003). Its genome size is approximately 5 000 million base pairs (Mbp) and contains an estimated 21 000 genes, thus an average distance of 240 kb between the barley genes (Dubcovsky *et al.*, 2001). However, Dubcovsky *et al.* (2001) confirmed that the average gene density in some genome regions was 12 times higher than the expected genome average, thus a gene every 20 kb, regions also termed 'gene islands'. Though the size of the barley genome might present a challenge to isolate genes at the molecular level, the considerable homology between small grain genomes enables to apply knowledge derived from gene discovery in barley to other small grain with less manageable genome sizes (Fig. 5) (Wise, 2000).

Resistance breeding

The primary means, and the most economically viable and environmentally acceptable method of disease control in sustainable cropping systems is through the incorporation of genetic resistance into commercial varieties (Backes*, et al.*, 2003; Shtaya*, et al.*, 2006; Williams *et al.*, 2002). Therefore, access to a diversity of exotic sources and the progress of genomics lead to better mapping and cloning possibilities of these resistance genes. A better understanding of the genetics

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[2] In June 2007, more than 437 700 entries were registered in the EST database of the National Center for Biotechnology Information.

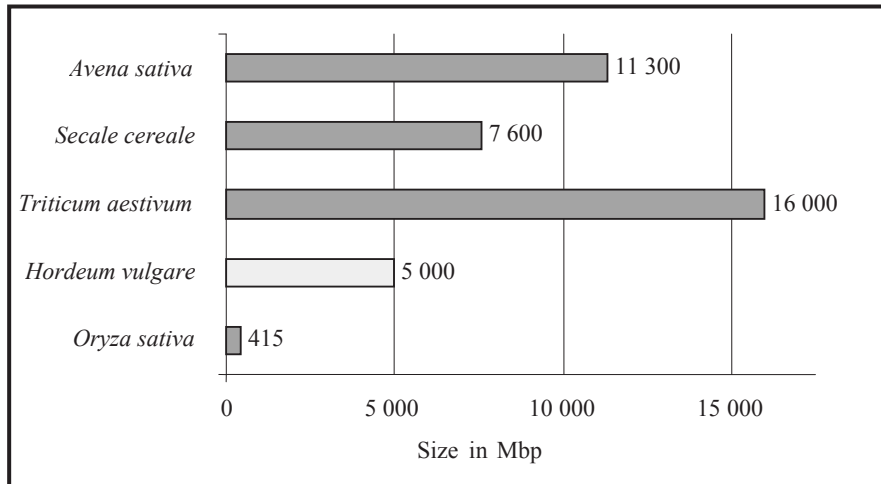governing these interactions thus provides a comprehensive toolbox to barley disease resistance breeding.

Fig. 5. Average genome size (in million base pairs) of small grain cereals. Early genomic research was initiated on small genome species such as rice. Barley as a true diploid, self fertile plant and a relatively manageable genome is advantageously serving as a model crop for small grain cereals; knowledge acquired from these model species has facilitated genomic efforts in cereal crops (see text).

Selection is commonly made on visual assessment of naturally occurring disease symptoms, plants are either qualitatively classified as resistant or susceptible, or the continuous variation in their response is quantitatively assessed. Both qualitative and quantitative data may be used to map resistance loci relative to molecular markers in plant genomes (Spaner *et al.*, 1998). In early studies, most major resistance genes were identified by using RFLP makers, which explains their strong occurrence in the overview presented in Table 2. They were later converted into PCR-based markers, with potential use in MAS and can be found in the GrainGenes database at http://wheat.pw.usda.gov.

Until recently, major genes for resistance efficiently controlled barley diseases but in the last decades several major resistance genes became ineffective due to the adaptation of the pathogens ("Boom and bust cycles", McDonald & Linde, 2002; Pink, 2002). Mapping the resistance genes in barley has revealed a rather narrow number of loci with major effects against important diseases and pests. This limits the number of genes that can efficiently be combined to produce durable resistance in breeding programmes (Williams, 2003). QTLs have also been found for resistance to all major diseases and may define major gene or race non-specific resistances (Qi *et al.*, 1999; Williams, 2003). A list of resistance QTLs and associated markers is available at http://barleyworld.org/. These non-specific resistances, also referred as partial resistance (PR) have been defined as resistance controlled by several genes (Parlevliet & Van Ommeren, 1975; Qi*, et al.*, 1999). They cause a reduced rate of epidemic development despite a high, susceptible, infection type (Parlevliet & Kuiper, 1977; Shtaya*, et al.*, 2006). Thus, two kinds of disease resistance have been described for barley 1) a single gene, race-specific

qualitative resistance, usually expressed as a hypersensitive host reaction with the formation of chlorotic and necrotic spots and 2) a partial or quantitative resistance (PR), which is polygenic and expressed as a reduced epidemic rate. While the genomic positions of these QTLs are presumably constant, the effects of QTL alleles may vary with the environment (Chelkowsky *et al.*, 2003). They are nonetheless considered today as a more durable source of resistance (Qi *et al.*, 2000; Qi*, et al.*, 1999; Shtaya*, et al.*, 2006). However, the necessity to identify novel major resistance loci and quantitative loci that can be combined with known genes is paramount for a sustainable resistance breeding (Williams, 2003).

At least 30 pathogens have been reported to affect barley (Williams, 2003) limiting its yield and quality, but because of their importance, focus has been made in this thesis on a couple of pathogens which have been subject to extensive studies.

Table 2. *Resistance genes to powdery mildew, leaf rust and nematode mapped or targeted with DNA-markers in barley. (Adapted from Backes et al., 2006; Chelkowsky, et al., 2003; Williams, 2003). This is not an exhaustive list, more marker-trait associations can be found at http://wheat.pw.usda.gov and http://barleyworld.org/. Marker definition: AFLP: amplified fragment length polymorphisms, CAP: cleaved amplified polymorphic sequence, RAPD: random amplified polymorphic DNA, RFLP: restriction fragment length polymorphisms, RGA: resistance gene analog, SCAR: sequence characterized amplified region, SNP: single-nucleotide polymorphisms, SSR: simple sequence repeats or microsatellites, STS: sequence-tagged-site*

Gene	Chromosome	Nearest marker(s)	Marker type	Reference
Powdery mildew resistance genes				
Race specific genes				
Mla	1HS	*Mla1*	Cloned gene	Zhou *et al* 2001
		Mla6	Cloned gene	Halterman *et al.* 2001
		cMWG645	RFLP	Graner *et al.* 1991
		MWG036	RFLP	Schüller *et al.* 1992
		MWG2191	RFLP	Schwarz *et al.* 1999
Mlk	1HS	MWG2083 & ABA004	RFLP	Jensen 2002
Mlnn	1HS	CD99 & ABG053	RFLP	Jensen 2002
MlGa	1HL	ABR377	RFLP	Jensen 2002
MlLa	2H	cMWG660 & MWG97	RFLP, STS	Hilbers *et al.* 1992
Mlhbl.a	2H	MWG682	RFLP	Pickering *et al.* 1998
Mlg	4HS	MWG032	RFLP	Görg *et al.* 1993
mlo	4H	*mlo*	Cloned gene	Büschges *et al.* 1997
		BAL88/2 & bAO11	RFLP	Hinze *et al.* 1991
		Bpm16, Bpm2 & Bxm2	AFLP	Simons *et al.* 1997
Mlj	5HL	MWG592 & MWG999	RFLP	Schönfeld *et al.* 1996
mlt	7HL	MWG035 & MWG999	RFLP	Schönfeld *et al.* 1996
Mlf	7HS	MWG053 & MWG539	RFLP	Schönfeld *et al.* 1996
Partial resistance genes				
qML2	2H	S-236	RGA	Backes *et al.* 2003
Rbgq1	2H	E38M54-390 & Bmag0125	AFLP, SSR	Shtaya *et al.* 2006
Rbgq2	3H	P15M51-342	AFLP	Shtaya *et al.* 2006
qMIL	3H	S-L8	RGA	Backes *et al.* 2003
Rbgq3	5H	E33M55-267	AFLP	Shtaya *et al.* 2006
qMl1	6H	MWG514	RFLP	Backes *et al.* 2003

Leaf rust resistance genes

Race specific genes

Rph4	1HS	Pic 18a & 5.2	RGA	Collins et al. 2001
Rph16	2HS	MWG874 & MWG2133	STS, CAPS	Ivandic et al. 1998
Rph.Hb	2H	MWG682	RFLP	Pickering et al. 1998
Rph6 (allelic to Rph5)	3HS	MWG2021 & BCD907	RFLP	Zhong et al. 2003
Rph5	3HS	ABG70	STS	Mammadov et al. 2005
Rph7.g	3HS	Hv3Lrk	SNP	Brunner et al. 2000
Rph7	3H	cMWG691	RFLP	Graner et al. 2000
RphQ, Rph2	5HS	CDO749 & ITS1	RAPD, STS, RFLP	Borovkova et al. 1997
Rph9	5HL	ABC155 & ABG3	STS	Borovkova et al. 1998
Rph12(=Rph9.z)	5HL	ABC155	STS	Borovkova et al. 1998
Rphx	7H	ABC310a & ABC461	RFLP	Hayes et al. 1996
Rph19	7H	HVM49 & HVM11	SSR	Park & Karakousis 2002

Partial resistance genes

Rphq6	2H	E41M32-83	AFLP	Qi et al. 1999
Rphq11	2H	E37M33-162	AFLP	Qi et al. 2000
Rphq12	2H	E38M54-134	AFLP	Qi et al. 2000
Rphq2	2H	E38M54-294	AFLP	Qi et al. 1999
Rphq10	4H	E38M54-144	AFLP	Qi et al. 1999
Rphq5	4H	E35M61-368	AFLP	Qi et al. 1999
Rphq4	5H	E38M54-247	AFLP	Qi et al. 1999
Rphq7	5H	E33M55-267	AFLP	Qi et al. 1999
Rphq3	6H	E37M33-574	AFLP	Qi et al. 1999
Rphq13	7H	E41M32-406	AFLP	Qi et al. 2000
Rphq1	7H	E38M32-195	AFLP	Qi et al. 1999
Rphq8	7H	E39M61-372	AFLP	Qi et al. 1999
Rphq9	7H	E33M61-173	AFLP	Qi et al. 1999

Nematode resistance genes

Ha2	2HL	AWBMA21 & MWG694	RFLP	Kretschmer et al. 1997
		EBmact0039	SSR	Karakousis et al. 2003
		Bmag0125	SSR	Barr et al. 2003
		Ha2S18	SCAR	Dayteg et al. 2008
Ha4	5HL	XYL	RFLP	Barr et al. 1998

Powdery mildew

The disease is caused by the obligate biotrophic fungus *Blumeria* (syn. *Erysiphe*) *graminis* f.sp. *hordei* Otth., and the main primary source of infection are wind borne *ascospores* that have survived on volunteer plants. The infection symptoms in the crop appear as fluffy white growth on the surface of the leaf, these are colonies of fungal spores known as *conidia* (Fig. 6). The colonies enlarge and come together, producing so many spores that the leaf appears powdery, they are themselves spread as the second infection by wind. Under favourable humid conditions the cycle of germination of spores, infection and subsequent infection can be completed within seven days, causing the characteristically rapid build-up of the disease in barley. The disease is most active early in the growing season and normally declines later in the spring. Infection leads to premature yellowing and

Fig. 6. Powdery mildew infection on barley. (Courtesy of Lise Nistrup Jorgensen).

later death of the entire leaf and severe early disease can induce tiller abortion and yield loss is mainly due to reduction in the number of ears (Jarosz *et al.*, 1989; Walters *et al.*, 1984). Yield may be reduced between 10 and 25 per cent depending on the severity and duration of mildew infection (Jayasena & Loughman, 2005; Young & Loughman, 1995). Powdery mildew is considered, in temperate climatic zones, the most important foliar disease on barley and has therefore been subject to intensive studies contributing to a good comprehension of its biology and epidemiology (Jørgensen, 1994; Wiberg, 1974; Williams, 2003). Jørgensen (1994) has classified the known types of powdery mildew resistance into 1) race-specific resistance (gene-for-gene system), 2) *mlo* resistance (effective against all known isolates) and 3) partial resistance (thought to be conferred by additively-acting genes with small effects). These types of resistance are not mutually exclusive e.g. *mlo* resistance may be partial or complete, and race-specific genes may have small effects, conferring partial resistance (Jørgensen, 1994).

To date 23 major resistance loci have been described for powdery mildew (Chelkowsky*, et al.*, 2003), Backes *et al.* (2006) reviewed the mapping efforts achieved for powdery mildew genes and reported the numerous major resistance genes that have been found and mapped on barley chromosomes (Table 2). Five major resistance genes *Mlra*, *Mla*, *Mlk*, *Mlnn* and *MlGa* have been identified and localized on chromosome 1H (see Backes*, et al.*, 2006). On chromosome 2H, the *MlLa* locus, originating from *H. laevigatum* (Hilbers*, et al.*, 1992) and *Mlhb*, transferred from *H. bulbosum* (Pickering *et al.*, 1995) have been identified as the only major resistance genes. Two resistance genes *Mlg* and *mlo* have been localized on chromosome 4H. Three resistance genes from wild barley lines

(*H. spontaneum*) were identified *Mlj* on 5H and *mlt* and *Mlf* on 7H (Schönfeld*, et al.*, 1996). Multiple allelism has been found on several loci, namely the *Mla*, *Mlp* and *mlo*. Because of their importance and their complex polymorphism, the *Mla* and the *mlo* loci have been subject to much interest (Büschges*, et al.*, 1997; Jahoor *et al.*, 1993; Jørgensen, 1992; Piffanelli *et al.*, 2004; Schwarz*, et al.*, 1999; Shen, 2004; Wei *et al.*, 1999; Wei *et al.*, 2002). Until now more than 32 alleles have been detected in the *Mla* locus, which is the highest number of different alleles identified among all known barley powdery mildew resistance genes, many of which were introduced from *H. spontaneum* (Kintzios *et al.*, 1995). The *mlo* gene, with 32 alleles described (Molina-Cano *et al.*, 2003), is the most famous, and used, as it gives a leaf-lesion phenotype and broad-spectrum resistance. Two major resistance loci to powdery mildew have been cloned and sequenced: *mlo* and *Mla*, and two genes *Ror1* and *Ror2* required for the full expression of *mlo* resistance have been identified by mutant analysis (Freialdenhoven *et al.*, 1996). Furthermore, two genes *Rar1* and *Rar2* (Required for *Mla*-mediated resistance) which are necessary for the function of multiple, but not all, resistance interactions at the *Mla*-locus have also been identified (Jørgensen, 1996) and *Rar1* has also been cloned (Shirasu *et al.*, 1999).

Several QTLs for mildew resistance have been mapped on all chromosomes (Backes*, et al.*, 2003; Heun, 1992; Shtaya*, et al.*, 2006; Williams, 2003; von Korff *et al.*, 2005; Yun *et al.*, 2005) some do coincide with major genes but others are localized in previously unreported chromosomal regions (Table 2).

Leaf rust

Leaf rust of barley is caused by the obligate biotrophic fungus *Puccinia hordei* f.sp. *hordei* Otth. The pathogen needs living barley host plants to survive and volunteer barley acts as a reservoir between cropping seasons. The rust spores are wind borne and may be introduced into a region on wind currents over long distances. Infection symptoms appear as round, light orange-brown pustules on the leaf (Fig. 7). Heavy infection results in early leaf yellowing with green specks around the pustules (so called "green islands"), which may be the most obvious symptom on older leaves. Old pustules turn dark and produce black spores (Jayasena & Loughman, 2005; Young & Loughman, 1995). Infection increases the plant's respiration and water-usage and decreases photosynthesis. If early infection occurs, yield may be reduced by more than 32 per cent (Griffey *et al.*, 1994). Grain quality may also be affected. Until the 1970's, this disease was considered unimportant in economic terms. Since, changes in cropping practices and the intensification of barley cultivation have resulted in an increase in the importance of leaf rust, with severe outbreaks occurring (Clifford, 1985).

As for powdery mildew, several resistance genes to leaf rust have been identified and localized on the barley genome. Several STS markers have been

Fig. 7. Leaf rust infection on barley. (Courtesy of Lise Nistrup Jorgensen).

developed to identify leaf rust resistance genes in barley accessions (Borovkova*, et al.*, 1998; Borovkova*, et al.*, 1997; Brunner*, et al.*, 2000; Ivandic*, et al.*, 1998; Mammadov*, et al.*, 2005). Twelve major race-specific resistance genes have been identified from barley and four from *H. spontaneum* (*Rph10*, *Rph11*, *Rph15* and *Rph16*) (Feuerstein *et al.*, 1990; Ivandic*, et al.*, 1998; Jin *et al.*, 1996) and designated as *Rph1* to *Rph16*, they have been assigned to barley chromosomes (Franckowiak *et al.*, 1997; Zhong*, et al.*, 2003) (Table 2). At the centromeric region of chromosome 2H the gene *Rph16* was mapped by Ivandic *et al.* (1998). Backes and colleagues (2006) reviewed the mapping efforts achieved for leaf rust resistance genes and reported the tagging on chromosome 3H, of *Rph6*, which is allelic to the previously localized *Rph5* and closely linked to *Rph7* (Brunner*, et al.*, 2000; Zhong*, et al.*, 2003). On the short arm of chromosome 5H both *RphQ* and *Rph2* (reviewed in Backes*, et al.*, 2006), which have been shown to be allelic, were mapped. *Rph9* and *Rph12* were mapped on the long arm of chromosome 5H (Borovkova*, et al.*, 1998). Both *Rphx* and *Rph19* were localized on chromosome 7H (Park & Karakousis, 2002). The remaining five however, have not yet been linked to any DNA markers, the *Rph4* (*Pa4*) gene on chromosome 1H, *Rph1* on 2H, *Rph10* on 3H, *Rph11* on 6H and *Rph3* on the long arm of 7H. Isozyme loci *EST2* and *Acp3*/*Dip2* have been linked to *Rph10* and *Rph11* respectively (Feuerstein*, et al.*, 1990). Recently, several of these major genes have become ineffective, this also includes genes considered to be the most effective and which were the most widely used in breeding programmes: *Rph3* has been overcome in Europe, *Rph12* in Europe and Australia and for *Rph7*, though still effective in Europe, the occurrence of virulence has been reported in Israel and Morocco and more recently in USA (Jin *et al.*, 1993; Steffenson *et al.*, 1993). Today wider,

more durable, resistance sources are sought to counteract the pathogen's adaptation but sources of leaf rust resistance that possess genes which are effective to a broad spectrum of *P. hordei* are rare (Brooks & Griffey, 1998).

Partial resistance to leaf rust in barley occurs very frequently in West-European spring cultivars (Parlevliet *et al.*, 1980) and Ethiopian barley landraces (Alemayehu & Parlevliet, 1996). Qi *et al.* (1999) have reported thirteen QTLs responsible for partial resistance designated as *Rphq1* to *Rphq13* in several barley populations and at several stages of plant development (Table 2).

Nematode

The cereal cyst nematode (CCN), or *Heterodera avenae* Wollenweber, is an obligate biotrophic parasite common in the cereal growing areas of the world. Before becoming adults the nematodes undergo three molts within the roots. Once fertilized the females, full of eggs, die and their bodies become a protective cyst for the eggs (Williamson & Gleason, 2003). The breakdown of the cell walls, to produce feeding sites, causes severe damage in cereal crops (Fig. 8) and important yield losses have been reported, as much as 30 per cent in Australia (Kretschmer, *et al.*, 1997; Taylor *et al.*, 1998; Williamson & Gleason, 2003).
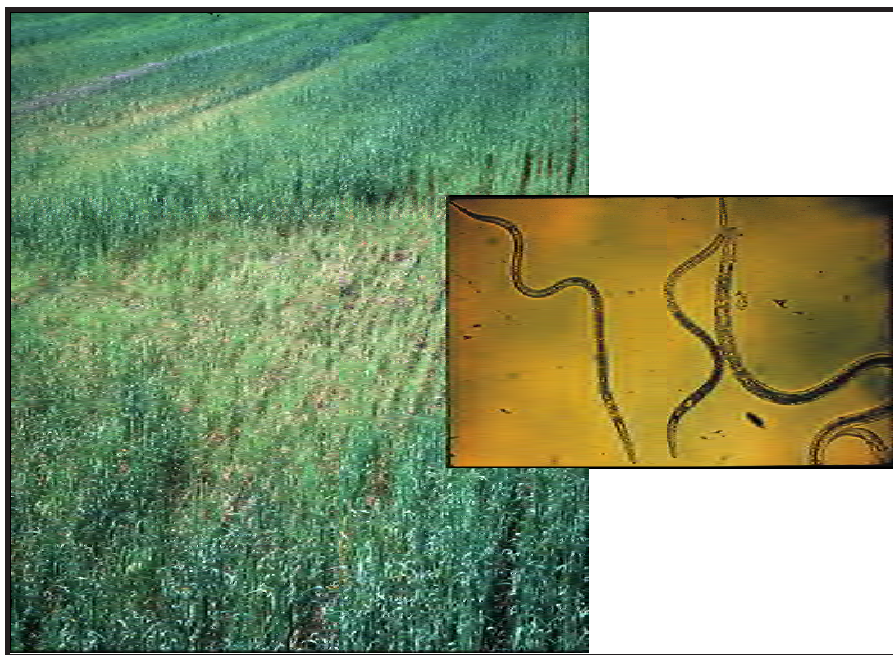

Fig. 8. Nematode infected barley field. (Courtesy of Sanja Mandurik). Microscope picture of cereal cyst nematodes (Source: Elaine R. Ingham).

The cultivation of nematode-resistant barley varieties not only circumvents the use of expensive and toxic nematicides, which with crop rotation and cultural practices are available methods to limit nematodes (Taylor, *et al.*, 1998), but is

also the most efficient soil-sanitation method as it reduces the CCN population (Andersson, 1982).

Studies on the inheritance of resistance to *H. avenae* in barley have revealed four major resistance genes, *Ha1*, *Ha2*, *Ha3* on chromosome 2H (Andersen & Andersen, 1973) and *Ha4* on chromosome 5H (Barr*, et al.*, 1998). Although these genes had been identified and localized, the selection of resistant genotypes continued to rely on the count of cysts infesting the roots of plants, a simple but laborious and thus expensive bioassay (Andersen & Andersen, 1973). Not for two decades would readily detectable linked-RFLP markers be identified (Table 2, Barr*, et al.*, 1998; Kretschmer*, et al.*, 1997). However, the technical complexity of these RFLP-based molecular tools limits their usefulness in practical plant breeding and PCR-based markers would be preferable for large scale MAS (Dayteg*, et al.*, 2007). The mapping of resistance gene analog (RGA) loci in the vicinity of *Ha2* (Madsen *et al.*, 2003; Seah *et al.*, 1998) and the identification of linked SSR markers (Barr*, et al.*, 2003; Karakousis*, et al.*, 2003) has opened the way for the use of such markers (Table 2). This is of particular interest as the *Ha2*-gene confers resistance to *H. avenae* race 1 and 2 (Andersen & Andersen, 1970) by degrading the feeding sites for female nematodes and thus stopping their development after 15 days (Williams & Fisher, 1993).

Automation at Svalöf Weibull (SW) laboratory

Though molecular markers are today well established practice in plant breeding the automation of the technology is still in its cradle. Readymade robotic applications can easily provide some answers to specific issues, i.e. sample extraction, sample preparation etc., if the investments are possible. However, we found these equipments often to be developed according to very specific protocols or routinely adapted to special commercial kits. They present, therefore, poor flexibility to already established in-house practices and more generally to the processes and economical constraints of practical breeding. There exists no ready-to-use automation solution. The concept of automation or high throughput (HT) in themselves remain largely in "the eye of the beholder" e.g. an increase from 50 to 100 DNA-extractions per day might be considered HT for some laboratories but insignificant for others. Therefore, in a more general manner HT should rather be seen as an appreciable increase of the productivity (in percent) and automation as an attempt to decrease manual labour from standardized workflows. This thesis does not claim to hold the ultimate key to automated HT applications in plant breeding but to simply lay down the principles used in a very practical approach which might be found useful for others (paper I).

It is primordial for plant breeding companies to keep focus on their main activities; it seemed therefore, more justified to adapt the molecular processes to the breeding programmes than *vice-versa*. Because of the cost involved in automation, it is of great importance to really understand the molecular needs and requirements necessary to achieve the goals set by modern plant breeding, and to carefully analyse the methodology for maintaining enough flexibility to be able to adapt to its challenges.

The whole molecular workflow was therefore first subjected to an "intellectual exercise" and the automation-possibilities were evaluated in a three step procedure as schematised in Figure 9. In the *analystic stage* the current state of the workflow is established and the prospective state characterised in terms of usage of the molecular tools (i.e. applications required in breeding programmes), identification of necessary molecular tools and expectation of the laboratory's capacity. The requirements needed to achieve this prospective goal, i.e.: all the required procedures in the process, are defined in the *definitional stage*. They are then detailed into operation-steps in the *descriptive stage*. Lydiate (1999) has described an efficient genomic research as a three steps procedure 1) automating what can be automated 2) speeding up the process 3) allowing molecular shortcuts. A similar approach was applied at this stage to identify all possible bottlenecks and to define possibilities of improvement at each step and subject them to an "automation-filter". This simply means that each of them are tested for their automation-ability, which is to evaluate if automation is feasible for this specific step in terms of robotic availability, staff skill and accessibility, cost/gain evaluation and if an eventual automation could present new bottlenecks (i.e.: extra procedures). This final evaluation is necessary to either redefine or accept the improved procedure (with or without automation).
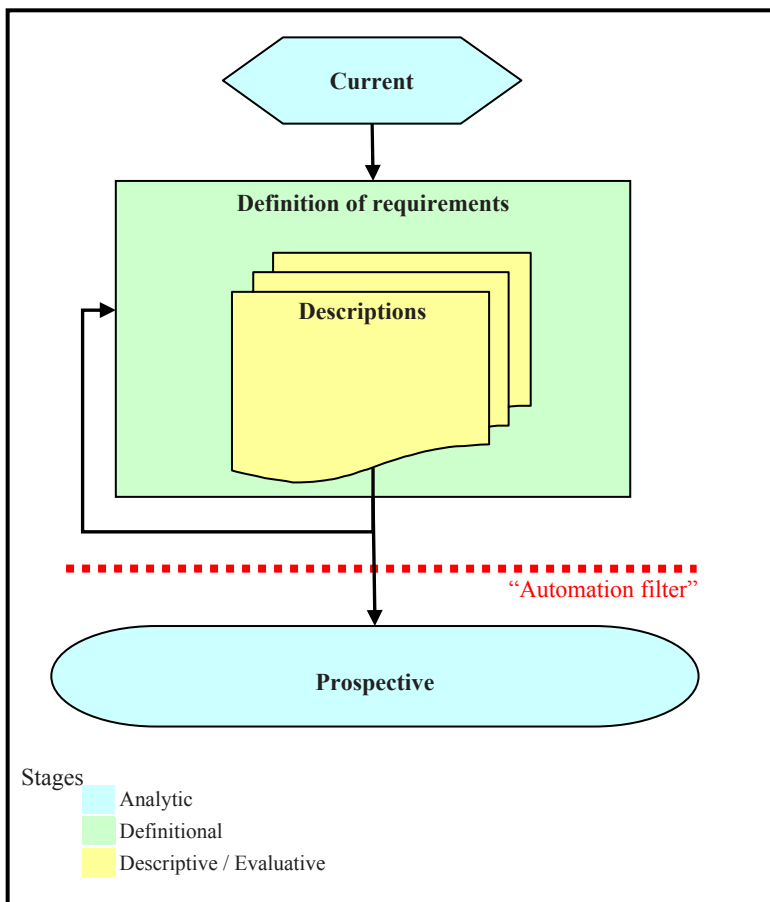
Fig. 9. Automation exercise. Mental process composed of three stages evaluating the automation possibilities of molecular workflows. The *analytic stage* establishes the current state of the workflow and characterizes the prospective state. The *definitional stage* defines the requirements needed to achieve the prospected goal. The *descriptive stage* details each of the defined steps and evaluates for each one visible bottlenecks and possibilities of improvement. These "improvements" are then subjected to the "*automation filter*". In that step the automation-feasibility is tested for each one of them and depending of the results the step can either be accepted in the prospective goal or redefined.

In regard to our plant breeding activities, we established that the application of molecular markers can be divided into two main groups when considering the relationship between the number of markers / number of individuals assayed, as seen in Figure 10. The choice of marker technologies was limited by focusing exclusively on PCR-markers because, as seen in Table 1, they fulfil most of the requirements necessary in practical plant breeding. They are easy to use, require small amount of crudely extracted DNA, enable automation and are relatively cheap. Within PCR-based markers, microsatellites (SSRs) are especially interesting as they are well spread on the genome, generally highly informative, widely available and well described in most of the crops. Their ease of detection via automated-systems makes them currently the most popular PCR-based marker

in cereal breeding (Korzun, 2003) and their flexibility allows their application in the two main groups described below.
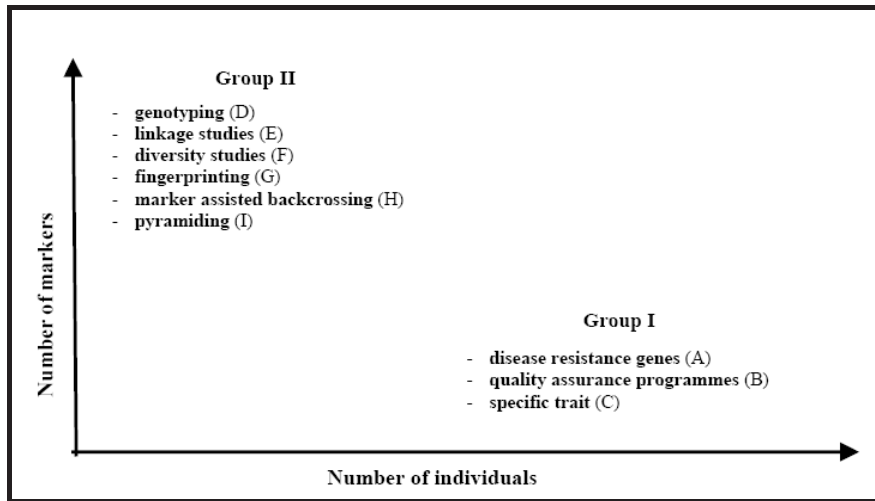

Fig. 10. Division of DNA-marker projects depending on the relationship between the number of markers / number of individuals (Dayteg, *et al.*, 2007). The ranking within groups has been made arbitrarily and may not be representative as the figures vary between crops and studies. (A) e.g. Phoma in Brassica (Foisset *et al.*, 1998), Barley Yellow Mosaic Virus (Ramsay *et al.*, 2000). (B) e.g. hybridity control, Adventitious Presence of Genetically Modified sequences (Delano *et al.*, 2003). (C) e.g. male sterility in Brassica (Primard-Brisset *et al.*, 2005) using internal markers and expression of final attenuation in malting (Frank Rath, personal communication). (D) to cover the entire genome e.g. association mapping (Ramsay, *et al.*, 2000). (E) to locate and link molecular markers to a trait of interest (Ivandic*, et al.*, 1998). (F) to assess genetic diversity in crops (Kolodinska-Brantestam, *et al.*, 2004). (G) to characterize varieties (Lombard, *et al.*, 2000). (H) to use a representative set of markers in order to efficiently select the recipient's genetic background in the offsprings when crossing with interesting exotic relatives (Åhman *et al.*, 2000). (I) to use a set of markers each specific to e.g. disease resistance genes in order to combine them in the same genotype (Werner*, et al.*, 2005).

The whole marker analyse-process was decomposed in a few components and each subjected to the approach in order to move samples numbers from tens of thousands to hundreds of thousands. All processes were standardized by working solely on microtiter-plate format from start to finish.

Sampling and DNA processing

Plant samples are collected in the field or in greenhouses using a paper punch devise and placed to the appropriate position in 96-well plates. Plates are kept cool during the collection process. Once collected, improvements to the in-lab procedures allow a rapid and efficient DNA-processing. The DNA isolation is generally performed according to a quick DNA-extraction protocol (Dayteg *et al.*, 1998) (Fig. 11) enabling the DNA to be processed within 10 min (theoretically more than 4 000 samples in a working day). For methods requiring larger DNA-quantity of better quality a "quick standard" method has been adapted from Cheung *et al.* (1993) enabling the extraction of ca 800 samples per day, by

processing the samples-plates in a robotic grinder devise. When handling samples from remote locations a seed-based DNA extraction protocol, as described by von Post *et al.* (2003), can be used to rapidly extract large amount of material (700 samples per day and person). The automation of these protocols has been successfully tested, on the system described below. Nonetheless, because they impeded the accessibility of the system to other, more demanding, procedures they remained principally manual. Parallel robotic equipment has been proposed as a possible solution. However, the marginal gain of time, and/or capacity, does not justify the investment costs, such decision has therefore been postponed.
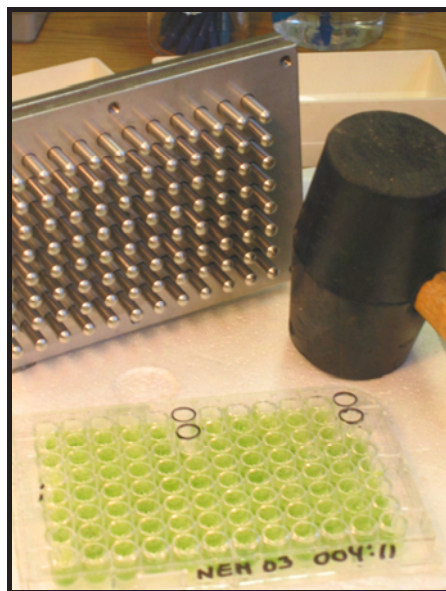
Fig. 11. Picture of a collection plate after a quick DNA-extraction. This simple procedure efficiently enable the crude extraction of thousand of DNA-samples in a working day for PCR-based molecular assays (Dayteg, *et al.*, 1998).

PCR procedure and data acquisition

Development of robotics for molecular analyses has been essential. In collaboration with Thermo CRS (Burlington, Canada), a fully automated system was developed with the main emphasis on flexibility and high throughput. The system, constituted of different peripherals, is served by a robotic arm as described in Figure 12. The components have not only been chosen for their individual automated performances but also because they all feature an open architecture that allows their nests to be accessible by the robotic arm, thus enabling full automation. Thermo CRS has supervised the integration of these peripherals into **one** core system.
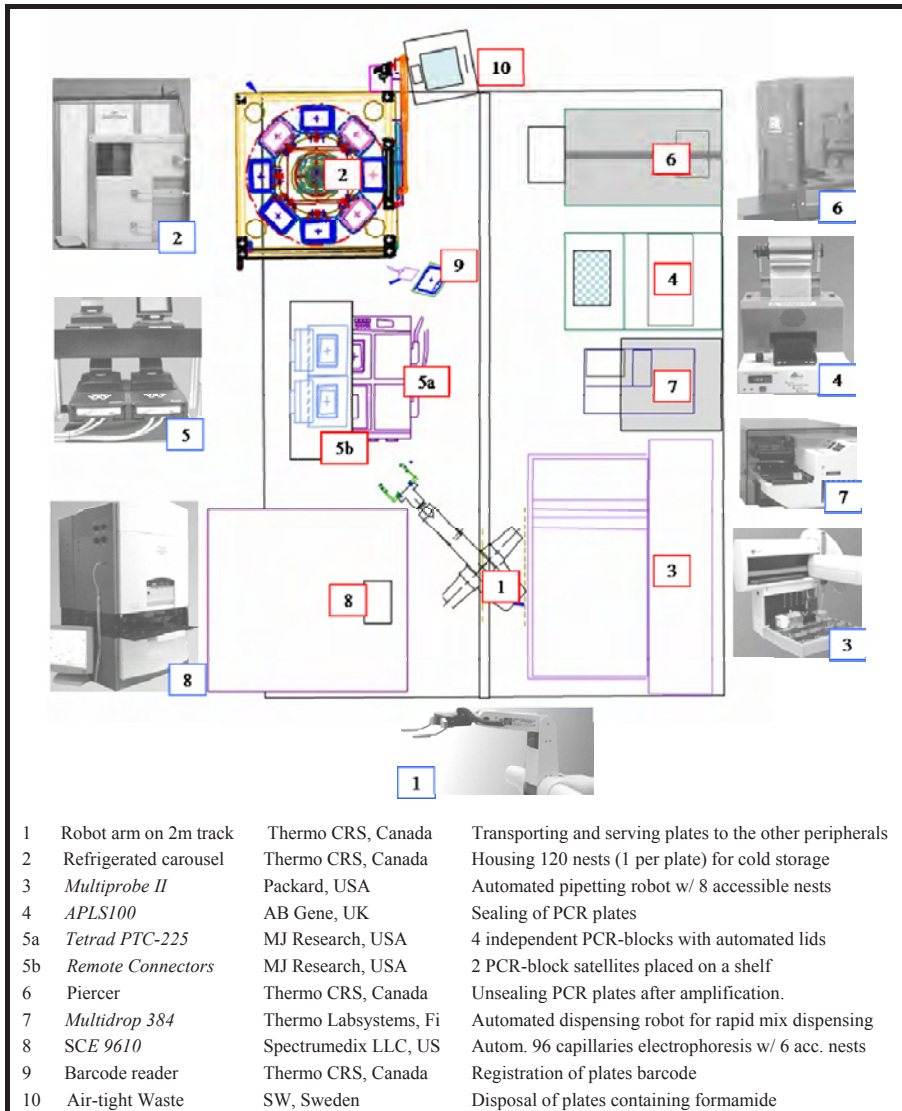
1	Robot arm on 2m track	Thermo CRS, Canada	Transporting and serving plates to the other peripherals
2	Refrigerated carousel	Thermo CRS, Canada	Housing 120 nests (1 per plate) for cold storage
3	*Multiprobe II*	Packard, USA	Automated pipetting robot w/ 8 accessible nests
4	*APLS100*	AB Gene, UK	Sealing of PCR plates
5a	*Tetrad PTC-225*	MJ Research, USA	4 independent PCR-blocks with automated lids
5b	*Remote Connectors*	MJ Research, USA	2 PCR-block satellites placed on a shelf
6	Piercer	Thermo CRS, Canada	Unsealing PCR plates after amplification.
7	*Multidrop 384*	Thermo Labsystems, Fi	Automated dispensing robot for rapid mix dispensing
8	*SCE 9610*	Spectrumedix LLC, US	Autom. 96 capillaries electrophoresis w/ 6 acc. nests
9	Barcode reader	Thermo CRS, Canada	Registration of plates barcode
10	Air-tight Waste	SW, Sweden	Disposal of plates containing formamide

Fig. 12. Illustrated blueprint of the fully automated molecular marker assay system developed at SW laboratory. (Dayteg, *et al.*, 2007).

Both main groups described above are accomplished using a common core set-up where the robot arm handles sample-plates as such: samples are moved to a pipetting devise for PCR-setup. While DNA-plates are moved back into cold-storage, the PCR-plates are sealed to avoid evaporation and placed in the thermocycler where the appropriated PCR is performed. They are then placed in cold-storage while waiting for visualisation. Prior to visualization, the PCR-plates are pierced, the samples are then either directly visualised with ethidium bromide on the 96-capillaries electrophoresis or first denatured with formamide containing a fluorescent internal line standard.

Focus was given to increasing the **throughput** and lowering the **cost** for the two groups and two specific sets of requirement could be defined and highlighted in Table 3. In Group I focus was given to decreasing procedure time for individual sample thus allowing a larger number of samples to be processed for a given time period. As few datapoints are gathered for each sample, neither DNA quality nor pipetting accuracy are a major issue and can therefore be simplified to their quickest, though reliable, form. Because the assays are well described data-acquisition can be focused and only limited to the expected fragments for more efficiency. In the case of Group II, focus was given to increasing the sample efficiency e.g. by multiplexing (simultaneous amplification of several molecular markers in a single reaction). This sets higher requirements on DNA quality, automated liquid handling and data-analysis (Mace *et al.*, 2003). In both cases, the capacity is further increased by the possibility of an extended assay capacity (overnight and week-end runs).

Table 3. *Set of requirements for the automation of DNA-markers.* ✚ *lower importance* ✚✚ *higher importance. Source: Dayteg et al. (2007)*

Requirements		Group I Many individuals / Few markers	Group II Few individuals / Many markers
Quality	DNA	✚	✚✚
	Pipetting	✚	✚✚
	Visualisation	✚	✚✚
Speed	DNA	✚✚	✚
	Pipetting	✚✚	✚
	Visualisation	✚✚	✚
High Throughput through		**Speed**	**Multiplex**

The use of a core system, besides its flexibility, increases the reliability of the system, reduces start-up time and enables different assays to be run simultaneously (Brandt, 1998). Furthermore it enables the whole process to be performed in a fully automated manner hence freeing such procedures from any human interventions, since it often is the major cost, a decrease in "hands-on" will also decrease the cost (Klapper *et al.*, 1998). The use of capillary electrophoresis increases not only the sample throughput but enhances the detection-sensitivity which allows multiplexing (using fluorescent dyes). The enhanced sensitivity also enables a decrease to $1/5^{th}$ of the reaction volume, which significantly lowers assay-costs.

Data handling and sample tracking in mass number

The development of specific softwares to handle electrophoresis-data has allowed the use of ethidium bromide and fluorescently labels for visualizing DNA-fragments which not only speeds up both data-acquisition and analysis time but also allows to efficiently cut down the analysis cost. Comparisons were made with a similar assay visualized with an agarose-gel based electrophoresis (Table 4). The assay-throughput, depending on the application, will range from ca 11 000 datapoints (dp)/week to more than 24 000 dp/week (using 4 multiplexed markers).

Table 4. *Comparison of the DNA-fragment visualization-methods. The values are calculated per plate (96 samples) at their respective capacity. The capillary electrophoreses have been performed on the SCE9610 (Spectrumedix LCC). All figures are given for a simple reaction (simplex). The extraction cost includes the material and consumable costs. The first two columns are for the quick-extraction protocol (4 000 samples capacity) while the last is for the "quick standard"-protocol (800 capacity). The PCR cost includes the material and consumable costs for the respective applications. The two last columns do not include the cost of tips as they are performed on the robot. Furthermore, their PCR-volumes have been decreased to $1/5^{th}$ and $1/3^{rd}$ respectively. The analysis cost includes the material (e.g. capillaries) and consumable costs. There are two figures for the last column, as the use of commercial or home-made ILS strongly influences the cost. Labour is calculated as the average time. Differences in the depreciation cost are due to the cost of the equipment. Source: Dayteg et al. (2007)*

	Agarose Electrophoresis	Capillary Electrophoresis Ethidium Bromide	Capillary Electrophoresis Fluorescent dyes
Extraction cost	1,5 €	1,5 €	82 €
PCR cost	20,5 €	6,2 €	8,3 €
Analysis cost	5,6 €	6 €	18,2€ † / 10,3€ ††
Depreciation ‡	0,9 €	9,3 €	9,3 €
Hands-on time (average)	55 min	16 min	50 min
Throughput (per day)	900 samples	2 200 samples	900 samples

‡ On a 10 year basis and 200 000 analyses per year.
† Use of commercial ILS
†† Use of non commercial ILS

In order to cope with this mass numbers of data, the laboratory has entered the next phase of development by integrating a sample tracking system to improve the monitoring samples from tissue arrival to data export which will allow a strong breeder-laboratory interface.

Barley mapping populations

To evaluate the automation of molecular markers in practical MAS schemes, four mapping-populations conferring resistance to the three pathogens previously described (powdery mildew, leaf rust and nematode) with a total of 7 putative disease resistance genes from different sources have been under investigation (Table 5).

Table 5. Øresund Food Network's barley mapping populations under investigation for MAS of resistance genes to powdery mildew, leaf rust and nematode

Mapping Population	Pedigree	Resistance genes to barley pathogens		
		B. graminis	*P. hordei*	*H. avenae*
MP 10	SW Buddy x SW Cecilia	*mlo*	*Rph7*	*Ha2*
MP 32A	Sebastian x (RS170-45 x Elgina x Roland x Caroline[2])	*Mla* (?) + unknown		
MP 32 B	Sebastian x (RS170-45 x Elgina x Koral[2])	*Mla13* + unknown		
MP 34	Sebastian x Risoe R2 (RS41-2)		unknown	

A population of 92 double haploid (DH) lines derived from the F_1 of a cross between the barley cultivars 'SW Buddy' and 'SW Cecilia' and designed MP10, was used to evaluate markers for powdery mildew resistance, leaf rust resistance and nematode resistance. Two other double haploid (DH) populations, designed MP32A and MP32B consisting of respectively 97 and 80 lines were derived from the F_1 of a cross between the cultivar 'Sebastian' and backcross lines from Risø derived from the Israeli *H. vulgare* ssp. *spontaneum* line 'RS 170-45' (kindly provided by Dr. A. Jahoor).
Their pedigrees are a follows:

MP32 A: Sebastian x ((((RS 170-45 x Elgina) x Roland) x Caroline) x Caroline)
MP32 B: Sebastian x (((RS 170-45 x Elgina) x Koral) x Koral)

Both lines combine *Mla*-resistance with exotic powdery mildew resistance and were used for investigating putative new powdery mildew resistance genes.

Another double haploid (DH) population, designed MP34 consisting of 91 DH-lines was derived from the F_1 of a cross between the cultivar 'Sebastian' and the Israeli *H. vulgare* ssp. *spontaneum* line 'RS41-2' ('Risoe R2' kindly provided by Dr. A. Jahoor). This line is known to have a good level of quantitative resistance against leaf rust and was used to investigate putative new leaf rust resistance genes.

Furthermore a collection of 96 barley lines either susceptible or resistant to race 1 and 2 of *H. avenae* (*Ha2*) was composed from 11 well known cultivars and 85 breeding lines from SW Seed's resistance and germplasm development programs. This collection, representing a broad genetic background, was used for marker validation.

Application I: For known resistance genes

"Text mining"

The barley mapping population MP10 was used to study the transferability of different published markers to an automated procedure and to evaluate their uses in practical breeding programmes.

MP10 possesses several known genes of interest (Table 5) for powdery mildew (*mlo*), leaf-rust (*Rph7*) and nematode (*Ha2*) resistance and linked markers have been reported to each one of them:

- *mlo*-resistance gene has been linked to 5 SSR markers on chromosome 4 (4H) (Piffanelli*, et al.*, 2004).
- *Rph7*-resistance gene has been linked to a SNP marker on chromosome 3 (3H) (Brunner*, et al.*, 2000).
- *Ha2*-resistance gene has been linked to a RGA marker (Madsen*, et al.*, 2003) as well as 2 SSR markers (Barr*, et al.*, 2003; Karakousis*, et al.*, 2003) on chromosome 2 (2H).

From a technical point of view, all markers worked satisfactorily without any necessary optimization and amplified strong signals. However, from a breeding point of view, only the *Rph7*-marker could be useful in practical programmes as its distance was calculated to be at 3.4 cM (Kosambi) from the resistance gene in SW-material. All of the SSRs identified for *mlo* were monomorphic; the fact that they were identified in a different genetic-background could explain this difference. In regards to the *Ha2*-resistance, both SSRs, identified on Australian material, revealed a distance to the gene impropriated for breeding purposes (>10 Kosambi centimorgan, Dayteg*, et al.*, 2008). The RGA-marker presented a distance of 9 cM to the gene (Dayteg*, et al.*, 2008) and used cross-specific dominant primers making improbable its practical use in large MAS programmes (Madsen L., personal communication).

Ha2-marker development

Attention was given to an important resistance character: the *Ha2*-mediated nematode resistance (paper II). From mapping information (Ramsay*, et al.*, 2000; Willsmore *et al.*, 2006) several SSR markers were chosen for their location in the vicinity of *Ha2* on chromosome 2 (2H). MP10, derived from the cross between the (cereal cyst nematode) CCN-susceptible barley variety 'SW Buddy' and the *Ha2*-carrying barley cultivar 'SW Cecilia' (CCN-resistant) was used. None of the markers showed a better linkage than the two published SSRs, *Bmag0125* (Barr*, et al.*, 2003) and *EBmatc0039* (Karakousis*, et al.*, 2003). While these PCR-based markers open the way for large-scale MAS of *Ha2*-nematode resistance, the low levels of polymorphism, exhibited in SW material, prohibits their use in breeding programmes. To overcome this issue, ISSR primers were used as their PCR amplification targets different SSR motifs which generate extended polymorphism although in quite complex profile (Zietkiewicz *et al.*, 1994). While the additional polymorphism on one hand simplified the identification of linked markers, the complicity of the profile on the other hand restricted the direct use of this method in MAS schemes. Therefore, a conversion from a multi-locus marker system into a locus specific marker had to be undertaken (Fig. 13) as it has been previously done with AFLP (Meksem *et al.*, 2001) or ISSR (Gold *et al.*, 1999).

From the isolated polymorphic band DNA sequence a 20-mer sequence characterized amplified region (SCAR) was designed. Its linkage to *Ha2* was
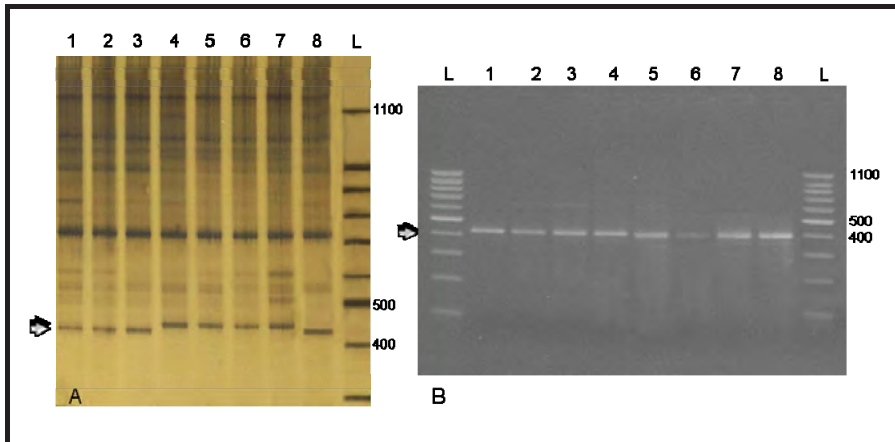
Fig. 13. **A.** Band profile of ISSR primer UBC9 #816 obtained on low-temperature PAGE-gel. *Lane 8*: SW Buddy (susceptible parent); *lane 7*: SW Cecilia (resistant parent); *lanes 4-6*: bulks resistant DH-lines; *lanes 1-3*: bulks susceptible DH-lines; *L*: 100-bp ladder. The *filled arrow* indicates the resistant allele and the *open arrow* shows the susceptible allele.
B. Band profile of the ISSR primer (UBC9 #816) re-amplification obtained on 2% (w/v) agarose. *Lanes 1-4*: eluted resistant fragments; *lanes 5-8*: eluted susceptible fragments; *L*: 100-bp ladder. The *filled arrow* indicates the resistant allele and the *open arrow* shows the susceptible allele.

evaluated on the whole MP10 mapping population and validated using the barley lines collection. The developed SCAR marker has been located to the long arm of chromosome 2H at a distance of 4,3 cM (Kosambi) to *Ha2* and co-segregates with resistant and susceptible phenotypes in 82,4% of the tested barley lines (Table 6). This new SCAR marker represents today, even without prior knowledge of the parental polymorphism, a valuable and cost efficient tool in high-throughput breeding schemes to develop nematode-resistant barley cultivars.

Application II: For mapping new resistance genes

Three DH-populations from crosses with *H. spontaneum* lines, i.e.: a total of 278 distinct DH-lines, were used to characterize inherited putative new sources of resistance to powdery mildew and leaf rust (papers III and IV).

From the SSR 'genotyping set' described by Macaulay and colleagues (2001), a subset of 42 mapped SSRs were chosen to screen each population in order to provide a good coverage of the whole genome, on average six SSR-markers per chromosome. Preference was given to robust markers which exhibited relatively high PIC (polymorphic information content) and with similar amplification conditions in order to maximize the genotyping throughput. To rapidly provide more data, two AFLP combinations were screened on all populations, the data generated was neither sufficient to provide a saturated map of the DH-lines nor definite results, but informative enough to identify chromosomal regions of interest. For leaf rust, two QTLs were identified on chromosome 2HS. Differentiation to the known *Rph1*-gene was not established but could provide the

basis to link molecular markers to this major gene. For powdery mildew, the presence of two *Mla*-alleles could be identified, one being *Mla13* and the other remaining unknown at this point, as well as 3 QTLs for the putative new source of resistance from *H. spontaneum*. Though portraying the possibility with modest mapping studies to identified chromosomal regions of interest, these results underline the necessity to possess well saturated maps and strong data for unequivocal QTL identification.

Discussion

The developed system enables the automation of PCR-based markers and provides a competitive platform for their large-scale use in plant breeding (paper I). However, the results of the first application (paper II) exemplify the difficulty, found in practical breeding, to directly use published marker-data. Technically the amplification of published markers is feasible and amenable to automation, yet the data observed might vary from the published ones. This is especially true for QTLs (Chelkowsky*, et al.*, 2003) but as we experienced even true for major genes. Most markers used nowadays are strong and stable, i.e.: exhibit good reproducibility between procedures and laboratories and amplify correctly even in suboptimal conditions. However, it is imperative, in order to be useful in breeding programmes, that their diagnosticity is tested on a wide genetic-background and not only on specific crosses (paper II).

Plant breeders are interested in diagnostic and stable markers which are amenable to high-throughput screenings. For that reason markers developed from defined sequences, like SCARs or STS, are especially interesting. Nonetheless, such markers are difficult to find for traits of practical breeding values, and finding or developing markers closely linked to these traits might be easier. In that aspect SSR-markers are considered a valuable tool and the system showed to be very useful to rapidly screen mapped microsatellite markers and evaluate their usefulness in this context. We believe that the system can provide a strong and powerful tool in linkage studies, enabling the mass-screening of numerous markers and genotypes, hence empowering mapping activities, provided a sufficient number of available markers. Unfortunately, none of the SSRs screened showed to be close enough to the genes under investigations to conclusively resolve the problems at hands. Additional SSR-markers are widely available (Pillen *et al.*, 2000; Ramsay*, et al.*, 2000; Willsmore*, et al.*, 2006) and will be used for future marker development. Nevertheless, in these studies this was resolved by using either AFLPs (papers III and IV) or ISSRs (paper II). AFLPs, though amenable to certain automation, are regulated under strict licensing and intellectual property rules which make them difficult and expensive to use in the private sector. ISSRs, on the other hand, are not under such regulations, but to date their amplification has not yet been transferred to the automated system. Nonetheless, except for the isolation step (impracticable because of the use of capillaries), no technical issues oppose its possible completion. Such application

would be very valuable to generate extended polymorphism with low input, which could be used in phylogeny studies or to develop molecular markers (paper II).

Automation of molecular markers in a plant breeding perspective is highly feasible and beneficial. Automated systems, such as the one developed, can even enhance the detection of linked markers. Nonetheless, today the rather limited amount of molecular markers, amenable to automation and diagnostically linked to genes of practical breeding interest, remains the major drawback of such application. Confidently, the current and rapid developments in the molecular field (e.g.: association mapping) will provide an appreciable quantity of molecular tools, adapted to practical plant breeding, that will increase the diversity of automated assays.

Conclusion

Plant breeders act in a very competitive market, continuously re-evaluating their products in an attempt to respond to ever-changing market demands and agricultural practices. The undeniable benefits of molecular markers and the constant decrease of PCR-costs provide considerable allies in such never-ending "selection-pressure". For these reasons, PCR-markers are considered a valuable tool in the most various, and even modest, breeding programmes. However, molecular technology is still often viewed as an additional cost in many breeding procedures. An improvement of the productivity, through procedure-optimizations and/or automation, will provide a further decrease of the assays cost and increase its availability. To date the costs for automating of marker technologies in applied plant breeding still represent an important investment. Nonetheless, it cannot be stressed enough, that automation is the ultimate steps in a series of optimizations of molecular (and breeding) procedures and that the automation concept itself very much depends of the beholder's perspectives. For our part, the main goal was to decrease to the maximum any human-interactions from tedious, unqualified, and non-rewarding, procedures thus liberating the highly qualified staff to perform more complex and demanding applications. The fully-automated system developed at SW, like any automated molecular screening settings, provides the ability to efficiently generate large dataset from either a large amount of material and/or markers. In turn, this allows the molecular description of distinct genetic material either as:
-more complex procedures i.e.: genotype characterization, phylogenic studies, mapping of genetically-unknown characters.
-single procedure using known linked markers in MAS and pyramiding of identified traits.

The described system is flexible enough to i) permit the development of linked markers i.e.: efficiently screen high numbers of markers ii) adapt to the requirements of practical breeding in regards to molecular analyses i.e.: to assay realistically high numbers of samples as promptly and as cheaply as possible and thus enabling practical "molecular-breeding".

Today, the increasing amount of sequence information and the determination of gene function is leading to the use of emerging marker types such as SNPs. They hold great promises of rapid and highly automated genotyping (Gupta, *et al.*, 1999; Korzun, 2003) and this technology may hold the key of an improved use of molecular markers in plant breeding in the future. However, the costs involved in the development and mechanization of SNPs suggest that our automated system will have some more golden hours. Nonetheless, it is important to keep in mind that automation of "molecular breeding" is an ongoing process, not only in terms of technical development but rather as a constant questioning of the different breeding-specific applications, trying to fulfil the main goal of breeders: to promptly release the best product quality at the lowest cost.

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  -study the genetics of allelopathy traits of wheat and barley and the possibilities to use marker assisted selection
  -identify markers linked to important disease resistance traits
  -develop a high throughput system for MAS of linked markers

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II

III

IV

Characterization and chromosomal location of a putative exotic barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) resistance in two crosses between a barley cultivar and wild barley (*Hordeum vulgare* ssp. *spontaneum*) derived line

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Abstract

The use of resistant cultivars is the most economical viable and environmental acceptable mean to control powdery mildew (*Blumeria graminis* f.sp. *hordei*) in barley (*Hordeum vulgare* L.). Until recently, breeding major resistance genes efficiently controlled the pathogen. However, resistance of most cultivar is rapidly overcome due to the constant adaptation of the pathogen populations. A rather narrow number of loci with major effects against powdery mildew limits the number of genes that can efficiently be combined to produce durable resistance in breeding programmes. Identification of new source of resistance, and of molecular markers closely linked to them, can greatly increase the efficiency of pyramiding new resistance genes in barley. The genetic basis of a putative novel resistance to powdery mildew was analysed using two double haploid barley populations developed from a *H. spontaneum* cross. Based on infection observations, the lines were classified into seven groups. Several putative loci were mapped with molecular markers. Two coincided with the previously mapped *Mla*-locus on chromosome 1H (5), one of them was identified as *Mla13*. Three other loci were identified by simple interval mapping of disease severity data from seedling test and field trial. Though not conclusive, the data presents a more complex resistance-loci interaction than initially believed and localizes 3 QTLs explaining between 17 to 34 per cent of the phenotypic variation observed. This study reports the first attempt into resolving the genetics and localizing these putative resistance-loci. It also demonstrates the usefulness of an integrated approach to identifying and mapping putative resistance loci using classification results from inoculated data and quantitative data.

Introduction

Powdery mildew, caused by the obligate biotrophic fungus *Blumeria* (syn. *Erysiphe*) *graminis* f.sp. *hordei* Otth., is one of the most important barley diseases worldwide mostly in cool and maritime climates. Concerns about crop production costs and environmental pollution has strengthened the use of resistant cultivars to control the disease (Jørgensen, 1993). Because of its economical importance, the barley-powdery mildew pathosystems has been one of the most explored in plants (Jørgensen, 1994; Wiberg, 1974; Williams, 2003). Jørgensen (1994) has classified the known types of resistance into 1) race-specific resistance (gene-for-gene system), 2) *Mlo* resistance (effective against all known isolates until now) and 3) partial resistance (thought to be conferred by additively-acting genes with small effects). These types of resistance are not mutually exclusive and to date 23 race specific loci, including *Mlo*, have been described and many mapped on barley chromosomes (Backes *et al.*, 2006; Chełkowsky, Tyrka & Sobkiewicz, 2003). Because of their importance and their multiple allelism, the *Mla* and the *Mlo* loci have been subject to much interest (Büschges *et al.*, 1997; Jahoor *et al.*, 1993; Jørgensen, 1992; Piffanelli *et al.*, 2004; Schwarz *et al.*, 1999; Shen, 2004; Wei *et al.*, 1999; Wei, Wing & Wise, 2002). Until now more than 32 alleles have been detected at the *Mla* locus, which is the highest number of different alleles identified among all known barley powdery mildew resistance genes and two genes *Rar1* and *Rar2* (<u>R</u>equired for *Ml<u>a</u>*-mediated <u>r</u>esistance) which are necessary for the function of multiple, but not all, resistance interactions at the *Mla*-locus have also been identified (Jørgensen, 1996). The *Mla1, Mla6* and *Rar1* genes have been cloned and sequenced (Halterman *et al.*, 2001; Shirasu *et al.*, 1999; Zhou *et al.*, 2001). The *mlo* gene, probably the most famous and used, as it gives a leaf-lesion phenotype and broad-spectrum resistance, has also been cloned. A total of 32 alleles have been described (Molina-Cano *et al.*, 2003), and two genes, *Ror1* and *Ror2*, required for the full expression of *mlo* resistance have been identified by mutant analysis (Freialdenhoven *et al.*, 1996). Finally, several QTLs for partial resistance have been mapped on all chromosomes (Backes *et al.*, 2003; Heun, 1992; Shtaya *et al.*, 2006; Williams, 2003; Yun *et al.*, 2005) some do coincide with major genes for powdery mildew but others are localized in previously unreported chromosomal regions.

   This abundance of knowledge and genetic resources does not however grant immunity, as powdery mildew resistance of most barley cultivar is rapidly overcome due to the constant adaptation of the pathogen populations (Hovmøller *et al.*, 2000), especially in cultivars carrying a single resistance gene (Jørgensen, 1993). Therefore the introduction of new source of resistance, such as it has

been done from *H. spontaneum* (Jahoor & Fischbeck, 1987), and the implementation of broad resistance strategies, such as pyramiding of different genes through the use of molecular marker technology (Chen *et al.*, 2000; Werner, Friedt & Ordon, 2005), are paramount to continually assure their effectiveness.

In the present study, two populations of double haploids derived from a cross with *H. spontaneum* were examined for powdery mildew resistance. The data obtained represents the first results in a program aimed at identifying and mapping putative novel sources of *B. graminis* resistance.

Material and methods

Plant material

Two double haploid (DH) mapping populations, consisting of 97 and 80 lines respectively, were derived from the F_1 of a cross between the cultivar 'Sebastian' and two backcross lines derived from the Israeli *H. vulgare* ssp. *spontaneum* line 'RS 170-45'. The DH lines were produced at Sejet Planteforædling, Denmark. Their pedigrees are as follows:

MP 32A: Sebastian x ((((RS 170-45 x Elgina) x Roland) x
       Caroline) x Caroline)
MP 32B: Sebastian x (((RS 170-45 x Elgina) x Koral) x Koral)

Disease Phenotyping

Seedling tests and field test were performed for powdery mildew resistance.

Seedling test were performed in glasshouse at Svalöf Weibull, Sweden. From the pedigree data, the presence of inherited *Mla13*-resistance gene from 'Koral' in MP32B was suspected and two distinct *B. graminis* isolates were therefore used. One isolate from the cultivar 'Goldie' (*Bg*Go), avirulent for the *Mla13*-resistance gene and one from the cultivar 'Meltan' (*Bg*Me) chosen for its virulence to *Mla13* were used on both populations. Disease severity was screened at the maximal stage of disease development in a scale from 0 (resistant) to 4 (susceptible).

Natural powdery mildew infection was observed in a field experiment in 2006 in Sweden. The DH-lines together with their parents were sown in 2 replicates as two rows per line with a susceptible spreader in the middle. Disease severity was screened at

the maximal stage of disease development in a scale from 1 (resistant) to 9 (susceptible).

SSR and AFLP amplifications

Genomic DNA was extracted from fresh leaf samples in 96 well plates according to a protocol derived from Cheung et al. (1993) for SSR amplification and using Quiagen's DNeasy® 96 Plant extraction kit according to the manufacturer's recommendations for AFLP amplifications.

Initially a bulked segregant analysis (BSA, Michelmore, Paran & Kesseli, 1991) approach was taken and 7 bulks of resistant and susceptible DH-lines were produced from the *Bg*Go and *Bg*Me seedling test results from both populations. From the SSR 'genotyping set' described by Macaulay and colleagues (2001), a subset of 42 mapped SSRs were chosen in order to provide a good coverage of the whole genome, on average six SSR-markers per chromosome. Preference was given to robust markers which exhibited relatively high PIC (polymorphic information content) and with similar amplification conditions in order to maximize the genotyping throughput. From the data obtained 10 candidate SSRs were chosen to screen both whole mapping populations (Table 1).

Table 1 *Map position of candidate SSR markers according to GrainGene's 2006 consensus map (Source: http://wheat.pw.usda.gov/GG2/index.shtml)*

SSR	Ch.	Pos. (cM)	SSR	Ch.	Pos. (cM)
Bmac0399	1H	28	Bmac0067	3H	49
Bmac0213	1H	30	Bmac0209	3H	57
HvM36	2H	31	Bmag0112	3H	64
Bmac0093	2H	65	Bmag0225	3H	75
EBmac0415	2H	132	HvCMA	7H	67

All microsatellite PCR-amplifications were performed according to Ramsay et al. (2000) using fluorescently labeled primers, which allows automated data capture and visualized by capillary electrophoresis on Spectrumedix SCE9610 (Pa, USA).

The AFLP protocol was applied as described by Backes *et al.* (2003) in order to provide further loci over the genome, the genomic DNA was digested with the restriction enzymes *MseI* and *PstI* and two primer combinations were used (Table 2) on both whole populations. AFLP marker names were according to the AFLP profiles of 16 reference barley lines (GrainGenes internet page, map data). The detection of AFLP-pattern was carried out on an ABI Prism 377 sequencer (Perkin-Elmer) using fluorescent dyes.

Table 2. *AFLP-primer combinations and number of loci generated in the different 'Sebastian' x 'RS 170-45'-backcross populations*

MseI primer	Selective bases	PstI primer	Selective bases	Nber of loci in MP32A	Nber of loci in MP32B	Fluorescent dye
M62	CTT	P32	AAC	5	5	FAM
M61	CTG	P31	CC	6	4	NED

Mapping and QTL analysis

The linkage groups were established by using LOD-data as calculated in an excel-macro developed by Gunter Backes (unpublished) to cluster the markers into linkage groups. Ordering the markers was carried out in GMendel 3.0 (Holloway & Knapp, 1993) using the 'kSAR' function. To avoid that the starting order of loci influence ordering, a Monte Carlo simulation was performed with 200 replications using the 'monte' function of the programme. Markers showing a high variance in this programme were checked for genotyping errors and some were excluded from the map. The resulting unambiguous map was controlled by using the 'SAL' function and distorted segregation of the markers investigated by a χ^2–test in GMendel. Trait data were inspected for outliers and the distribution over the lines of the populations. An interval mapping approach using PLABQTL 1.2 (Utz & Melchinger, 2003) was used for mapping putative QTLs. A LOD of 2.5 was chosen as significance threshold value for declaring a QTL.

Results and discussion

Trait data

When screened with the *B. graminis* isolate from the cultivar 'Goldie', the population MP32B did not exhibit the segregation ratio between resistant and susceptible individuals expected from a DH-population containing a resistance gene (1:1, $\chi^2 = 2.32$). Intermediary resistance reactions (score '1' and '2' with necrotic spots) were recorded, when computed together into a fictive "intermediary" score, a Mendelian fraction of 2:1:1 ($\chi^2 = 0.3$) could be observed (Fig. 1). This segregation ratio normally exhibited in DH-population containing 2 resistance genes, supports the assumed inherited presence of the *Mla13*-resistance gene in the MP32B population. To test this hypothesis, both populations were therefore also screened with a *Mla13*-virulent strain of *B. graminis*, isolated from the cultivar 'Meltan'. The MP32B population exhibited only two types of reactions: susceptible and partially resistant (score '2'

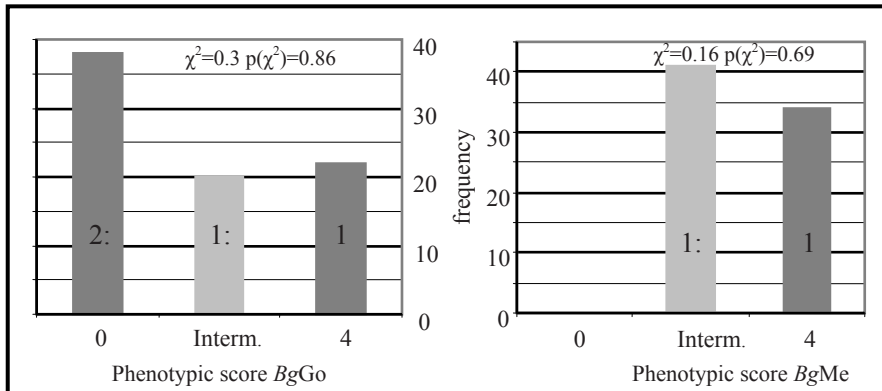with necrotic spots) segregating in a 1:1 ratio ($\chi^2 = 0.16$) as seen in Figure 1.

Fig. 1. Phenotypic segregation of MP32B when screened with two different *B. graminis* isolates. *Bg*Go is a *Mla13*-avirulent isolate from the cultivar 'Goldie'. *Bg*Me is a *Mla13*-virulent isolate from the cultivar 'Meltan'. Individuals showing a hypersensitive resistance reaction have a phenotypic score of '0', individuals exhibiting partial resistance have been gathered in the 'intermediary' class while individuals susceptible to powdery mildew have a score of '4'. The χ^2 and $p(\chi^2)$ have been calculated for respectively 2:1:1 and 1:1 segregation ratio.

This result indicates the presence of a second powdery mildew resistance gene different to the *Mla13*-resistance gene inherited from the cultivar 'Koral'. The assumption, as schemed in Table 3, is that the *Mla13*-resistance gene, alone and/or in combination with the exotic-resistance gene from 'RS 170-45', produces the hypersensitive reaction observed with *Bg*Go. However the *Mla13* gene is silenced with *Bg*Me, allowing only the *H. spontaneum*-inherited resistance to exhibit its partial resistance.

Table 3. *Schematic assumption explaining the difference in phenotypic segregation ratio exhibited with the different B. graminis isolates used to screen MP32B (see text). BgGo is a Mla13-avirulent isolate from the cultivar 'Goldie' which induced a 2:1:1 segregation ratio in MP32B. BgMe is a Mla13-virulent isolate from the cultivar 'Meltan' which induced a 1:1 segregation ratio*

B. graminis isolate	Resistant (score 0)	Intermediary	Susceptible (score 4)
BgGo (Mla13 avir.)	50	25	25
Resistance genes	Mla13 Mla13 + Exotic	Exotic	None
BgMe (Mla13 vir.)		50	50
Resistance genes		Exotic Mla13 + Exotic	None Mla13

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As seen in Figure 2, the MP32A population, when screened with *Bg*Go, exhibited a slightly skewed 1:1 segregation ratio (1.34:1). However, when screened with *Bg*Me, the previously resistant individuals divided into two resistance reactions: a hypersensitive reaction (scored '0') and a '1t' reaction. These results, though excluding the presence of the *Mla13*-resistance gene, indicate the presence of two distinct resistance genes, differentiated by *Bg*Me, also in MP32A.
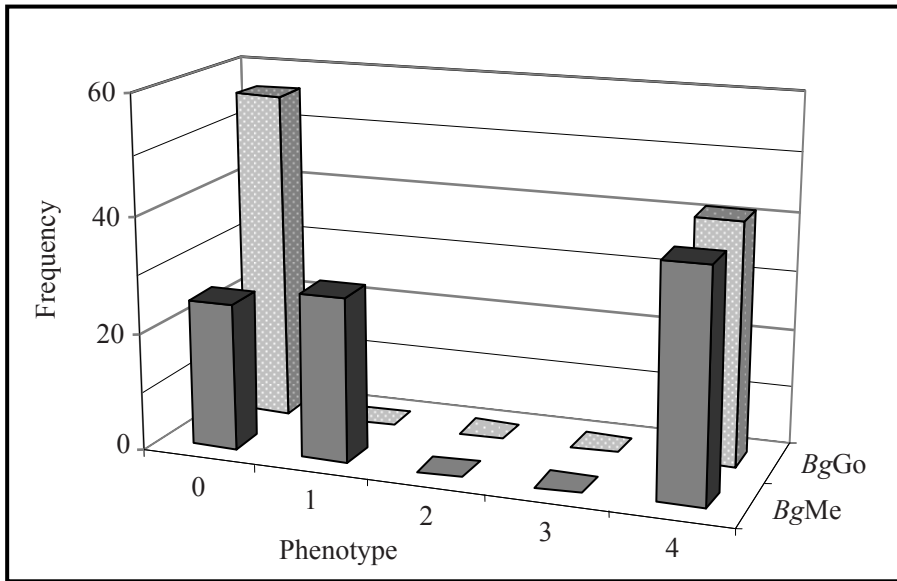

Fig. 2. Phenotypic segregation of MP32A when screened with two different *B. graminis* isolates. *Bg*Go is a *Mla13*-avirulent isolate from the cultivar 'Goldie'. *Bg*Me is a *Mla13*-virulent isolate from the cultivar 'Meltan'. Individuals showing a hypersensitive resistance reaction have a phenotypic score of '0', while individuals susceptible to powdery mildew have a score of '4'.

Symptoms were quite severe in the field evaluation performed in the summer of 2006 in Svalöv, Sweden. Both populations exhibited a good overall resistance to natural powdery mildew infection, though MP32A showed a slightly better resistance frequency than MP32B. The distribution of the disease observation in the field is shown in Figure 3. The observations showed a normal distribution for MP32B.
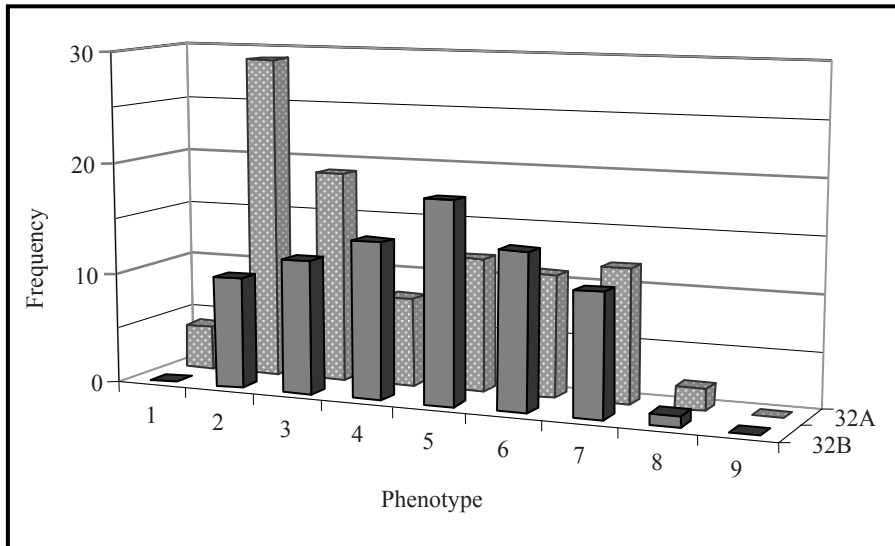
Fig. 3. Phenotypic segregation for natural *B. graminis* infection in field. Disease severity was screened at the maximal stage of disease development in a scale from 1 (resistant) to 9 (susceptible).

Linkage map and QTL study

A BSA approach was initially taken to identify putative chromosomal regions of interest. For MP32A, three bulks were formed according to the *Bg*Me seedling data, namely a hypersensitive-resistant (score '0'), a partial resistant (score '1t') and a susceptible bulk. For MP32B four bulks representing all gene combinations were made according to the assumptions presented in Table 4.

Table 4. *Resistance gene(s) present in MP32B bulks, according to the individual seedlings data obtained with the two different B. graminis screenings. Individuals exhibiting hypersensitive resistance reaction were scored 0, individuals exhibiting partial resistance, other than 2 with necrotic spots (2nc), are here represented as interm. while the susceptible ones were scored 4.*

Bulk	Scored w/ *Bg*Go	Scored w/ *Bg*Me	
1	0	4	*Mla13*
2	0	2nc	*Mla13* + exotic
3	Interm.	2nc	Exotic
4	4	4	None

From the 42 SSRs used to screen the bulks, 10 were selected (Table 1) for the polymorphism they exhibited between the bulks and screened on the whole populations. Of these markers only two, *Bmac0399* and *Bmac0213* both located on the short arm of chromosome 5 (1HS), showed to be linked to the two MP32B bulks containing the *Mla13*-gene, as well as the MP32A bulk exhibiting the '0' reaction. Both the *Mla13*- resistance gene and the *Mla*-locus

have previously been mapped on chromosome 1HS (Wei, *et al.*, 1999; Yun, *et al.*, 2005) and as we know that MP32A does not contain the *Mla13*-gene it would seem that, in this study, these markers are not only linked to *Mla13* but rather to the whole *Mla*-locus. Furthermore, this indicates that one of the resistance gene present in the MP32A, the one responsible for the '0' reaction with *Bg*Me-isolate, is an *Mla*-allele other than the *Mla13*. Unfortunately, no further conclusive marker-associations could be made with the other resistance bulks. Both populations were therefore also assayed with AFLP-markers to allow a QTL-approach to be taken.

A total of 38 marker loci were available for the map construction, 20 for MP32A and 18 for MP32B. Of these, 32 were assigned to linkage groups. During mapping, further loci were excluded as they either showed too few or no recombination event with other loci and/or the Monte Carlo simulation in GMendel revealed that they led to ambiguities in the ordering, thus either indicating potential errors in genotyping or erroneous attachment to a group. The largest reduction happened in MP32A's linkage group 3H where no markers remained; on the other hand a linkage exclusively composed of AFLP-loci could be assigned (designed as linkage group 4). The final map consisted of 14 loci: 5 SSR loci and 9 AFLP loci for population MP32A and of 15 loci: 8 SSR loci and 7 AFLP loci for population MP32B. All chromosomal regions under investigation, other than MP32A's chromosome 3H, were represented by one coherent linkage group. Overall the markers showed a distorted segregation from the expected 1:1 segregation for DH population. Both microsatellite and AFLP markers distorted toward the allele from 'Sebastian'. The predominance of this allele was stronger marked with AFLP markers. In crosses between cultivars and wild species, as well as in crosses between subspecies, distorted segregation was often encountered. Several factors such as hybrid sterility, incompatibility and nuclear cytoplasmic interaction are believed to be the cause (Backes, *et al.*, 2003). Furthermore, distorted segregation ratios have also been previously observed in several DH populations suggesting indirect selection during the DH-production (Kretschmer *et al.*, 1997).

In the *Mla*-containing bulks of both populations a strong QTL was found on the short arm of chromosome 1H as seen in Table 5. In both cases the QTL was linked to the microsatellite marker *Bmac0399* and the allele for powdery mildew resistance came from *H. spontaneum* parent, confirming the BSA-results. The *Mla*-locus has previously been linked to the microsatellite marker *Bmac0213* (Yun, *et al.*, 2005) which is in the close vicinity of *Bmac0399* (Fig. 3) and described as a 261-kb resistance gene cluster including 32 predicted genes, 15 of which are associated with plant defense responses and 6 which are involved with response to powdery

mildew infection (Wei, Wing & Wise, 2002). In MP32A bulk 1, this QTL explained 25% of the field-score phenotypic variation and 32% of the observed phenotypic variation in the seedling test, no effect difference between the isolates phenotypic reaction could be observed (Table 5). These results confirm the presence of a *Mla*-allele in these individuals, test crosses will however be needed to characterise its nature. In MP32B, these results confirm the presence of the *Mla13*-resistance gene in bulks 1 and 2. However the strong effect of this QTL expressed in the second bulk, with the *Bg*Me-isolate (*Mla13*-virulent), cannot be attributed to the presence of *Mla13* but perhaps to a powdery mildew induced-response, such as the presence of the *Rar2*-locus which is required to some *Mla*-resistance reactions (Jørgensen, 1996) or, considering that its nature is equal to the one present in MP32A, the occurrence of the unknown *Mla*-allele found in MP32A. Indeed, the two genes segregating in MP32A could actually be the exotic-gene in MP32B. Further studies will be needed to resolve and clarify the nature of this exotic-gene and the effect of the QTL in the *Mla13*-virulent phenotype.

In the second bulk of MP32A, one QTL was found on 2H and 2 on linkage group 4, their main effects explained respectively 17.3%, 41,8% and 32,6% of the phenotypic variation observed in the seedling test (Table 5), they are all originating from the *H. spontaneum* parent. The field-scored data showed one QTL on linkage group 4 which, though linked to the same marker, was different to the others (Fig. 4), its main effect explained 33% of the phenotypic variation. Positional comparison with known qualitatively and quantitatively inherited resistance against powdery mildew where not conclusive as the only resistance described on 2H either originated from *H. bulbosum* (Pickering *et al.*, 1998) or *H. Laevigatum* (*MlLa*, Hilbers, Fischbeck & Jahoor, 1992). However, the *Rar1*-locus has previously been associated to several *Mla*-resistances and has been located on 2H (Jørgensen, 1996; Shirasu, *et al.*, 1999). This could indicate that the *Mla*-allele present in MP32A could therefore also be a *Rar1*-dependant resistance source. The lack of common markers between QTLs on linkage group 4 and others studies made comparisons impossible.

The results of the MP32B exotic-gene bulk (bulk 3) attributes 44% of the field-scored phenotypic variation to a QTL located on 3H and flanked by the SSR markers *Bmac0067* and *Bmac0209* (Fig. 4). The chromosomal position of the QTL localized was compared with those of known loci for qualitatively and quantitatively inherited resistance against powdery mildew, only a quantitative resistance locus has previously been described on 3H (*Rbgq2*, Backes, *et al.*, 2003; Shtaya, *et al.*, 2006), however, the differences in germplasm and environment and the lack of common markers between studies

make comparisons of common QTL difficult. The presence of this QTL could not be confirmed in the seedling tests or in bulk 2, this could be due to the restricted marker coverage, to the limited size of the population, the limited phenotypic evaluations, and/or because of faulty bulk assignation, and for the same reasons any other putative QTLs also remained undetected in this experiment. Therefore further studies will be required before this QTL can categorically be assigned to the MP32B's exotic-resistance gene. This lack of data complicates any comparison with the second bulk of MP32A, and the lack of common marker does not allow, nor contest, the possibility that linkage group 4 could actually be on 3H. Further experiment would be required to clearly identify and localize this QTL.
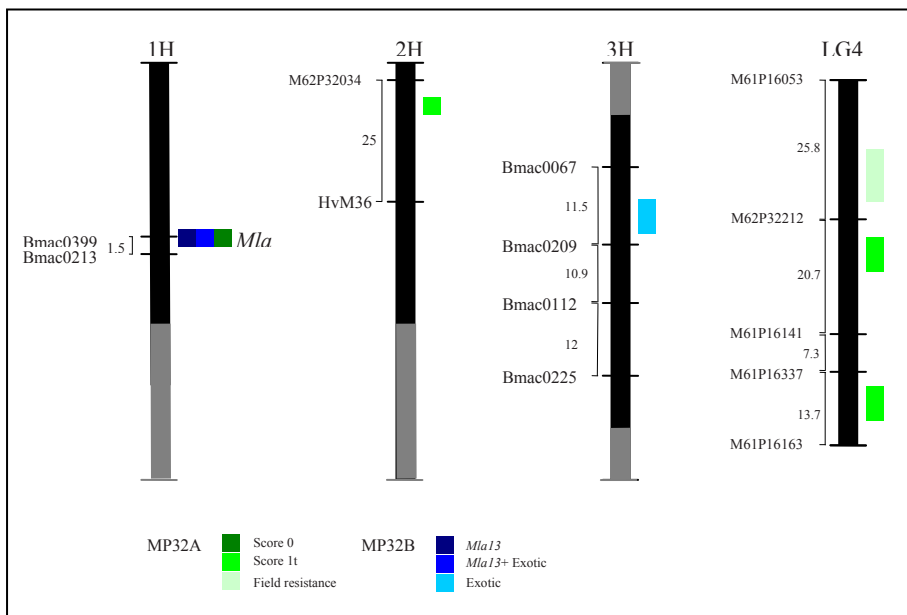
Fig. 4. Local map of barley linkage groups containing molecular markers linked to powdery mildew resistance loci, based on 'Sebastian' × 'RS 170-45' DH populations MP32A and MP32B. Marker identifications are provided on the left side of the map with the calculated genetic distance in Kosambi centimorgans, the identified QTLs for each resistance source are on the right. Note that the distances between microsatellites, mostly on 3H, vary compared to the ones positioned in the GrainGene's consensus map due to the broad nature of the crosses. Correspondence with know known resistance gene has been annotated in italics.

Table 5. List of detected QTLs in a simple interval mapping for QTL main effects using a threshold LOD of 2.5. The name of the closest flanking marker is displayed, the LG indicates the linkage group to which the identified marker is assigned, the LOD score is calculated from the F-value in the multiple regressions and Var. is the percentage of the scored phenotypic variance explained by the putative QTL. The H. spontaneum parent carries the favorable allele for powdery mildew resistance under study

Identifier	QTL BgGo	LG	LOD	Var.	QTL BgMe	LG	LOD	Var.	QTL Bg Field	LG	LOD	Var.
MP32A	**Phenotype BgMe**											
Bulk 1	0											
	Bmac0399	1H	2.74	31.8	Bmac0399	1H	2.74	31.8	Bmac0399	1H	2.85	25.3
Bulk 2	1t											
	M62P32034	2H	2.64	17.3	M62P32034	2H	2.64	17.3	M62P32212	4	4.07	32.9
	M62P32212	4	5.52	41.8	M62P32212	4	5.52	41.8				
	M61P16337	4	4.02	32.6	M61P16337	4	4.02	32.6				
MP32B	**Genotype**											
Bulk 1	Mla13	Bmac0399	1H	10.98	85.7	-				-		
Bulk 2	Exotic+ Mla13	Bmac0399	1H	5.83	55.7	Bmac0399	1H	2.68	31.2	Bmac0399	1H	3.33	37.1
Bulk 3	Exotic	-				-				Bmac0209	3H	3.02	44.0

Conclusion

We initially believed that the powdery mildew resistance inherited from the *H. spontaneum* line 'RS 170-45' could simply be resolved by a BSA. Beside the presence of *Mla13* in MP32B, the seedling test results made us envisage that only 2 resistance-genes could be present. Even after an unpretentious QTL-study the results showed to be insufficient in order to resolve the ambiguity, however they allowed us to hypothesize a more complicated picture. Assuming that MP32A and MP32B inherited the same resistance background, the results could be interpreted as follows. Firstly, it would seem, considering the results obtained in MP32A, that the 'RS 170-45'-powdery mildew resistance reaction is the product of several interacting resistance loci: one determined as an *Mla*-allele and at least 2 others: one located on 2H and one on an undetermined linkage group. Then, considering that these loci remained in linkage, and did not segregate in MP32B, would explain the *Mla*-reaction observed in the second bulk with the *Mla13*-virulent isolate. Finally, that linkage group 4 in MP32A actually is on chromosome 3H and coincides with the loci expressed in MP32B. However, we will need to answer some key-issues before considering these many assumptions: 1) access the identity of the MP32A *Mla*-allele 2) define the nature of the *Mla*-response in MP32B i.e.: presence of *Mla*-induced interactions 3) increase the resolution of the QTLs found on both populations 4) investigate any correlation between the QTLs found on 3H and linkage group 4. A new approach needs to be taken allowing a full-scale QTL study to be performed, this might require the production of new populations differentiated by the *Bg*Me-isolate and fully segregating for all the loci involved.

This resistance source may provide an interesting case study for genes interaction and because of the good field performances exhibited, a valuable source of broad-resistance for the constantly demanding resistance-breeding activity.

Acknowledgment

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