# Barley-*Puccinia* rusts: a model system to study the genetics, evolution and mechanisms of nonhost immunity in plants

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This thesis is dedicated to my dear parents, my dear wife and my lovely children: Negin and Yashar

# CHAPTER 1

## General introduction and scope of thesis

## Introduction

The genetic basis of nonhost immunity is one of the most intriguing topics in the field of plant pathology. The inheritance is very hard to study, since it typically requires interspecific crosses between host and nonhost species. Until now, mutagenesis and transformation have lead to the discovery of major key-genes in nonhost defence, but it is not clear what role, if any, those key-genes play in natural differences in host status between plant species. The work presented in this thesis is the first comprehensive forward genetics study on nonhost immunity which involves more than one heterologous (nonhost) pathogen species. Our system, barley-*Puccinia* rust fungi, focuses on natural variation in the plant species and is therefore evolutionary relevant. This model is based on an experimental barley line with exceptional susceptibility to several heterologous rust fungi. In this chapter the terms and concepts commonly used in plant immunity and defence are introduced followed by an explanation of the molecular basis, the genetics of nonhost resistance and the association between host and nonhost resistance. Finally the lay-out and scope of this thesis is presented.

## Innate and acquired immunity

Innate immunity is a well-described phenomenon in vertebrates and insects (Nurnberger et al. 2004). This type of immunity is based on recognition of pathogen-associated molecular patterns (PAMPs) and is induced upon infection by all pathogens (Boller 1995; Felix et al. 1999). Acquired immunity in animals, however, is based on recognition of highly specific antigens by highly specialized antibodies, induced upon infection by specialized pathogens and is carried out by specialized cells of the immune system (Boller 1995; Nurnberger et al. 2004).

Plants are not able to form antibodies. Consequently, the acquired immunity with specificities as strong as observed in animals (characterized by the creation of antigen-specific receptors through somatic recombination in maturing lymphocytes) does not exist in plants and this is one of the major differences between the immunity system in plants and animals. Innate immunity in plants is based on elicitor-induced defence which is activated upon infection by all microbes and is based on recognition of general elicitors (=ubiquitous molecules characteristic for whole groups of microbes). One of the important features of this kind of defence is rapid activation of defence responses which is generally without hypersensitivity (Boller 1995; Felix et al. 1999; Gomez-Gomez and Boller 2002; Nurnberger et al. 2004). In recent publications the word PAMPs is being used instead of general elicitors since PAMPs is a well-described term in animals and using this term in plants facilitates communication among scientists in both animal and plant science. Innate immunity in plants and animals both are therefore induced upon perception of PAMPs by specific and conserved molecules named Pattern Recognition Receptors (PRRs) (Felix et al. 1999; Gomez-Gomez and  Boller 2000). Interestingly, recent studies revealed striking similarities of innate immunity in plants and animals (Gomez-Gomez and  Boller 2002; Parker 2003). Remarkable similarity has been shown on the molecular building blocks of PAMP-induced signaling cascades, leading to the transcriptional activation of immune response genes, which are shared among plants and animals (Nurnberger et al. 2004). Bacterial flagellin is a PAMP by which gram-negative bacteria are perceived by plants and is an important virulence factor for those bacteria (Felix et al. 1999). FLS2 as a PRR is a part of the flagellin perception complex in plants. Interestingly, this protein shares a similar modular structure with receptors in Drosophila (Toll-like receptors) and in human (TLRs) (Gomez-Gomez and  Boller 2000).

### Heterologous and homologous pathogens

The majority of plant pathogens can only infect one or a few, mainly closely related, plant species or genera. The pathogen species that are not adapted to a plant species are known as heterologous (= inappropriate or nonhost) pathogens. The word heterologous refers to the fact that any potential pathogen has a limited range of plants on which it can cause a disease and often only plants of a single genus or species are host for a particular pathogen and all other plant species, i.e. the vast majority of species, are durably and effectively resistant. When the requirements for a pathogen for growth and development are met and the pathogen overcomes, tolerates or suppresses the general defence reactions of plants, then the plant species is considered as a host to the given pathogen which is called a homologous pathogen. The spectrum of plant species that a given pathogen species can infect is the host range of that pathogen.

### Basal defence, nonhost, near –nonhost and host resistance

Basal defence is not a well-defined word in plant pathology. One definition of basal (= basic) defence is related to the concept of basic compatibility. When a parasite has sufficient pathogenicity factors to successfully parasitize a plant species it has achieved a basic compatibility with that plant species (Heath 1991). In case of nonhost interaction where the host species of the pathogen is taxonomically distinct from the nonhost species, resistance is thought to be due to absence of a basic compatibility between the plant and the pathogen (Heath 1991). Heath ( 2000) has defined basal defence as the resistance that occurs when the pathogen lacks the required pathogenicity factors to achieve colonisation of the plant species and the plant does not provide the environment required by the pathogen for successful infection. The second definition of basal defence is based on inhibition of pathogen spread after successful infection and onset of disease. The existence of this type of defence is inferred from the identification of mutants that are more susceptible to a virulent pathogen than the wild type (Dangl and Jones 2001). The latter definition of basal defence pertains to a compatible interaction where a plant reacts inefficiently or too late to the pathogen. Such a resistance is considerably less obvious than that against avirulent pathogens that evoke a hypersensitive response. In this compatible interaction however, the plant still has some defence strategies to restrain the progress of a virulent pathogen. This type of defence is sometime referred to as basal defence or to polygenic, horizontal resistance that acts in slowing down of the disease development.

The two different definitions of basal defence explained above do not necessarily exclude each other. In some plant pathosystems such as barley-leaf rust (Niks 1982; Zhang et al. 1994; Hoogkamp et al. 1998) and barley-powdery mildew (Asher and Thomas 1983; Jorgensen 1994) resistance to heterologous pathogens and partial resistance (PR) are both based on poor haustorium formation, which in case of non-adapted pathogens is almost entirely prevented, while in PR haustorium formation is only hampered to some extent. Therefore it is likely that genes governing PR to a homologous pathogen play similar roles as genes for basal resistance to heterologous pathogens (Niks 1982; Hoogkamp et al. 1998). PR in barley has a polygenic inheritance, is governed by minor genes and is not based on hypersensitivity (Parlevliet 1975; Rubiales and Niks 1995).

The most durable, complete and common type of immunity of plants to potential pathogens is called nonhost resistance which by definition occurs in all genotypes of a plant species to all genotypes of a pathogen species (Heath 2000). None of the individuals of nonhost plant species allow any member of a potential pathogen species to successfully reproduce (Elmhirst and Heath 1987). This strict definition of nonhost resistance is however not easily applicable in practice. It has been shown that some genotypes of nonhost species can be infected by some heterologous pathogens to a limited extent and under special circumstances (Niks 1987). A few barley accessions, for example, are in the seedling stage somewhat susceptible to rust species like the wheat leaf rust fungus (*P. triticina*) and the wall barley leaf rust (*P. hordei-murini*) (Niks 1987; Niks 1996; Zhang et al. 1994; Hoogkamp et al. 1998). Near nonhost (=intermediate host) status has been proposed when only few accessions are at most only moderately susceptible to a normally heterologous pathogen (Niks 1987).

Immunity of a plant accessions to certain pathogen species can also be due to other types of resistance rather than nonhost and near-nonhost resistance. During evolution, some pathogens have evolved the capacity to breach nonhost defence mechanisms and to reproduce on a particular plant species, establishing a compatible interaction with the host plant, the so-called basic-compatibility (Neu et al. 2003). In such a compatible host-pathogen interaction the pathogen can be stopped in certain plant genotypes that possess one or more race specific resistance genes. Such genes act in a gene-for-gene interaction with effector genes (see below) in the pathogen, called in this context avirulence factors.

Race specific immunity is the well-described type of host resistance in plants, and is governed by major genes (qualitative) and is triggered upon direct or indirect recognition of avirulence gene products of a pathogen by race–specific resistance (*R*) proteins of host plant. This type of immunity is usually associated with HR reaction and in biotrophic fungi occurs after haustorium formation (=posthaustorial). Therefore, a host species can have two types of host resistance, viz (1) the prehaustorial partial resistance, which can be considered a form of basal resistance or reduced basic compatibility, and (2) the posthaustorial hypersensitivity resistance.

**Molecular basis of nonhost resistance**

The first line of defence in plants is the presence of both physical and chemical preformed or constitutive factors. Plant cell wall and cuticule are the first physical line of defence. Cell wall structures have an important role as physical barriers against fungal infections (Thordal-Christensen 2003). Preformed chemical defence, which comprises phenols and alkaloids, are constitutively present and can prevent establishment of infection units of a potential pathogen (Dixon 2001; Heath 2000; Kamoun 2001; Nurnberger and Lipka 2005). Although constitutive plant features contribute to nonhost resistance, they rarely account completely and solely for the nonhost status of a plant species (Heath and Skalamera 1997).

Active defence responses such as cell-wall apposition and papilla formation (Figure 1) are other lines of defence in plants (Wolter et al. 1993). When a pathogen manages to negate the two earlier lines of defence (physical / chemical barriers and cell wall) it may become subject to perception at the plasma membrane of plant cells. Perception of such signals by plant receptors leads to activation of defence responses through HR reaction as the next line of defence (Gomez-Gomez and Boller 2002; Jones and Takemoto 2004). Interestingly, Christopher-Kozjan and Heath (2003) collected some cytological and pharmacological evidence suggesting that biotrophic fungi trigger different cell death execution processes in host and nonhost cells during the hypersensitive response. PAMP-based microbe recognition and defence activation together with preformed, constitutive defence barriers are suggested to explain the molecular basis of nonhost resistance (Nurnberger and Lipka 2005). See Figure1. Therefore nonhost resistance is thought to be a multi-component phenomenon (Heath 2001).

Attempts by microbial invaders to infect nonhost plants trigger the defence-signaling network. In general three major pathways are known to be involved in plant defence; the Salycilic Acid (SA), Jasmonic Acid (JA) and Ethylene (ET) pathways. The SA pathway plays an important role in defence responses initiated by *R*-genes (Glazebrook 2001). The analysis of mutants impaired in hormone homeostasis revealed that all three pathways are not only crucial to cultivar-specific host plant resistance, but probably are indispensable for the maintenance of nonhost resistance in specific plant–microbe combinations (Mysore and Ryu 2004). For example, SA as a key signaling molecule in plant defence to host pathogens has been implicated in nonhost resistance in *Arabidopsis* to the cowpea rust fungus, *Uromyces vignae* (Mellersh and Heath 2003). Tobacco plants impaired in ethylene perception lacked nonhost resistance to some species of *Pythium* (Knoester et al. 1998) while nonhost resistance of *Arabidopsis* to *Alternaria brassicicola* depends on JA perception (Thomma et al. 1998).

Adapted pathogens evolved the ability to suppress the active defence that is triggered by PAMPs. In *Arabidopsis* plants, for example, active defence to a bacterial pathogen *Pseudomonas syringae* is suppressed soon after initiation of a defence response (Kang et al. 2003) probably by injection of a large number of effector proteins into the plant cells (Kang et al. 2003; Kim et al. 2005).

The crucial role of suppression has been demonstrated in particular for rust and powdery mildew pathogens that form long-term biotrophic relationships with their host plants and for plant pathogenic bacteria (Mellersh and Heath 2001; Panstruga 2003; Nomura et al. 2005).

### Association between host and nonhost resistance

The idea that “plants defend to host and nonhost pathogens in the same way” has received supporting evidence in plant biology. Many inducible defence responses are involved in the expression of both host and nonhost resistance, suggesting that host and nonhost resistance can be elicited by both parasite-specific and parasite-non-specific signals (Heath 2000).

Gene expression studies in the *Arabidopsis–P. syringae* pv. *Phaseolicola* nonhost

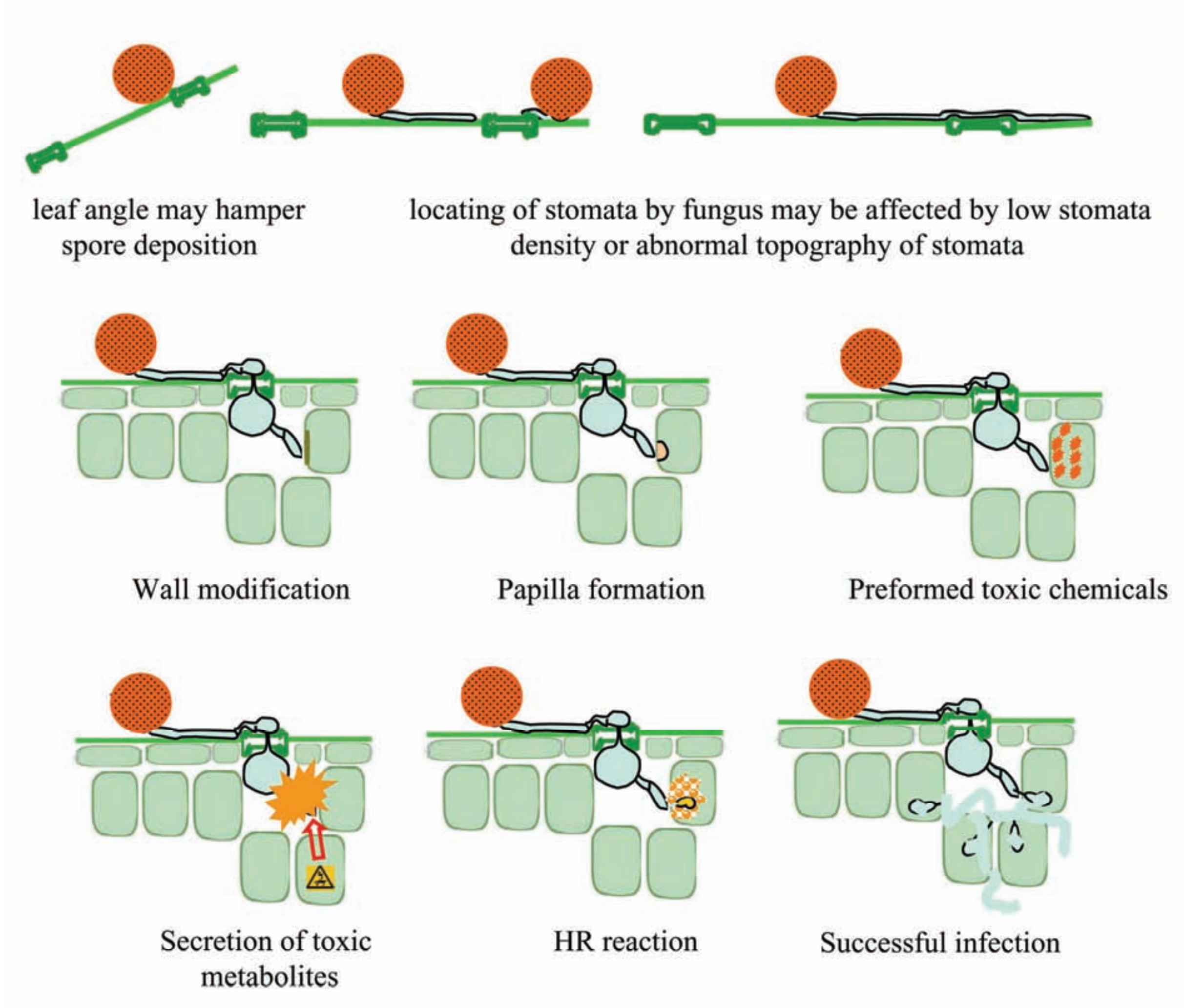

**Figure 1.** Cartoon illustrating the summary of performed and inducible defence responses in plants that are potentially involved in nonhost resistance. Plants may have one, two or more defensive features against nonhost pathogens. Successful infection occurs when a pathogen can negate all potential defence mechanisms.

interaction showed that a wide array of plant defence genes was activated, and this array is similar to that induced during a gene-for-gene interaction with host pathogens (Tao et al. 2003; Peart et al. 2002). The *EDS1*gene (enhanced disease susceptibility 1) in *Arabidopsis*, is shown to be involved in host resistance. The *Arabidopsis eds1* mutant, however, is partially susceptible to different isolates of two heterologous pathogens viz. *Peronospora parasitica* and *Albugo candida*. (Both *P. parasitica* and *Albugo candida* are pathogens of *Brassica oleracea*) (Parker et al. 1996).

In barley, the mechanisms of partial resistance to the homologous rust *P. hordei* not only are similar to that of nonhost resistance to heterologous rusts, but also there seems to be a moderate association between level of partial resistance and level of resistance to two heterologous rust fungi (Hoogkamp et al. 1998; Zhang et al. 1994).

## Genetics of nonhost resistance

Our knowledge about the genetics behind nonhost resistance is negligible mainly due to lack of proper genetic systems to study the genetic basis of this phenomenon. By definition, all individuals of the nonhost plant species are immune to the pathogen (Heath 2000) and members of plant species typically do not exhibit genetic variation for nonhost resistance. Therefore, study of the genetics of nonhost resistance would require interspecific crosses between host and nonhost species. There are very rare examples of successful crosses between host and nonhost species (Jeuken and Lindhout 2002). Progeny from interspecific crosses, if obtained at all, frequently suffer from sterility, low seed viability and abnormalities in chromosome pairing during meiosis. Mutagenesis is frequently and successfully used to find genes that affect the level of nonhost resistance. Genes that have been identified in this way may play a role in perception of PAMPs, in the signal transduction or as compatibility factors (Table 1), but it is doubtful whether in nature these genes determine whether a plant is a host or a nonhost to a particular pathogen. Therefore such genes are not evolutionary relevant to understand the genetic basis of host status. An alternative to investigate the genetics of nonhost resistance is use of the near-nonhost resistance systems assuming that the results can be extrapolated to full non-host systems.

## Barley-rust pathosystem as a model to study nonhost resistance

Barley exhibits a complete resistance to the many heterologous rust pathogens such as rye leaf rust fungus (*P. recondita*). However, some barley lines are at the seedling stage somewhat susceptible to heterologous leaf rust fungi such as the wheat leaf rust and the leaf rust fungus of *Hordeum murinum* (Niks 1983; Niks 1996; Hoogkamp et al. 1998). Although one may argue that such a plant species cannot be defined as a nonhost in the strict sense, it is likely that in such “near-nonhost” plant-pathosystems similar types of resistance occur as in full nonhosts, and they can therefore be used as a model to study the genetic basis of this type of resistance. In addition, barley as a diploid crop with a rather simple genome and chromosome homology to common wheat (Moore 1995) seems to be an ideal model crop to study the genetics of nonhost resistance and to extrapolate the results to the other cereals, like wheat, with more complex genomes. Another advantage of using barley is the availability of massive information on the genetics of host resistance, including partial and hypersensitive resistance, to *P. hordei*. At the Laboratory of Plant Breeding of Wageningen University, there is a long tradition of research on partial resistance that retards epidemic development of disease although plants show a compatible (non-hypersensitive) infection type (Parlevliet

1975). During the last decades, many aspects of partial resistance have been determined in barley and by using classical genetics, molecular markers, field studies, QTL mapping, and microscopy, comprehensive information has been collected about the genetics and mechanisms of PR in barley to leah rust (*P. hordei*) (Parlevliet, Niks and associates, Qi et al. 1998; Qi et al. 2000). The histological observations on infected barley lines suggested that PR and nonhost resistance might be due to the same mechanism (Niks 1983). Study of nonhost resistance in barley therefore may provide clues to the nature of PR to and also raises the question whether genes for PR to leaf rust are also part of the defence system of barley to heterologous rust fungi.

The majority of rust fungi can only infect one or a few closely related plant species or genera. This high degree of specialisation implies that plant species that are host to one pathogenic rust fungus are nonhost to most other rust fungal species, even to rust fungi that are pathogenic to a closely related plant species (Zhang et al. 1994). It is a fascinating question which genetic factors both in plants and pathogens determine whether a plant species is a host or nonhost for a pathogen species. This question may be addressed using such a model system in which pathogens are extremely host specific. The high specificity of rusts along with the large number of rust species on cereals and related grasses, allow studies with rusts that vary in their degree of phylogenetic relationship, and to relate pathogenicity characteristics of rusts to the degree of relationship between their respective host species.

## Scope of the thesis

This thesis presents an extensive genetic study on nonhost resistance of barley to different heterologous rust fungal species by using a forward genetic approach to answer the following questions: (1) What is the genetic basis of nonhost immunity and is that based on quantitative (QTLs) and/or qualitative resistance genes? (2) How rust species specific are the genes involved in nonhost immunity and is there any association between host resistance (HR and partial) and nonhost resistance? (3) Is the immunity of barley accessions to heterologous rusts mainly due to genes that they have in common, or does each immune barley accession have a different set of genes causing the immunity?

**Chapter 2:** We first focused on host range quantification of barley to different heterologous rust species. We exposed 109 barley accessions in the seedling stage to a collection of 19 rust species and isolates of cereals and grasses. We determined the relationship between level of susceptibility at seedling and at adult plant stage and also the rust and isolate specificity of the resistance of barley accessions to heterologous rust species

**Table 1**. Mutagenesis, transformation, intraspecific and rarely interspecific crosses have been used for genetic dissection of nonhost resistance to fungi and bacteria in different pathosystems during the last decade

| **Plant pathosystem** | **Approach** | **Genetics** | **Reference** |
|---|---|---|---|
| *Triticum aestivum/Puccinia striiformis* f. sp. *hordei* | Intraspecific cross | One single *R*-gene confers resistance in wheat to the barley stripe rust | (Pahalawatta and Chen 2005a) |
| *T. aestivum/Puccinia striiformis* f. sp. *hordei* | Intraspecific cross | Four QTLs were mapped for nonhost resistance of wheat to barley stripe rust | (Rodrigues et al. 2004) |
| *Hordeum vulgare/P. striiformis* f. sp. *tritici* | Intraspecific cross | Two major race-specific genes control resistance in barley to wheat stripe rust | (Pahalawatta and Chen 2005b) |
| *Lactuca saligna/Bremia lactucae* | Interspecific cross | 3 QTLs and one *R*-gene involved | (Jeuken and Lindhout 2002) |
| *Arabidopsis/Blumeria graminis* f. sp. *hordei* | Mutagenesis | *PEN1* gene in *Arabidopsis* affects penetration by barley powdery mildew | (Collins et al. 2003) |
| *Arabidopsis /Pseudomonas* | Mutagenesis | *NHO1* is required for general resistance against *Pseudomonas* bacteria | (Lu et al. 2001) |
| *Maize/Xanthomonas oryzae* pv. *oryzicola* | Transformation | Nonhost resistance in maize to the *X. oryzae* is under the control of a single gene named *Rxo1* | (Zhao et al. 2004) |
| *Nicotiana benthamiana/ Pseudomonas , Xanthomonas* | Transformation | *SGT1* is required for host and nonhost disease resistance in plants | (Peart et al. 2002) |
| *Hordeum vulgare/P. triticina* | Gene expression (SSH) | Two genes, including a barley ortholog of the rice resistance gene *Xa21*, and another novel gene are involved in nonhost resistance to *P. triticina* | (Neu et al. 2003) |
| *Arabidopsis/Pseudomonas* | Mutagenesis | Using an *Arabidopsis* defence-related gene (*SID2*) showed that SA is synthesized from chorismate and that SA is required for both local and systemic acquired responses | (Wildermuth et al. 2001) |
| *Arabidopsis/Peronospora parasitica-Albugo candida* | Mutagenesis | *Arabidopsis eds1* mutant is partially susceptible to several isolates of *Peronospora parasitica* and *Albugo candida* for which *Arabidopsis* is a nonhost | (Parker et al. 1996) |
| *Arabidopsis/Albugo candida* | Mutagenesis | *Rac4*, an *Arabidopsis* nonhost resistance gene is effective to the *Brassica oleracea* pathogen *Albugo candida*. | (Holub 2002) |

was investigated. Then we developed two research lines fully susceptible to *P. triticina* and to *P. hordei-murini* called SusPtrit and SusPmur respectively, by accumulating genes for susceptibility (Figure 2). In the seedling stage these accessions were as susceptible as the host species to the target rusts and also showed unusual susceptibility to some other heterologous rusts and therefore we used the line SusPtrit to develop two mapping populations.

**Chapter 3:** We developed a mapping population from a cross between the research line SusPtrit and the regular, immune, cv Vada. After seven generations of single seed descent, from 200 $F_2$ plants a population of 152 $F_8$-drived Recombinant Inbred Lines (RILs) was obtained and genotyped with AFLP and SSR molecular markers (Figure 2), in order to construct a linkage map. Seedlings were phenotyped with 8 isolates belonging to five heterologous and two homologous (host) rust species. By QTL analysis, chromosomal regions implicated in resistance were mapped on the barley genome. The number and specificity of QTLs for different rust species and also association of host and nonhost resistance were studied in this chapter. Furthermore, the internal transcribed spacer sequences (ITS) of rust fungi were analysed as a measure of taxonomic relationship between the rust fungal species applied in the study. This allowed us to determine whether or not QTLs with double or multiple effectiveness tend to be effective to rust species that are phylogenetically related.

**Chapter 4**: We developed a second mapping population by crossing SusPtrit with immune barley, cv Cebada Capa. We constructed a molecular marker map and phenotyped the RILs at the seedling stage with four heterologous rust fungal species and host pathogen *P. hordei.* The Oregon Wolf Barleys population, which showed unexpected segregation for resistance to heterologous rusts, was also phenotyped with those four heterologous rust fungal species (Figure 2). The availability of three populations segregating for resistance to heterologous rust fungi allowed addressing the question whether barley accessions commonly share resistance genes to heterologous rust species. The positions of chromosomal regions contributing to nonhost immunity were mapped through QTL analysis and were compared with those mapped in the Vada x SusPtrit population using an integrated consensus map. Furthermore, with this extensive data set we were able to test whether the genes involved in nonhost immunity co-locate with loci for partial resistance of barley to the pathogenic barley leaf rust fungus (*P. hordei*) and with defence gene homologues (DGHs) loci that possibly play a role in plant defence.

**Chapter 5:** Host status of barley to *P. hordei-bulbosi (Phb)* was studied using a set of 105 barley accessions. Barley is considered as a near-nonhost to the *Phb*. The host of *Phb* is the closest relative of *H. vulgare/spontaneum*, and the rust is closely related to the homologous *P. hordei* and the heterologous rust *P. hordei-murini*. This made it very interesting to compare the association of resistance to the various rust species for which the three mapping populations segregated. QTL analysis in three mapping populations was carried out based on macroscopic visible infection sites and the map position of QTLs for resistance to *Phb* in three maps were compared using an integrated consensus map. We studied the histology of infection to *Phb* and mapped QTLs involved in various components of infection, including colony size, early abortion without necrosis and the percentage of infection units associated with necrotic plant cell(s), in Vada x SusPtrit population.

**Chapter 6:** We provided a summarizing discussion of the thesis. Our finding about specificity, diversity, mechanisms and evolutionary aspects of nonhost immunity are discussed in this chapter. The possible role and nature of QTLs mapped for resistance to heterologous rust fungi as well as the future use of the barley-*Puccinia* model system are explained.

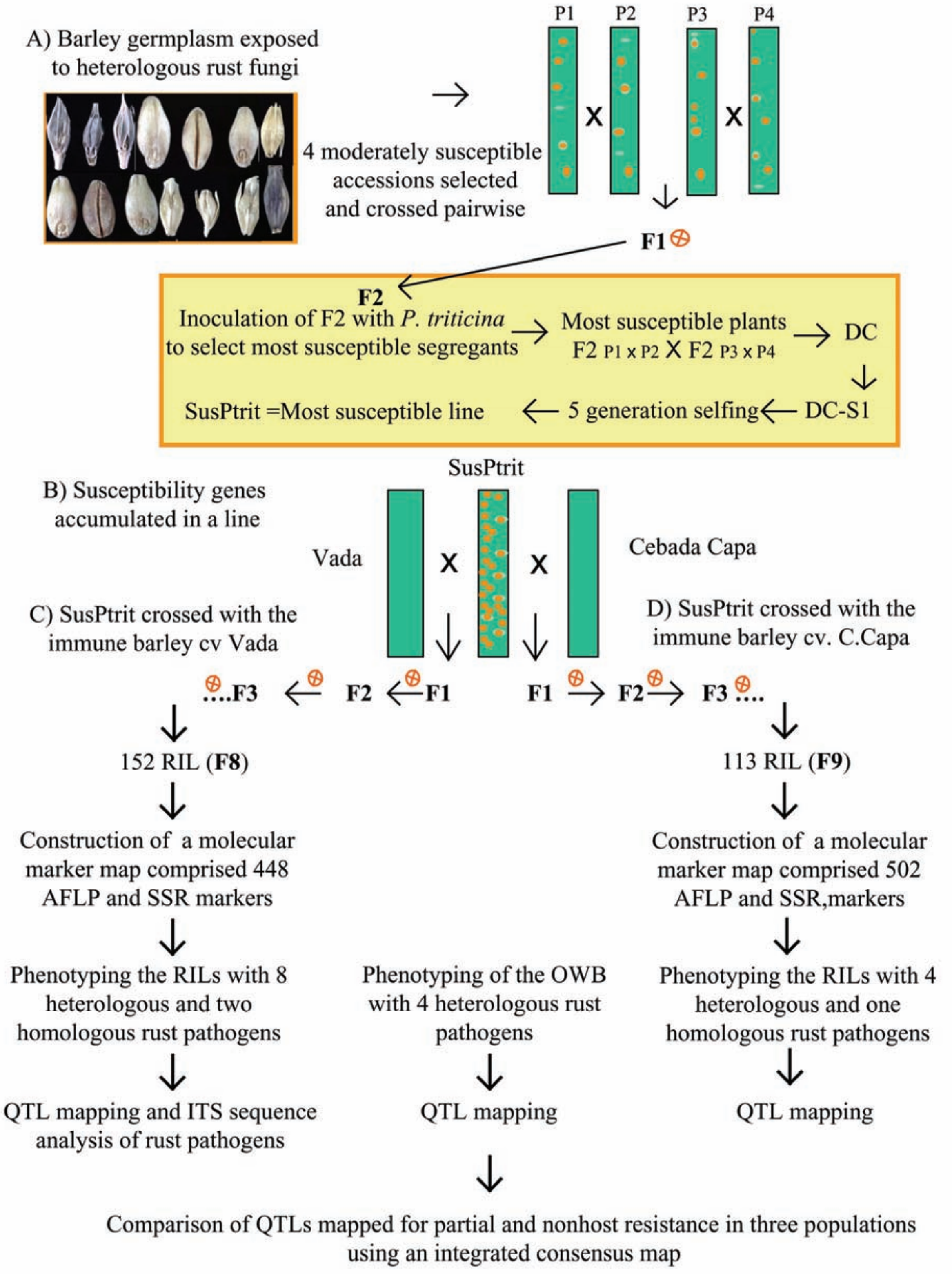

**Figure 2**. Working plan to study the genetics of nonhost immunity using the barley-*Puccinia* rust model

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# CHAPTER 2

# Accumulation of genes for susceptibility to rust fungi for which barley is nearly a nonhost results in two barley lines with extreme multiple susceptibility

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## ABSTRACT

Nonhost resistance is the most common type of resistance in plants. Understanding the factors that make plants susceptible or resistant may help to achieve durably effective resistance in crop plants. Screening of 109 barley accessions at the seedling stage indicated that barley is a complete nonhost for most of the heterologous rust fungi studied, while it showed an intermediate status in relation to *P. triticina*, *P. hordei-murini*, *P. hordei-secalini*, *P. graminis* f. sp. *lolii* and *P. coronata* ff. spp. *avenae* and *holci*. Accessions that were susceptible to a heterologous rust at the seedling stage were much more or completely resistant at the adult plant stage. Differential interaction between barley accessions and heterologous rust fungi was found, suggesting the existence of rust-species-specific resistance. In particular, many landrace accessions from Ethiopia and Asia and naked-seeded accessions tended to be susceptible to several heterologous rusts, suggesting that some resistance genes in barley are effective against more than one heterologous rust fungal species. Some barley accessions had race-specific resistance against *P. hordei-murini*. We accumulated genes for susceptibility to *P. triticina* and *P. hordei-murini* in two genotypes called SusPtrit and SusPmur, respectively. At the seedling stage these accessions were as susceptible as the host species to the target rusts. The accessions also showed unusual susceptibility to other heterologous rusts. These two lines are valuable assets to further experimental work on the genetics of resistance to heterologous rust fungi.

**Keywords**: *Hordeum vulgare*. Nonhost resistance. *Puccinia*. Rust fungi

## INTRODUCTION

Haustorium-forming fungi like rusts and powdery mildews can cause economically important losses. Cereals and grasses are hosts of several species and forms of these fungi. Understanding the factors that make plant genotypes and crop species susceptible or resistant may help to achieve durably effective resistance against such pathogens. Haustorium-forming fungi are characterised by extreme host specificity. The majority of these pathogens can only infect one or a few closely related plant species or genera. Resistance shown by all the individuals of a plant species to a specific parasite or pathogen species is known as nonhost resistance (Heath 1981; Elmhirst and Heath 1987). The pathogens involved in nonhost resistance are known as heterologous, inappropriate or nonhost pathogens (called “heterologous” in this paper). Nonhost resistance is the most common type of disease resistance in plants since only a few pathogen or parasite species within the myriad of potential ones develop a successful infection.

More is known about the mechanism of nonhost resistance than about its genetics. Various responses have been described, as reviewed by Heath (2000). Examples of responses in nonhost infections are the formation of callose deposits (Perumalla and Heath 1989; Vleeshouwers et al. 2000) and the deposition of phenolic compounds or silica in the cell wall (Heath and Stumpf 1986). The actin cytoskeleton (Kobayashi and Hakuno 2003; Mellersh and Heath 2003; Yun et al. 2003) and the adhesion between plasma membrane and cell wall (Heath 2002; Mellersh and Heath 2001) have recently been reported as relevant aspects in the defence against heterologous pathogens.

Gene expression studies have proven to be a useful tool to understand which genes are involved in defence reactions against heterologous pathogens (Neu et al 2003; Tao et al. 2003). Recently, Neu et al. (2003) have studied the *Hordeum vulgare-Puccinia triticina* interaction at the molecular level and have found four genes that are differentially expressed during this nonhost interaction. However, it is not known why such genes for defence are activated in nonhost species, and apparently not in susceptible host genotypes. It is likely that plants also contain genes, encoding “compatibility factors” (Panstruga 2003) or *R*-genes, that perceive the invading potential pathogen and determine whether or not defence genes will be activated. Such genes might be discovered in inheritance and mapping studies. However, genetic studies are difficult to perform since by definition all the individuals of the nonhost plant species are completely resistant to the pathogen. Therefore, the study of the genetics of this type of resistance would require interspecific crosses between host and nonhost species followed by the development of backcross inbred lines (Jeuken et al. in press) or of chromosome addition lines (Johnson et al. 1994; Martens et al. 1983; Riley and Macer 1966) or crosses between *formae speciales* (Tosa et al. 1987; Cotter and Roberts 1963). Approaches based on the use of mutants to investigate signaling pathways are also used (Mellersh and Heath 2003). If applied to susceptible host plants, mutagenesis may lead to the detection of putative compatibility factors (reviewed by Panstruga 2003).

We propose to follow a genetic approach, by studying the genetics of resistance in a species that is intermediate between host and nonhost status. It has been pointed out that the distinction between host and nonhost status is not always clear (Heath 1985; Niks 1987). The genetics of such near-nonhost resistance can be investigated, by crossing some rare genotypes showing moderate susceptibility to some regular completely resistant genotypes (Niks et al. 1996). We suppose that the genetics of near-nonhost resistance is relevant to understanding the genetics of full nonhost resistance (Zhang et al. 1994). Because of its near-nonhost status for some heterologous rust species, *Hordeum vulgare* L. is a useful model crop to study the genetics and the mechanisms of resistance against heterologous rust fungi such as *P. triticina* and *P. hordei-murini*. To our knowledge, there are few reports on the nonhost status of barley to heterologous pathogens (Mains 1933; Hassebrauk 1932). The objectives of the present work are fourfold. First, to quantify the host status of barley for different heterologous rust fungi. Second, to determine the relationship between the level of susceptibility in seedling and in adult plant stages. Third, to investigate the rust- and isolate-specificity of the resistance of barley accessions to heterologous rust species. Fourth, the development and evaluation of barley lines fully susceptible to *P. triticina* and *P. hordei-murini.*

## MATERIAL AND METHODS

### Plant material

A collection of 109 spring barley accessions from diverse geographic origins was subjected to infection experiments. The set contained modern and old cultivars from various continents, landraces and wild (*H. vulgare* ssp. *spontaneum*) accessions. Passport data were obtained from Centrum voor Genetische Bronnen, Nederland (CGN) located in Wageningen (http://www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources/Collections) and The Germplasm Resources Information Network (GRIN) located in the USA (http://www.ars-grin.gov/npgs/acc/acc_queries.html) and occasionally from other sources.

### Selection of barley genotypes to develop research lines with full susceptibility to *P. triticina* and *P. hordei-murini*

Earlier screens of barley accessions for susceptibility to *P. triticina* and *P. hordei-murini* (Niks et al. 1996; Hoogkamp et al. 1998) allowed identification of several accessions that showed some degree of susceptibility to these rust fungi. Barley accessions Menelik, L100, Trigo Biasa and Nigrinudum which had exhibited either a relatively high number of pustules or a high infection type were considered suitable for developing a research line highly susceptible to *P. triticina.* Line 52, Trigo Biasa, Nigrinudum and PI391136 (*H. vulgare* ssp *spontaneum)* were selected as lines relatively susceptible to *P. hordei-murini*. These susceptible accessions were crossed pairwise to start the selection programme.

### Pathogen material

Several heterologous rust pathogens were used (Table 1). These pathogens were multiplied on their respective host species as listed in Table 1. The complete set of 109 accessions, to which SusPtrit, SusPmur and the appropriate host species were added, was tested against each of these isolates. The test for susceptibility to *P. coronata* was performed first by inoculating with a mixture of four *P. coronata* formae speciales (ff. spp.) as listed in Table 1, to identify susceptible accessions. The accessions showing some degree of susceptibility were tested again for each *forma specialis* separately. This pooled testing was used because, on the basis of earlier unpublished tests, the *P. coronata* isolates were expected to produce hardly any successful infection in the accessions tested. For the same reasons seven other rust fungi were tested in two sets of mixtures. In one experiment the barley accessions were exposed to four rust species including: *P. bromina* (Dutch isolate), *P. recondita* of rye, *P. holcina* and *Uromyces dactylidis*; a few accessions that showed at least one pustule or many flecks were tested for each rust species individually. In another experiment three other rust fungi, viz. *P. vulpiana*, *P. recondita alopecuri*, and *P. bromina* (Spanish isolate), were used in a mixture as described previously. Inoculum of wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*) was also multiplied on susceptible host plants and was used in a separate experiment.

### Inoculation

Seedlings were grown in boxes (37 x 39 cm). Each line was represented by mostly three seedlings, each box containing 15 lines, including line L94 and the appropriate susceptible host as listed in Table 1 as reference. Urediniospores were produced on the appropriate host species and stored at -80 ºC.

The inoculations were carried out with freshly collected spores, in some cases supplemented with spores from the freezer to have a sufficient amount of inoculum, i.e. about 10 mg spores per plant box, resulting in a deposition of about 600 urediniospores per cm$^2$. An inoculation with the barley pathogen *P. hordei* was carried out using about 3 mg spores per plant box. Approximately a ten-fold greater volume of *Lycopodium* spores was added to the urediniospores to homogenise the distribution of the inoculum. Twelve days after sowing, completely unfolded primary leaves were fixed in a horizontal position, with the adaxial surface facing up. The inoculum was applied in a settling tower (Hoogkamp et al. 1998). The spores of wheat powdery mildew were administered directly onto barley leaves by shaking the infected host plants, which were sporulating abundantly. The plants were incubated overnight in a dew chamber for 9.5 hours (17-18 ºC) at 100% relative humidity. In the morning, the boxes were transferred to a greenhouse compartment at about 22/18ºC (day/night). Inoculation occurred with only one pathogen species per day.

To prevent cross contamination, the settling tower and other tools were cleaned with 96 % ethanol. Furthermore, atypical large and compatible pustules were considered contamination by *P. hordei*. In the cases of *P. coronata* and *P. graminis*, colour and spore morphology were also used to discriminate probable contamination by other rust species.

### Study at the adult plant stage

To study the level of susceptibility at the adult plant level, we used the *P. triticina* isolate ‘Flamingo’, the *P. hordei-murini* isolate ‘Rhenen’ and *P. agropyrina*. The plant material was selected on the basis of the results obtained at the seedling stage including resistant, moderately susceptible and susceptible accessions for each of the pathogens. In addition, SusPmur, SusPtrit and L94 were included in all the inoculations. Plants were grown in pots (16 cm diameter). For each accession, two pots each containing five plants were used. One repetition experiment was performed. Inoculation was performed on flag leaves and leaves immediately under the flag leaves using a mixture of rust and *Lycopodium* spores (1:10 w/w) on the leaves. Three mg of rust spores was used and five leaves per pot were inoculated by dusting the inoculum over them. This inoculation was not performed in the settling tower. Host plants were added as controls. Evaluation was performed as described for the seedling stage.

**Table 1.** Pathogen material used in host range quantification of barley

| **Pathogen/Isolate or *formae specialis*** | **Host plant** | **Common name** | **Place of collection** |
|---|---|---|---|
| *P. agropyrina* Eriks. | *Agropyron repens* (L.) Beauv | Wheat grass leaf rust | Wageningen (The Netherlands) |
| *P. triticina* Eriks./ Isolate Flamingo | *Triticum aestivum* L. ‘Little Club’ | Wheat leaf rust | Wageningen |
| *P. triticina* Eriks. /Isolate B9414-1cA3 | *Triticum aestivum* L.‘Little Club’ | Wheat leaf rust | France |
| *P. hordei-secalini* V.-Bourgin | *Hordeum secalinum* Schreb. | Meadow barley leaf rust | Mesquer (France) |
| *Puccinia graminis* subsp. *graminicola* Z. Urban | *Lolium westerwoldianum* | Rye-grass stem rust | Wageningen |
| *P. hordei-murini* Buchwald/Isolate Rhenen | *Hordeum murinum* L. | Wall barley leaf rust | Rhenen (The Netherlands) |
| *P. hordei-murini* Buchwald/ Isolate Córdoba | *Hordeum murinum* L. | Wall barley leaf rust | Córdoba (Spain) |
| *P. coronata* Cordaf. sp. *festucae* | *Festuca gigantea* (L.) Vill. | Fescue Crown rust | Wageningen |
| *P. coronata* Corda f. sp. *avenae* | *Avena sativa* L. cv.Cebeco | Oat crown rust | Wageningen |
| *P. coronata* Corda f. sp. *holci* | *Holcus mollis* L. | Yorkshire fog crown rust | Wageningen |
| *P. coronata* Corda f. sp. *lolii* | *Lolium westerwoldianum* | Rye-grass crown rust | Wageningen |
| *P. bromina Eriks./* Isolate Wageningen | *Bromus sterilis* | Bromus leaf rust | Wageningen |
| *P. bromina Eriks./* Isolate Spain | *Bromus sp.* | Bromus leaf rust | Barcelona (Spain) |
| *P. recondita* Roberge f. sp. *recondita* | *Secale cereale* L. | Rye leaf rust | Wageningen |
| *P. holcina Eriks.* | *Holcus mollis L.* | Yorkshire fog leaf rust | Veenendaal (The Netherlands) |
| *Uromyces dactylidis* Otth | *Dactylis glomerata* L. | Cock’s-foot rust | Rhenen |
| *P. recondita* Rob. ex Desm. f. sp. *alopecuri* | *Alopecurus pratensis* L. | Fox tail leaf rust | Wageningen |
| *P. vulpiana* Guyot | *Vulpia myuros* | Vulpia rust | Wageningen |
| *Blumeria graminis* (DC.)E.O.Speer f. sp. *tritici* Em.Marchal | *Triticum aestivum* L.‘Little Club’ | Wheat powdery mildew | Zelder BV, Gennep, (The Netherlands |

## RESULTS

### Host status of barley to different heterologous rust pathogens

There were several heterologous rust pathogens that could not infect any of the barley accessions. This was the case for *P. bromina* Eriks (Dutch and Spanish isolates), *P. holcina* Eriks., *P. recondita* Roberge f. sp. *recondita*, *P. coronata* Corda f. sp. *lolii*, *Uromyces dactylidis* Otth, *P. recondita* Rob. ex Desm. f. sp. *alopecuri* and *P. vulpiana* Guyot. Also the wheat powdery mildew fungus *B. graminis* (DC.) E.O. Speer f. sp. *tritici* Em. Marchal did not successfully reproduce on any of the barley accessions. Barley germplasm showed an intermediate susceptibility for the rest of pathogens (Tables 2, 3).

For any of the heterologous rusts, full susceptibility (scores 4 and 5 of the susceptibility scale) occurred on less than 10% of the accessions. In contrast, *P. hordei* infected 92% of accessions with score values 4 or 5 at three times less inoculum (Table 2). A very low susceptibility was found for a mixture of four formae speciales of *P. coronata* Corda (Table 1) that only marginally infected 3% of the accessions (Table 3).

**Table 2.** Percentage of barley accessions per susceptibility scores 0 – 5 for 10 heterologous rust species, one heterologous powdery mildew and *Puccinia hordei* at seedling stage

| | **Susceptibility score[a]** | | | | | |
|---|---|---|---|---|---|---|
| ***Pathogen*** | 0 | 1 | 2 | 3 | 4 | 5 |
| *P. agropyrina* | 39 | 7 | 9 | 37 | 8 | 0 |
| *P. triticina* Flamingo | 55 | 10 | 19 | 13 | 3 | 0 |
| *P. triticina* French | 54 | 10 | 17 | 19 | 0 | 0 |
| *P. hordei-secalini* | 30 | 37 | 9 | 16 | 7 | 1 |
| *P. graminis* f. sp. *lolii* | 72 | 0 | 14 | 11 | 3 | 0 |
| *P. hordei-murini Rhenen* | 59 | 27 | 3 | 3 | 8 | 0 |
| *P. hordei-murini* Córdoba | 31 | 11 | 44 | 8 | 6 | 0 |
| *P. coronata* f. sp. *festucae* | 67 | 32 | 1 | 0 | 0 | 0 |
| *P. coronata* f. sp. *avenae* | 67 | 29 | 4 | 0 | 0 | 0 |
| *P. coronata* f.sp. *lolii* | 67 | 33 | 0 | 0 | 0 | 0 |
| *P. coronata* f. sp. *holci* | 67 | 31 | 2 | 0 | 0 | 0 |
| *Uromyces dactylidis* | 96 | 4 | 0 | 0 | 0 | 0 |
| *P. holcina* | 97 | 3 | 0 | 0 | 0 | 0 |
| *P. bromina* | 98 | 2 | 0 | 0 | 0 | 0 |
| *P. recondita* f. sp. *recondita* | 100 | 0 | 0 | 0 | 0 | 0 |
| *Blumeria graminis* f. sp. *tritici* | 100 | 0 | 0 | 0 | 0 | 0 |
| *P. hordei (1.2.1)*[b] | 3 | 0 | 0 | 5 | 66 | 26 |

[a] Susceptibility score (per leaf):
0: Immune or near immune (less than 3 pustules and few flecks)
1: Less than 3 pustules and medium or many flecks    2: 3-10 pustules
3: 10-100 pustules    4: More than 100 pustules
5: More than 500 pustules

[b] Amount of inoculum was three times less than for heterologous rusts

This low susceptibility was probably due to infection by *avenae* and *holci* formae speciales (Table 2). The other heterologous rust species caused some infection (score 2 or higher) on up to 58% of the accessions (Table 3). The highest susceptibility was observed for *P. hordei-murini*, isolate Córdoba, and *P. agropyrina.* The data (Table 3) indicate that naked-seeded barley accessions are more susceptible to heterologous rust fungi than others.

Susceptibility in landraces of African and Asian origin and in wild barley was relatively common and obviously higher (scores 3 and 4) than in modern cultivars of European origin. Examples of such accessions with relatively high susceptibility to several heterologous rusts are L94 and Trigo Biasa (Table 4). Many accessions, like Cebada Capa, were fully resistant to all heterologous rust species. These results indicate genetic variation in barley for resistance to heterologous rust species and that none of the heterologous rust fungi can infect barley as successfully as the barley pathogen *Puccinia hordei*.

### Development of a research line with full susceptibility to *P. triticina*

The $F_2$ lines were tested at the seedling stage for susceptibility to *P. triticina*, and those derived from the crosses Menelik × L100 and Trigo Biasa × Nigrinudum were the most susceptible. Transgressive segregation for number of pustules per leaf and infection type was clearly observed. Infection type ranged from immune to fully compatible but also included several degrees of hypersensitivity. In both $F_2$ lines the most susceptible plants were selected and grown to adult plant stage and crossed between the two crossing combinations to obtain double cross (DC) plants. Each DC plant was grown to develop DC-$S_1$ lines by selfing. The most susceptible plants within the most susceptible DC-$S_1$ lines were selected. After several cycles of selfing without selection, DC-$S_5$ lines were obtained and evaluated for susceptibility to *P. triticina*. The DC-$S_5$ line with the highest number of pustules per leaf and the highest IT was selected and named SusPtrit. Seed of this research line is derived from a single DC-$S_5$ plant.

### Development of a research line with full susceptibility to *P. hordei-murini*

In the same way six $F_2$ lines were obtained from crossings between accessions that showed some degree of susceptibility to *P. hordei-murini*. The two $F_2$ populations with the highest susceptibility to *P. hordei-murini* were selected, and from those the most susceptible plants. Selection criteria for susceptibility were as in the procedure to obtain SusPtrit. A DC population was obtained from the accessions Trigo Biasa × Line 52 and Nigrinudum × PI391136 (*H. vulgare* ssp *spontaneum*). Line 52 is a Recombinant Inbred Line derived from the cross L94 × C123 (Hoogkamp et al. 1998). The same generation-selection process as described above for *P. triticina* was followed here, except that selection was not only for susceptibility to the target rust *P. hordei-murini*, but also against some inconvenient agronomic traits of the *H. vulgare* ssp *spontaneum* ancestor, viz. seed dormancy, narrow first leaves and brittle spikes. The most susceptible DC-$S_5$ line was selected and named SusPmur. Like with the development of SusPtrit, transgressive segregation and a gradual increase in susceptibility occurred during the process of development of SusPmur.

**Table 3.** Level of susceptibility[a] (%) of barley germplasm (n=109) to 7 heterologous rust fungi

| | **Number of accessions** | *P. hordei-murini* 'Cordoba' | *P. hordei-murini* 'Rhenen' | *P. agropyrina* | *P. triticina* 'Flamingo' | *P. hor.-secalini* | *P. graminis lolii* | *P. coronata*[b] |
|---|---|---|---|---|---|---|---|---|
| **Level of agronomic application** | | | | | | | | |
| Wild species (*H. spontaneum*) | 6 | 40 | 17 | 50 | 83 | 50 | 83 | 17 |
| Line from land race and research lines | 30 | 80 | 33 | 50 | 57 | 60 | 45 | 3 |
| Cultivar released before 1945 | 17 | 70 | 0 | 70 | 0 | 18 | 18 | 0 |
| Cultivar released in 1945 or later | 44 | 45 | 7 | 55 | 25 | 16 | 16 | 2 |
| Unknown | 12 | | | | | | | |
| **Origin**[c] | | | | | | | | |
| Europe | 56 | 48 | 5 | 59 | 13 | 30 | 16 | 2 |
| North America | 7 | 85 | 0 | 33 | 50 | 33 | 33 | 0 |
| South America | 13 | 46 | 0 | 50 | 33 | 25 | 17 | 0 |
| Africa | 10 | 77 | 70 | 20 | 80 | 50 | 30 | 0 |
| Asia | 12 | 91 | 23 | 85 | 70 | 92 | 54 | 0 |
| Unknown | 5 | | | | | | | |
| **Morphological traits**[c] | | | | | | | | |
| 6-row | 35 | 71 | 17 | 54 | 40 | 45 | 34 | 3 |
| 2-row | 68 | 56 | 12 | 54 | 26 | 24 | 20 | 1 |
| Naked seeded | 10 | 90 | 60 | 50 | 80 | 80 | 70 | 0 |
| Covered seeded | 93 | 59 | 9 | 55 | 26 | 26 | 19 | 2 |
| Black seeded | 7 | 85 | 71 | 0 | 71 | 57 | 43 | 14 |
| White seeded | 96 | 46 | 9 | 58 | 25 | 29 | 23 | 1 |
| **All accessions** | 109 | 58 | 14 | 54 | 36 | 32 | 29 | 3 |

[a] Susceptibility means at least 3 pustules per leaf

[b] Mixture of 4 *formae speciales*

[c] Excluding the *H. spontaneum* accessions

### Susceptibility of SusPtrit and SusPmur to heterologous rust pathogens

SusPtrit and SusPmur showed a much higher susceptibility than any of the 109 accessions studied in this research. SusPtrit was the accession with the highest susceptibility to *P. triticina*. Its level of susceptibility was as high as that of the accession of the susceptible host species (Fig. 1), and higher than that of each ancestor (Table 4). Interestingly, SusPtrit showed full susceptibility not only to the target rust, but also it was the line with the highest susceptibility to several non-target rusts like *P. agropyrina, P. hordei-secalini* and *P. graminis* f. sp. *lolii* (Table 4). The susceptibility of SusPtrit and SusPmur also seemed to have a rust-species-specific component, since SusPtrit was much less susceptible to *P. hordei-murini* than to *P. triticina*, and for SusPmur the reverse was true (Fig. 1, Table 4). SusPmur was fully susceptible to the target rust *P. hordei-murini*. In addition, SusPmur was also susceptible to some non-target rusts like *P. hordei-secalini, P. graminis* f. sp. *lolii,* and *P. coronata* f. sp. *lolii*, but rather resistant to *P. agropyrina* and *P. triticina* (Table 4). Both SusPtrit and SusPmur fulfil the objective for which they were developed, i.e., full susceptibility to *P. triticina* and *P. hordei-murini*, respectively.

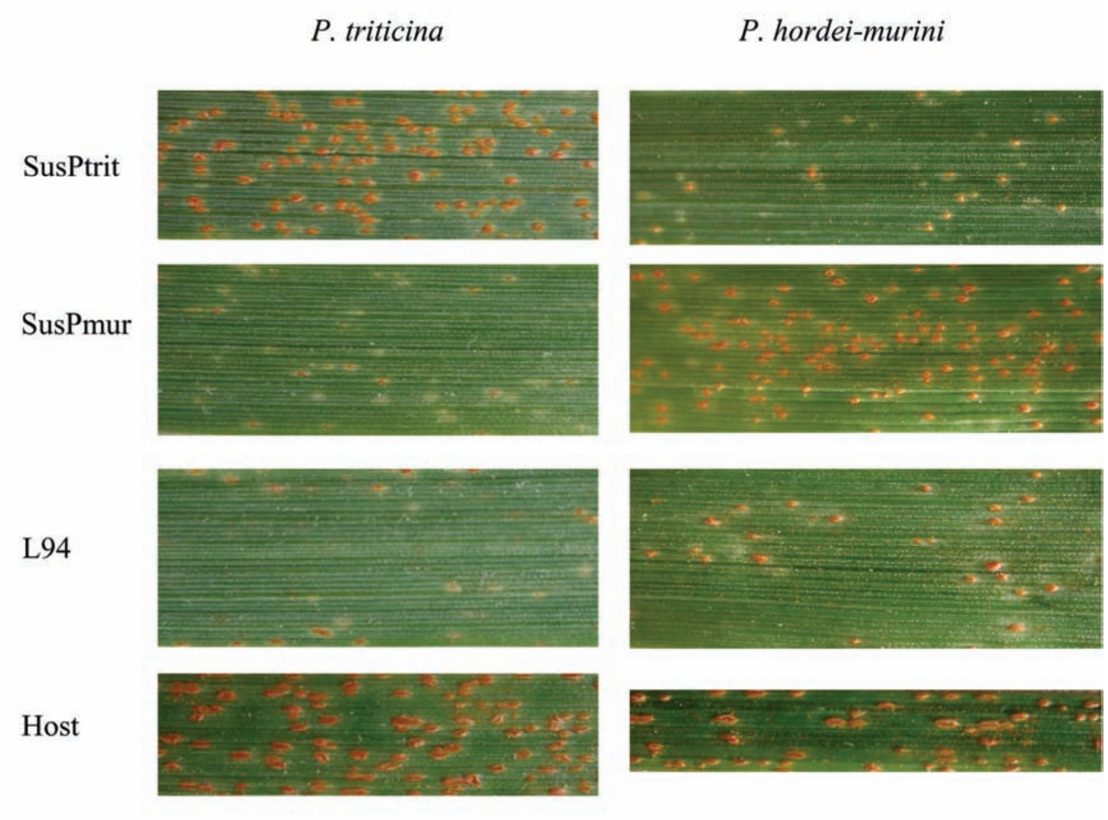

**Figure 1**. Urediosores of *Puccinia triticina* and *P. hordei-murini* on L94 and two lines, SusPtrit and SusPmur, with accumulated gene dose for susceptibility to these two heterologous rusts, as compared with the infection on susceptible host plants.

### Relation between susceptibility at the seedling and adult plant stages

We used *P. triticina* ‘Flamingo’, *P. hordei-murini* ‘Rhenen’ and *P. agropyrina* to evaluate several accessions at the adult plant stage selected on the basis of the results at the seedling stage. Resistant, moderately susceptible and susceptible accessions for each pathogen were included. SusPtrit, SusPmur and L94 were evaluated against the three rusts. Most of the accessions were fully resistant in the adult plant stage and only five accessions, including research lines SusPtrit and SusPmur, showed some susceptibility to heterologous rusts. The corresponding host species showed full susceptibility in all the cases except *P. agropyrina*.

The use of a different accession of *Agropyron repens* for seedling and adult plant studies may explain the difference in susceptibility of this host species between the two studies. These observations indicate that, even in barley accessions with the highest susceptibility to heterologous rusts, a very high level of resistance appears as the plants reach the adult plant stage. To evaluate to which extent resistance to heterologous rust fungi may be isolate-specific, we used two different isolates of *P. triticina* and *P. hordei-murini* (Table 1). In the case of *P. triticina*, the percentage of susceptible accessions was the same for both isolates (Table 2) and there was no strong evidence indicating isolate specificity for this rust fungus.

There was a great difference between the two isolates of *P. hordei-murini*. About 58% of barley accessions showed susceptibility (score value at least 2) to the isolate collected at Córdoba, while the isolate from Rhenen produced a susceptible reaction in 14% of the accessions. In spite of this strong pathogenicity of the Córdoba isolate, a few barley accessions were resistant to this isolate but susceptible to the Rhenen isolate. This differential behaviour is clear in only a few lines, most notably in Japan 1 and L92 (Table 4). This suggests that some of the resistance to heterologous rust fungi is race-specific and may depend on the particular isolate of the rust taxon.

## DISCUSSION

### Host status of barley for the different heterologous rust pathogens

The first objective of this research was to determinate the nonhost status of barley for different heterologous rust pathogens (Table 1). Several of the pathogens studied could not cause any susceptible reaction in any of the accessions studied and therefore, barley may be considered a true nonhost to them. Barley may be considered a near-nonhost for *P. triticina*, *P. hordei-murini*, *P. agropyrina*, *P. hordei-secalini* and *P. graminis* f.sp. *lolii.*

**Table 4.** Susceptibility (number of pustules per leaf) of barley lines SusPtrit and SusPmur and some accessions of the tested barley germplasm to some heterologous rust fungi in seedling stage

| | **Heterologous rust** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Barley line** | *P. triticina* Flamingo | *P. hordei-murini* Rhenen | *P. hordei-murini* Córdoba | *P. agropyrina* | *P. hordei- secalini* | *P. graminis lolii* | *P. coronata f.sp. holci* | *P. coronata f. sp. lolii* | *P. coronata f. sp. avenae* | *P. coronata f. sp. festucae* |
| Vada | 0 | 0 | 2 | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cebada Capa | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Japan 1 | 32 | 125 | 0 | 3 | 6 | 0 | 1 | 1 | 1 | 1 |
| Nigrinudum | 9 | 135 | 180 | 0 | 162 | 18 | 0 | 0 | 0 | 0 |
| L92 | 87 | 360 | 12 | 2 | 163 | 0 | 1 | 1 | 1 | 1 |
| Trigo Biasa[a] | 134 | 245 | 200 | 192 | 432 | 158 | 1 | 0 | 0 | 1 |
| Hassan | 0 | 0 | 3 | 139 | 0 | 1 | 0 | 0 | 0 | 0 |
| L94[a] | 89 | 352 | 133 | 2 | 194 | 23 | 23 | 1 | 4 | 5 |
| SusPtrit | 708 | 575 | 225 | 438 | 657 | 166 | 10 | 0 | 5 | 5 |
| SusPmur | 19 | 700 | 517 | 37 | 475 | 142 | 6 | 56 | 5 | 1 |
| Control = host[b] | 618 | 138[c] | 694 | 609 | 344 | 49[d] | 300 | 396 | 500 | 32 |

[a]Line used to develop SusPtrit
[b]The respective host for each heterologous rust is indicated in Table 1.
[c]Low number of pustules due to small and narrow leaves of host plants
[d]Low number of pustules caused by systemic infection, leading to merging of pustules and colonies in host plants

To our knowledge, this is the first time that the host status of barley for the latter three pathogens has been established. Our results agree with reports by Mains (1933) and Hassebrauk (1932) indicating that *P. triticina* can infect a low percentage of barley accessions to some degree. Anikster (1989) reported that *P. hordei-murini* is confined to wall barley (*H. murinum*) and should not be pathogenic on cultivated barley. However, Niks et al. (1996) studied 212 cultivated barley accessions and reported a susceptible reaction on 16% and 8% of these lines for *P. triticina* and *P. hordei-murini*, respectively. We have used the same isolates as these authors (‘Flamingo’ for *P. triticina* and ‘Rhenen’ for *P. hordei-murini*). Also, one additional isolate for each pathogen was included in this study (Tables 1, 2, and 4). The number of barley accessions that showed susceptibility (score value of 2 or higher) to those pathogens in this research was higher than in the previous work reported by Niks et al. (1996). These differences may be caused by the higher quantity of inoculum used in this work compared to the previous one. It may be possible that our barley collection is slightly biased toward susceptibility to *P. triticina* and *P. hordei-murini* since the collection's size is smaller than that used by Niks et al. (1996) with some higher representation of exotic landraces. In any case, barley may be considered a near-nonhost for both rust species.

Susceptibility to heterologous rusts tended to be associated with uncovered seeds and Asian or African landrace origin (Table 3). This supports the conclusion that there is a linkage between the gene for naked seeds (on chromosome 1) and allele(s) for susceptibility to heterologous rusts as reported by Niks et al. (2000).

Modern cultivars often possess a fair level of partial resistance to *P. hordei* and may also carry one or more major genes for race-specific resistance to that rust (Niks et al. 2000). In two mapping populations there was a positive correlation between level of partial resistance to *P. hordei* and level of resistance to heterologous rusts in barley (Hoogkamp et al. 1998 and Zhang et al. 1994). This suggests that minor genes for resistance may also have a role against some heterologous rusts. It has been demonstrated that the race-specific resistance gene *Rph7* of the barley line Cebada Capa does not contribute to resistance against *P. triticina* (Neu et al. 2003; Niks and Rubiales 1994), but there is evidence that this gene was effective against *P. hordei-murini* (Niks and Rubiales1994). It has been suggested that individual *R*-genes are 're-used' again and again in plants and that plant species can have functional homologues of a particular *R*-gene and consequently the same *R*-gene may act towards different heterologous pathogens (Thordal-Christensen 2003). Therefore it may be speculated that resistance to heterologous rust fungi in modern cultivars is partly a consequence of accumulation of this type of major and several minor genes involved in resistance to rust fungi.

**Are different genes controlling the resistance against different pathogens?**

One of the most important questions regarding nonhost resistance in barley is whether the same or different resistance genes are controlling different pathogens. As a first step towards the elucidation of this point, we have compared the reaction of 109 accessions against different heterologous rusts that have low pathogenicity to barley. Barley exhibited different levels of susceptibility to heterologous rust fungi, depending on the rust species. Some lines were susceptible to one heterologous pathogen and immune or completely resistant to others. In several cases differential interaction occurred between barley accessions and heterologous rust, as shown in Table 4 for Hassan and L94. It seems that nonhost resistance of barley to heterologous pathogens is rather pathogen-species-specific. In spite of this obvious specificity, some accessions that were susceptible to one heterologous rust were also frequently susceptible to other heterologous rusts, like Trigo Biasa and L94 (Table 4). This suggests that barley may possess genes for general defence that protect against several heterologous rust fungi. It is interesting that even some evidence was obtained for race-specific resistance against a heterologous rust (Table 4, Japan 1 and L92 with the two isolates of *P. hordei-murini*). In conclusion, the results suggest that in barley both genes for general and genes for specific resistance against heterologous pathogens exist.

The transgressive segregation and gradual increase in susceptibility observed during the process of development of SusPtrit and SusPmur suggest a quantitative inheritance of the resistance against *P. triticina* and *P. hordei-murini*. In hardly any of the barley accessions did a sufficient gene dose occur to cause a high susceptibility (score value 5) to a heterologous rust.

**SusPtrit and SusPmur are valuable materials as the basis for experimental work**

To study the genetics of nonhost resistance of barley to heterologous rust fungi, accessions with susceptibility as high as in host plants are required to be intercrossed with regular, completely resistant accessions. Our host range quantification (Tables 2, 3 and 4) of barley to heterologous rust pathogens showed that full susceptibility in barley to heterologous rusts was rare or absent. This prompted us to develop some fully susceptible barley material, allowing subsequent studies on the inheritance of resistance to heterologous rusts. The gradual increase in level of susceptibility in the generations leading to SusPtrit and SusPmur suggests that the resistance against the heterologous rust is based on quantitative genes, each with probably relatively low effect. From a cross between a barley with a moderate gene dose of such genes and a normal, fully resistant cultivar, we expect the large majority of the progeny to be fully resistant and phenotypically identical, irrespective of whether they carry no, one or maybe two such quantitative genes for susceptibility. Only the relatively few progeny combining three or four such alleles would be phenotypically distinct and such a low number might be insufficient to determine the loci involved. In the RIL population derived from a cross between L94 as a line that is medium susceptible to *P. triticina* (Fig. 1 and Table 4) and the fully resistant line (Vada), the large majority of RILs was indeed fully resistant to *P. triticina* (unpublished data). SusPtrit and SusPmur were at the seedling stage as susceptible as accessions of the host species, not only for the target rusts *P. triticina* and *P.*

*hordei-murini*, respectively (Table 4), but also for several non-target rusts (Table 4). Therefore, those two lines are suitable to study the genetics of resistance against *P. triticina*, *P. hordei-murini*, *P. agropyrina*, *P. hordei-secalini*, *P. graminis* f. sp. *lolii* and *P. coronata* f. sp. *lolii*. The minor genes involved in resistance to those pathogens can be mapped in a RIL population derived from a cross between SusPtrit and resistant lines. Such mapping populations are being developed at present and will allow us to compare the inheritance of resistance to heterologous rust species with that of resistance to the barley leaf rust pathogen *P. hordei*.

## ACKNOWLEDGMENTS

S. G. Atienza and H. Jafary contributed equally to the work.

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# CHAPTER 3

## Innate nonhost immunity in barley to different heterologous rust fungi is controlled by sets of resistance genes with different and overlapping specificities

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## ABSTRACT

We developed an evolutionary relevant model system, barley – *Puccinia* rust fungi, to study the inheritance and specificity of plant factors that determine to what extent innate nonhost immunity can be suppressed. A mapping population was developed from a cross between an experimental barley line (SusPtrit) with exceptional susceptibility to several heterologous (nonhost) rust fungi and regular, immune, cv Vada. Seedlings were inoculated with five heterologous and two homologous (host) species of rust fungi. Resistance segregated quantitatively for each of the rust fungi. In total 18 chromosomal regions were implicated. For each rust species, a different set of genes was effective. Eleven chromosomal regions were significantly effective to only one rust species, seven regions were effective to more than one rust species, implying genetic linkage or pleiotropy. One *R*-gene for hypersensitive resistance to *P. hordei-secalini* was mapped, suggesting occasional contribution of *R*-genes to nonhost resistance in barley. QTLs with effects to multiple rust fungi did not tend to be particularly effective to rust species that were phylogenetically related, as determined from their ITS sequence. We suggest that the QTLs described here play a role as specific and quantitative recognition factors that are specifically negated by the rust to successfully suppress innate immunity.

**Additional keywords**: basal resistance, *Hordeum vulgare*, partial resistance, Rust fungi

## INTRODUCTION

In plants and animals, innate immunity, in particular resistance to nonhost pathogens, is induced by recognition of pathogen associated molecular patterns (PAMPs) (Zipfel and Felix 2005) which are generally occurring and conserved compounds, like chitin in fungi and flagellins of bacteria. The innate immunity system of animals and plants recognise PAMPs through pattern recognition receptors (PRRs) like FLS2 in *Arabidopsis* which is involved in flagellin perception of gram-negative bacteria and has a highly conserved structure (Gomez-Gomez and Boller 2000). One of the most abundant and conserved bacterial proteins, elongation factor Tu (EF-Tu), can also be recognized as a PAMP by *Arabidopsis* plants (Kunze et al. 2004). Zipfel et al. (2006) recently showed that a receptor kinase called EFR is essential for EF-Tu perception in Arbidopsis. Since host pathogens also contain PAMPs, they apparently have developed a way to suppress basal defence in their susceptible host plant. For example, *Pseudomonas syringae* pv. *tomato* contains flagellin, which indeed briefly initiates a basal defence, but within about 6 hours suppresses this response (Kang et al. 2003) probably by delivering a large number of effector proteins into the plant cells (Kang et al. 2003; Kim et al. 2005). Rust fungi probably suppress or prevent elicitation of basal defence in their hosts by interfering with communication between host plant plasmalemma and cell wall (Mellersh and Heath 2001). Basal defence of plants to rust fungi in many cases expresses itself as failed haustorium formation, without induction of a hypersensitive response (HR) (Mellersh and Heath 2001). If PAMPs occur universally in whole classes of pathogens, and PRRs that perceive PAMPs are conserved in plants (Gomez-Gomez and Boller 2000; Kunze et al. 2004; Zipfel and Felix 2005), then one of the challenges is to understand how pathogens are able to suppress basal defence in their host plant species, but not in closely related nonhost species. Given the presumed conservation of PAMPs and PRRs it is surprising for example that the rye leaf rust fungus *Puccinia recondita* is able to suppress basal defence in its host species rye (*Secale cereale*), but not in wheat (*Triticum aestivum*) (Niks and Dekens 1991) although rye and wheat are closely related and are able to hybridize. Obviously there is a range of specific factors in the pathogen and in the plant that determine the extent to which basal defence can be suppressed. Even within a host species there appears to be genetic variation in the degree to which basal defence can be suppressed by the pathogen. For example in the barley (*Hordeum vulgare*)/ barley leaf rust *(P. hordei*) system (Niks 1986a), some barley cultivars have a high level of non-HR polygenically inherited resistance, which has been coined "partial resistance" by Parlevliet (1975). In such partially resistant barley genotypes the barley leaf rust fungus seems to be less effective in suppressing the basal resistance reaction.

The objectives of this study were to investigate the inheritance and specificity of plant factors that determine the degree of suppression of basal defence by host and (near) nonhost pathogens and to determine whether there is a correlation between phylogenetic relationship of the fungal pathogens and specificity of these plant factors. We followed a forward genetics approach, using the barley cultivar Vada and recently developed experimental line SusPtrit.

The latter accession which is fully susceptible to the wheat leaf rust fungus *P. triticina,* was developed by a selection program for increased susceptibility to *P. triticina* (Atienza et al. 2004). Interestingly, this line was partially or fully susceptible to some other heterologous rust fungal pathogens as well. Rust fungi used in this study represent three taxonomic groups: i) *P. hordei* complex (*P. hordei* and *P. hordei-murini*); ii) *P. recondita /P. persistens* complex (*P. hordei-secalini, P. persistens, P. triticina* (from bread wheat) and *P. triticina-"duri"* (from durum wheat) and iii) *P. graminis (P. graminis* f. sp. *lolii* and *P. graminis* f. sp. *tritici).* Cultivated barley (*H. vulgare*) is a host for *P. hordei* and *P. graminis* f. sp. *tritici* but a near-nonhost for the other rust fungal species and *formae speciales* i.e. only a small fraction of barley accessions allowed some reproduction of these rusts fungi (Atienza et al. 2004).

## RESULTS

### Marker analysis and map construction

A total of 448 markers (AFLP, SSR and two morphological markers) were assembled into 18 linkage groups at a LOD threshold of 8.0, and could be united into 7 linkage groups with a total map length of 972 cM. The average marker distance on the extracted skeletal map used for QTL mapping was 5.4 cM. For those markers that had been mapped in L94 x Vada or other populations, the order and relative position in the linkage groups was consistent with previously published maps (Qi et al. 1998a; Ramsay et al. 2000; Liu et al. 1996)

### Disease tests with rust species and isolates to which barley is a near-nonhost

For all pathogens tested, frequency of visible infection sites (FVIS; the number of both flecks and pustules per $cm^2$), infection frequency (IF; the number of pustules per $cm^2$) and relative infection frequency (RIF; the number of pustules per $cm^2$ relative to the IF of the susceptible parent SusPtrit in the same box), showed continuous and quantitative segregation, indicating polygenic inheritance of the resistance. Frequency distributions of phenotypes for FVIS are shown in Figure 1.

The infection phenotypes for four heterologous rust fungi (*P. triticina, P. persistens, P. hordei-murini* and *P. hordei-secalini*) were nearly immune for Vada. SusPtrit was the most susceptible accession. However, in the case of *P. graminis* f. sp. *lolii* some RILs showed higher levels of susceptibility and resistance compared to the respective parental lines indicating that the two parents carried both susceptibility and resistance alleles to this rust fungus.

Resistance to *P. triticina* (three isolates), *P. persistens, P. hordei-murini* and *P. graminis* f. sp. *lolii* was not associated with a clear hypersensitive reaction (HR). For *P. hordei-secalini*, 59% of RILs showed HR response with hardly any pustule development (Figure 2Ad). The remaining RILs varied in level of non-HR resistance (Figure 2Aa, 2Ab and 2Ac). The HR phenotypes varied from restricted chlorosis (chlorotic spots) to extensive chlorosis (chlorosis of most of the leaf) and from small brown lesions (Figure 2Ad) to large confluent necrotic flecks. The levels of infection established by *P. triticina* and *P. persistens* ranged from immune (no pustules and less than 3 flecks per $cm^2$) to fully susceptible (maximum 92 and 76 sporulating pustules per $cm^2$, respectively). However, for *P. hordei-murini* a lower level of susceptibility was observed in SusPtrit and in the most susceptible RILs compared to the other heterologous rust fungi.

### Disease test with two rust species to which barley is a host

The RIL population showed a quantitative segregation for susceptibility to *P. hordei* isolate 1.2.1 (Figure 1G). The relative latency period (RLP) values of RILs in three replications did not indicate transgressive segregation in this population. SusPtrit was as susceptible as our long term most susceptible standard line L94 and in some cases slightly more susceptible.

The mapping population showed a quantitative segregation for susceptibility to *P. graminis* f. sp. *tritici* as well (Figure 2B). The pustules of neighbouring infections in more susceptible RILs often merged and, in more resistant RILs, single infection sites often produced two or more tiny pustules, making infection frequency (IF) an inaccurate measure for the level of resistance to *P. graminis* f. sp. *tritici* (Figure 2B). Therefore the area covered with lesions, including the pale green/yellowish areas surrounding pustules, was used as an alternative index to quantify susceptibility of RILs. In a random subset of RILs correlation between IF and area covered with lesions was positive and highly significant (r= 0.91).

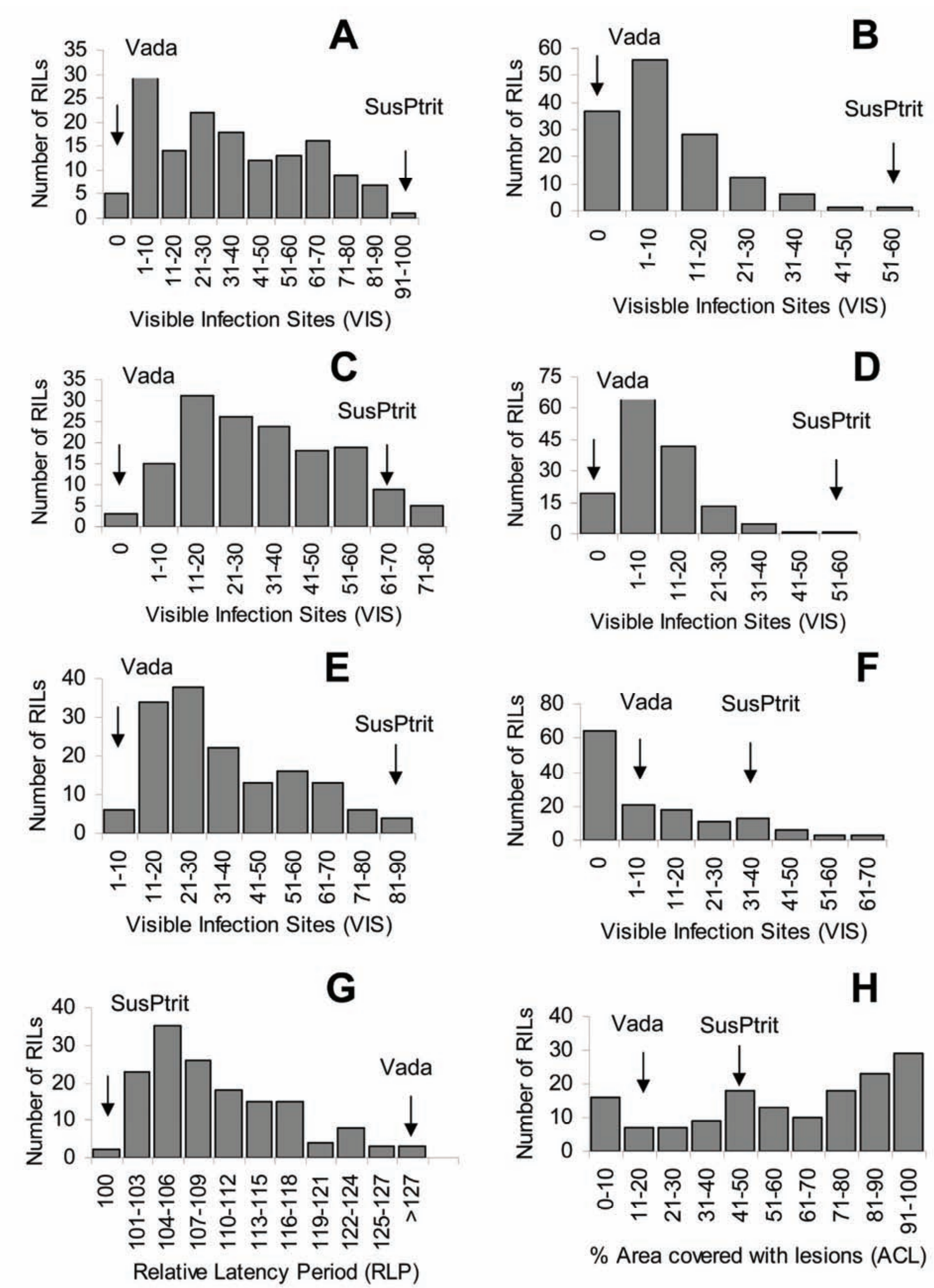

**Figure 1.** Frequency distribution of phenotypes for resistance to eight rust species in barley mapping population Vada x SusPtrit. Values of the two parental lines are shown by arrow
A) *P. triticina* (Flamingo isolate) B) *P. triticina* from *T. durum* C) *P. persistens*
D) *P. hordei-murini* E) *P. hordei-secalini* F) *P. graminis* f. sp. *lolii*
G) *P. hordei* H) *P. graminis* f. sp. *tritici*

Some of the individuals showed higher susceptibility or resistance compared with the two parental lines indicating transgressive segregation to *P. graminis* f. sp. *tritici* in this mapping population.

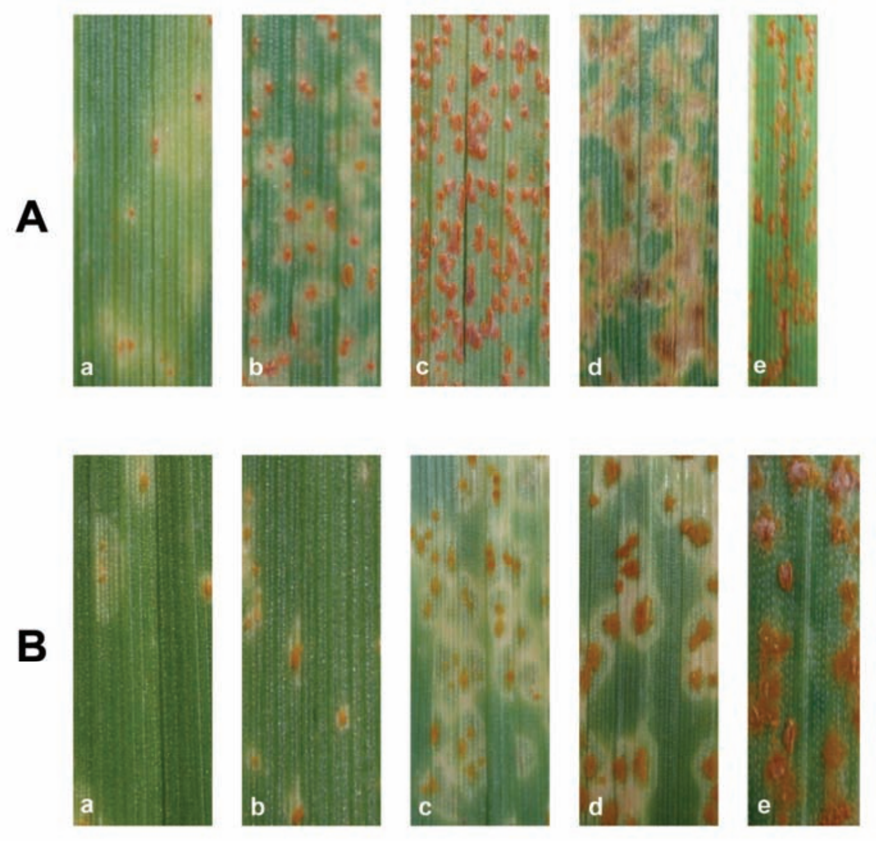

**Figure 2.** Segregation of RILs (Vada x SusPtrit) for nonhost resistance to *P. hordei-secalini* and host resistance to *P. graminis* f. sp. *tritici*
A- Quantitative (a, b and c) and qualitative (d) segregation to *P. hordei-secalini* (e=host plant)
B- Quantitative segregation (a, b and c) to *P. graminis* f. sp. *tritici* (d=abaxial side of infected leaf, e= susceptible wheat line "Morocco"

## Phylogenetic analysis of rust fungi

Parsimony analysis of ITS sequence data from 20 rust isolates resulted in four optimal trees with a tree length of 262 (Figure 3). Distance analysis using the Neighbor Joining method (Kimura 2-parameter) resulted in a tree with identical topography. DNA sequences from rust samples used in this study, grouped into three distinct and well-supported clades (Figure 3). Clade I consisted of the barley leaf rusts *P. hordei* (*H. vulgare*) and *P. hordei-murini* (*H. murinum*). These two rusts are closely related to onion and garlic rust (*P. allii*), which forms a sister group within Clade I. The remaining barley leaf rust, *P. hordei-secalini*, (*H. secalinum*) grouped in Clade III, which also contained *P. persistens*, *P. triticina*, and *P. triticina-"duri"*. *P. bromina* (*Bromus* sp.) and *P. cerinthes-agropyrina* (*Agropyron* sp.) formed a sister group within Clade III.

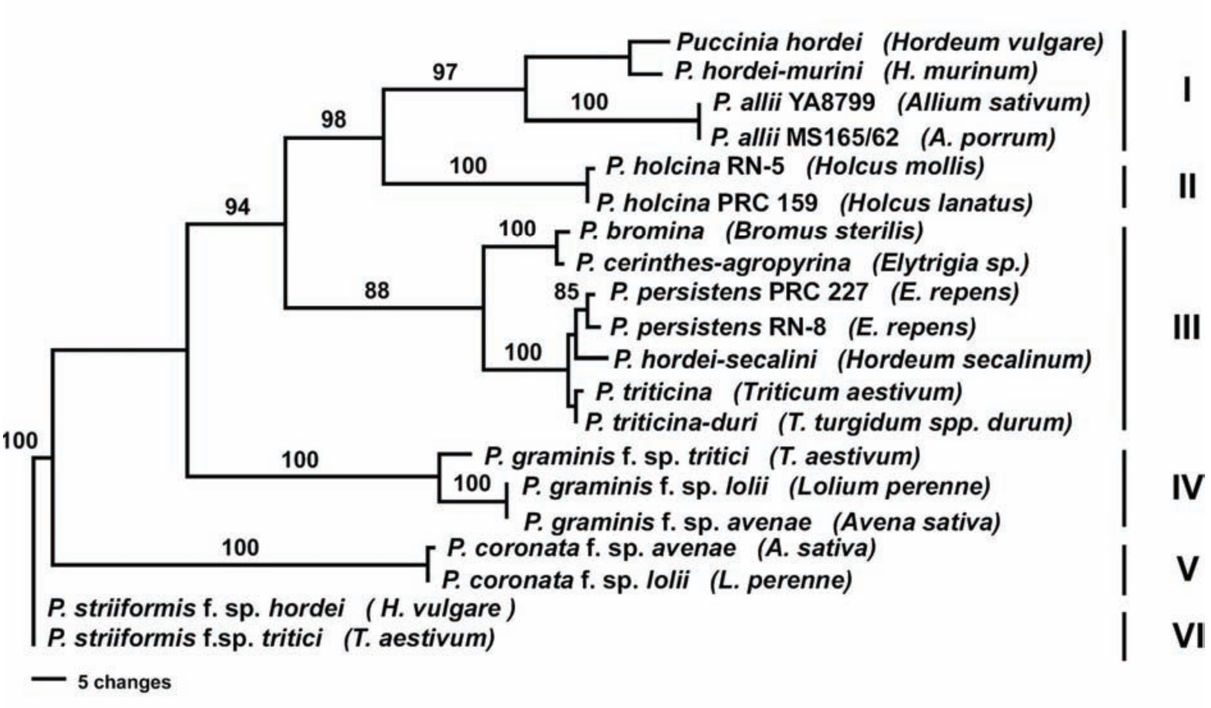

**Figure 3**. Parsimony tree from the analysis of nuclear ribosomal internal transcribed spacer (ITS) sequence data of selected cereal and grass rusts. Phylogenetic analysis resulted in four optimal trees, one of which is shown (tree length of 262 steps, CI = 0.7634, HI = 0.2366, RI = 0.8839 and RC = 0.6747). Numbers above branches indicate percentage of congruent clusters in 1,000 bootstrap trials and only values above 80% are shown. Rust hosts are indicated in parentheses. Clades are indicated along the right hand side. *P. striiformis* DNA sequences were used as out-group.

Three collections of stem rust (*P. graminis*) formed Clade IV, which was divided into two subgroups, *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *lolii* and *P. graminis* f. sp. *avenae*. *P. holcina*, *P. coronata* (crown rust) and *P. striiformis* (stripe rust) formed distinct clades, II, V and VI, respectively.

### Correlations among quantified traits for different rusts

Correlation coefficients (*r*) among the infection parameters measured in the mapping population for nonhost and partial resistance to 10 isolates representing seven rust species are shown in Table 1. In the case of association, a negative correlation would be expected between RLP to *P. hordei* and FVIS of other rust fungi. The highest negative correlation was between RLP to *P. hordei* and FVIS to *P. persistens* (*r*= -0.40), indicating that at least some of the genes for resistance to these rusts are either linked or have pleiotropic effects. The highest positive correlation was observed between different isolates of *P. triticina* (*r*=0.62-0.73). Correlation between two *formae speciales* of *P. graminis* was intermediate (*r*=0.43). Among the three phylogenetic groups of rusts, the *P. triticina* and the *P. graminis* group showed the highest correlation (Table 1).

**Table 1**. Correlation coefficients (*r*) among infection parameters expressing level of resistance of 152 RILs of Vada x SusPtrit, for 10 isolates belonging to seven rust species

| **Criteria measured** | **RLP** (*P. hordei*) | **FVIS** (*P. hordei-murini*) | **FVIS** ( *P. triticina*) Flamingo | **FVIS** ( *P. triticina*) Swiss | **FVIS** ( *P. triticina*) French | **FVIS** (*P. triticina-“duri”*) | **FVIS** (*P. persistens*) | **IF** (*P. hordei- secalini*) | **Inf. Area** (*P. graminis* f. sp. *tritici*) | **FVIS** (*P. graminis* f. sp. *lolii)* |
|---|---|---|---|---|---|---|---|---|---|---|
| **RLP** (*P. hordei*) | | | | | | | | | | |
| **FVIS** (*P. hordei-murini*) | -0.11 | | | | | | | | | |
| **FVIS** ( *P. triticina*) Flamingo | -0.22 | 0.34 | | | | | | | | |
| **FVIS** ( *P. triticina*) Swiss | -0.30 | 0.37 | **0.73** | | | | | | | |
| **FVIS** ( *P. triticina*) French | -0.30 | 0.33 | **0.70** | **0.69** | | | | | | |
| **FVIS** (*P. triticina “duri”*) | -0.27 | 0.33 | **0.62** | **0.69** | **0.50** | | | | | |
| **FVIS** (*P. persistens*) | -0.40 | 0.36 | 0.49 | **0.54** | **0.53** | **0.51** | | | | |
| **IF** (*P. hordei- secalini*) | -0.14 | 0.22 | 0.47 | 0.44 | 0.40 | **0.48** | **0.46** | | | |
| **Inf. Area** (*P. graminis* f. sp. *tritici*) | -0.12 | 0.28 | 0.44 | **0.45** | 0.34 | 0.34 | 0.42 | 0.16 | | |
| **FVIS (***P. graminis* f. sp. *lolii)* | -0.14 | 0.20 | 0.34 | 0.33 | 0.35 | 0.21 | 0.17 | 0.17 | 0.43 | |

**RLP**: Relative Latency Period
**FVIS**: Frequency of Visible Infection Site
**IF**: Infection Frequency
**Inf. Area**: % of Area Covered by Lesions
Correlation coefficients 0.45 and higher are highlighted in **bold**

## QTLs mapped for nonhost and partial resistance

QTL mapping for nonhost resistance was performed using 8 isolates belonging to 5 heterologous rust species and three evaluated traits: IF, FVIS and RIF. In all cases FVIS and IF were closely correlated (r = 0.73 to 0.99), resulting in the same QTLs identified for both parameters. Due to homogeneous distribution of spores on the leaves of inoculated plants the same QTLs were identified for Relative Infection Frequency (RIF) and IF traits as well. The LOD scores for QTLs were in general higher for FVIS than for IF and therefore only mapping data for parameter FVIS are presented (Table 2 and Figure 4). For all heterologous rust species except *P. graminis* f. sp. *lolii*, alleles for resistance originated from Vada and susceptibility alleles from the susceptible parent SusPtrit. For the latter rust species, one QTL allele for resistance originated from Vada and two from SusPtrit (Table 2). The qualitative segregation for hypersensitivity against *P. hordei-secalini* (Figure 2A) allowed mapping of this feature by Joinmap. This demonstrated that a major gene at 58 cM on chromosome 5 (1H) was responsible for the hypersensitive reaction.

Four QTLs were found to be effective to all three *P. triticina* isolates, and one QTL seemed effective to only the Flamingo isolate since the LOD profile in that region was flat in the tests with both other isolates of this rust species. The contribution of seven barley chromosomes in resistance to the rust species varied from one QTL effective to only one rust species (chromosome 4) to four QTLs effective to seven different rust species (chromosome 1). In the same way as described above, QTL mapping was performed for RLP of *P. hordei*. The most effective QTL to *P. hordei* was mapped at the bottom of chromosome 2 (Table 2; Figure 4). This QTL was mapped to a similar position as a QTL reported by Qi et al. (1998b) with identical flanking markers. Two other QTLs with minor effect were mapped on chromosome 1 and 3. Their confidence interval did not overlap with QTLs mapped in the L94 x Vada mapping population. Five QTLs were mapped for resistance to *P. graminis* f. sp. *tritici;* two for resistance were contributed by SusPtrit and three by Vada (Table 2). Two QTLs for partial resistance to *P. hordei* and three QTLs for resistance to *P. graminis* f. sp. *tritici* co-located with QTLs effective to heterologous rusts (Table 2 and Figure 4). Three chromosomal regions were mapped with LOD scores lower than the LOD threshold but significant to other rusts (Table 2).

## DISCUSSION

### “Barley-rust pathosystem” as a model to study the genetics of nonhost resistance

There is a lack of good genetic systems to study the genetic basis of nonhost resistance. Mutagenesis and transformation are at present the main approach to study the genetic basis of nonhost resistance in the model plant *Arabidopsis thaliana*. By mutagenesis major genes have been demonstrated to be involved in nonhost resistance (Lu et al. 2001; Wildermuth et al. 2001). A forward genetics approach, however, has the advantage to allow identification of evolutionary relevant qualitative and quantitative factors that determine the elicitation of resistance or the degree of suppression of basal defence by host and nonhost pathogens. However, crossings between host and nonhost species are by definition interspecific crosses that, if feasible at all, nearly always suffer from sterility, abnormal growth and poor seed viability and are therefore unsuitable to study the genetics of nonhost resistance. We therefore make use of the fact that barley is a marginal host to some heterologous rust species. Only few, mainly exotic, *Hordeum vulgare* accessions are at the seedling stage somewhat susceptible to some grass and cereal rust species (Atienza et al. 2004). This allowed us to develop a barley research line, named “SusPtrit”, in which susceptibility genes to heterologous rust species were accumulated (Atienza et al. 2004). This line was used as one of the parents in the presently analyzed mapping population.

**Table 2**. LOD values of regions on barley chromosomes conferring resistance to 8 different rusts (LOD value 2.7 and higher is considered as a QTL)

| **Locations of affecting regions** | | **Rust Species** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Chromosome** | **Position(cM)** | *P. hordei* | *P. hordei-murini* | *P. triticina*[a] | *P. triticina-“duri”* | *P. persistens* | *P. hordei-secalini* | *P. graminis* f. sp. *tritici* | *P. graminis* f. sp. *lolii* | Total number of rusts |
| Chromosome 1 (7H) | 65-75 | | | | | | 4.2 | | | 1 |
| | 85-100 | | | 8.0 | | 4.4 | | | | 2 |
| | 105-110 | 2.7 | | | | | | | | 1 |
| | 120-135 | | 5.0 | 7.0 | 7.0 | 4.2 | | 5.0 | | 5 |
| Chromosome 2 (2H) | 90-100 | | | 3.9[b] | | | | | | 1 |
| | 130-140 | 12.0 | | | | 5.6 | | | | 2 |
| Chromosome 3 (3H) | 1-20 | | | | | 3.2 | **2.1** | **2.2** | | 3 |
| | 40-60 | | 3.1 | | | | | | | 1 |
| | 100-120 | 3.1 | | 3.8 | | | | | | 2 |
| Chromosome 4 (4H) | 15-30 | | | | | 2.8 | | | | 1 |
| Chromosome 5 (1H) | 20-35 | | 4.0 | | | | | | | 1 |
| | 50-60 | | | | | | 11 [c] | | | 1 |
| | 90-100 | | | | | | | 3.0 | | 1 |
| | 110-122 | | | | | | | | 2.8[d] | 1 |
| Chromosome 6 (6H) | 50-65 | | | 11.0 | 5.7 | **2.5** | | 6.4 | 5.5 | 5 |
| Chromosome 7 (5H) | 40-50 | | | | | | | 4.5[d] | | 1 |
| | 120-130 | | | | | 3.5 | 6 | | | 2 |
| | 155-170 | | | | | | | 4.2[d] | 3.5[d] | 2 |
| Total number of chromosomal regions | | 3 | 3 | 5 | 2 | 7 | 4 | 6 | 3 | |

Regions with LOD values between 2 and 2.7 (highlighted **bold)** were included only when they were implicated in resistance to other rust fungi.

[a] Results for three isolates (collected in the Netherlands, France and Switzerland) identified the same QTLs with similar LOD scores. We present here the data obtained for the Flamingo isolate

[b] This QTL was found to be effective to Flamingo and not to the two other isolates.

[c] The qualitative segregation for hypersensitivity against *P. hordei-secalini* allowed mapping of this feature by Joinmap. This demonstrated that the QTL at 50-60 cM on chromosome 5(1H) was responsible for the hypersensitive reaction.

[d] QTLs with resistance allele contributed by SusPtrit

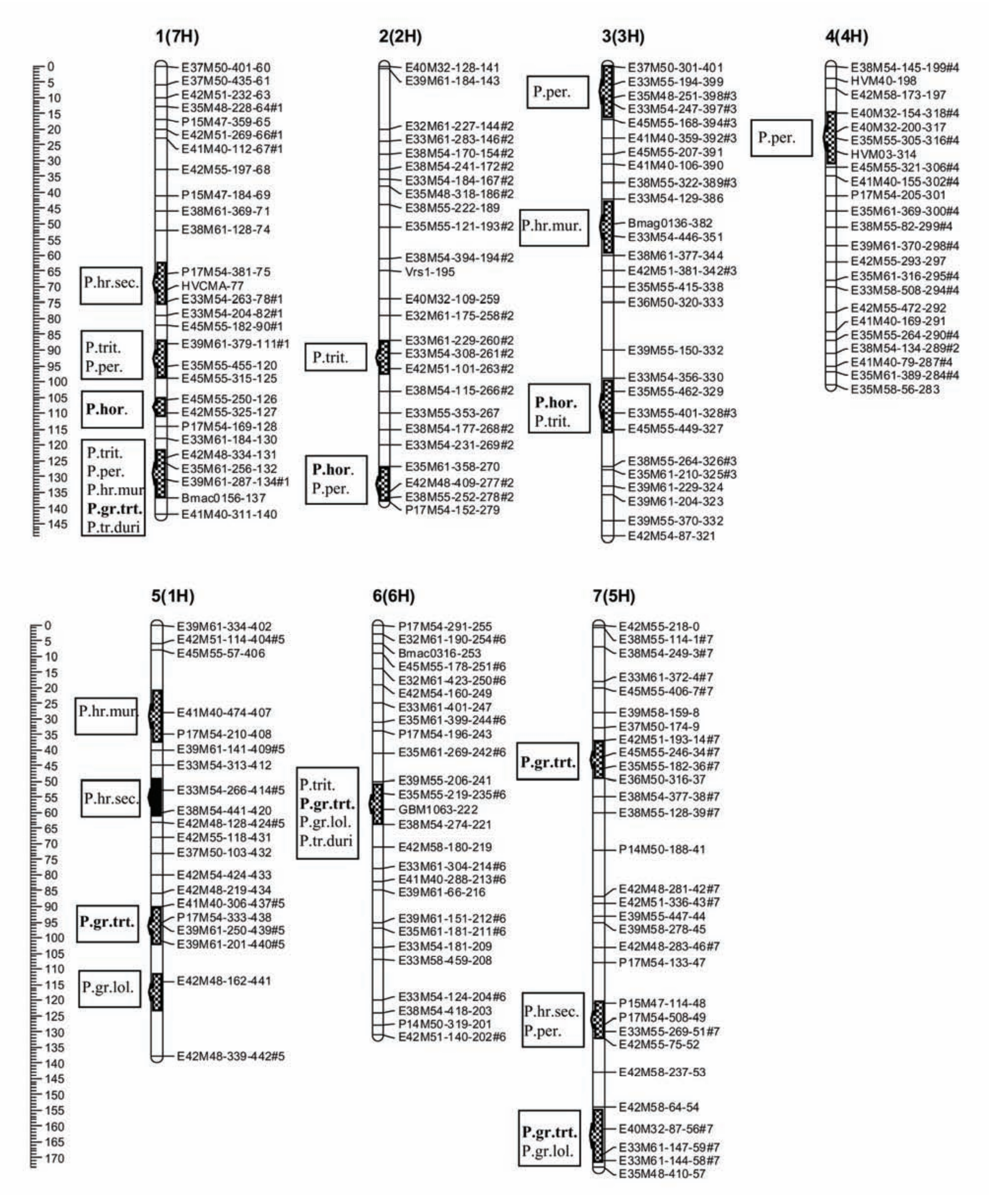

**Figure 4.** Locations of QTLs mapped for host and nonhost resistance to eight rust species and isolates in barley mapping population Vada x SusPtrit (P.trit. =*P. triticina* Flamingo isolate, P.pers. =*P. persistens*, **P.hor**. = *P. hordei*, P.hr.sec. = *P. hordei-secalini*, P.hr.mur. =*P. hordei-murini*, **P.gr.trt**. = *P.graminis* f. sp. *tritici*, P.gr.lol= *P. graminis* f. sp. *lolii*, P.tr.duri=*P. tirticina* from *T. durum*). QTL bars indicate approximate position of two LOD support intervals and the ruler at the left margin shows the distance in centiMorgans. QTLs effective to host pathogens are highlighted **bold**

▨ QTL with single / multiple effects ▮ QTL coincide with major gene to *P. hordei-secalini*

## Genetics of nonhost resistance

In total 18 chromosomal regions segregated for resistance in the studied mapping population of which eleven were significantly effective to only one rust species. These regions were spread all over the genome, but tended to map especially to chromosomes 1 (7H) and chromosome 5 (1H) (both four regions), and least to chromosome 4 (4H) (1 region). Two regions, one at the bottom of chromosome 1 (125-140 cM) and another at the centromeric region of chromosome 6 (50-65 cM) showed multiple effects to five and four rust species respectively (Figure 4). Effects to two or more rust species can either be due to close linkage of genes or to pleiotropy. Basal resistance to heterologous pathogens has also been reported to inherit polygenically in wild lettuce (*Lactuca saligna*) which is nonhost to *Bremia lactucae* (Jeuken and Lindhout 2002). Previous evidence already suggested that nonhost resistance of barley is due to additive effects of genes with effectiveness to several heterologous rusts, and to genes with a rust species specific effect (Hoogkamp et al. 1998; Atienza et al. 2004). The present research indicates that also linkage between rust specific genes may have contributed to an apparent wide spectrum of effectiveness.

## Reliability of QTL mapping

In order to evaluate the reliability of QTL detection we performed three replications for disease test experiments and compared QTLs detected in each replication. Only repeated detection of QTLs was considered as reliable. One QTL for *P. persistens* (LOD value 2.9) only occurred in one replication and therefore was not included. Four QTLs effective to different isolates of *P. triticina* (Table 2) were mapped nine times (three isolate x three replications) at identical positions indicative of a high repeatability of QTL analysis. In addition, for each rust species we used three parameters (IF, FVIS and RIF) to estimate resistance of RILs and we mapped the same QTLs for all three parameters with only some small changes in LOD values. For all the mapped QTLs the parental provenance of the resistance allele was as expected, i.e. from Vada, except in case of *P. graminis* f. sp. *lolii* where transgression occurred. This lends confidence that the QTLs found are not due to random experimental errors.

## Host and nonhost resistance: Possible associations in mechanisms and genetics

Heterologous rust fungi that are pathogenic to Gramineae are generally well able to find and penetrate stomata on nonhost grass and cereal species (Niks 1986b) but usually are hardly able to form haustoria (Niks 1983; 1986b ; Niks and Rubiales 2002). It is likely that prevention of haustorium formation, which is associated with papilla formation, is indicative for lack of basic-compatibility (Panstruga 2003). Niks (1989) showed that the heterologous rye leaf rust fungus *P. recondita* could form haustoria in barley mesophyll cells only when *P. hordei* had already formed a haustorium in that cell. This suggests that pathogens should suppress innate immunity, as put forward by Mellersh & Heath (2001). Also in some host genotypes haustorium formation by its own regular pathogen can be relatively poor. *P. hordei* is much more successful in formation of haustoria in the susceptible line L94 than in the cultivar Vada (Niks 1983; 1986a). Interestingly, there seemed to be a moderate association between level of partial resistance to *P. hordei* and level of resistance to two heterologous rust fungi (Hoogkamp et al. 1998; Zhang et al. 1994). In the present study two QTLs (out of three) for partial resistance to *P. hordei* and three QTLs (out of five) for *P. graminis* f. sp. *tritici* showed double/multiple effectiveness to some heterologous rust species (Table 2; Figure 4) suggesting a genetic association between host and nonhost resistance in barley, both acting at the level of basic-compatibility and its complementary phenomenon, basal resistance.

Our data suggest that *R*-genes may also contribute to nonhost resistance. The *R*-gene that we mapped on chromosome 5(1H) for *P. hordei-secalini* showed similarities in its phenotype (HR reaction) and the genetics (monogenic inheritance) with the *R*-genes, named *Rph*, in barley to *P. hordei* that cause a posthaustorial hypersensitive response. Remarkably, in that region no *Rph* genes to *P. hordei* have been reported. In some barley germplasm accessions (Atienza et al. 2004) many visible infection sites were found after inoculation with heterologous rust species, suggesting a fair colonisation, and hence a relatively successful haustorium formation, but followed by HR. These accessions may contain a relatively low gene dose for basal resistance, but may harbour a back-up resistance through one or more *R*-genes like the gene that Vada possesses against *P. hordei-secalini*. We will address the role of *R*-genes in nonhost resistance in a future study.

### Evolutionary perspective of innate immunity in barley

Adaptation of biotrophic pathogens to a restricted number of host species can be regarded as a consequence of the co-evolution of these pathogens with their host plant species (Thordal-Christensen 2003). Therefore one may wonder about a possible association of evolutionary distance and host status to a certain potential pathogen. In case the systematic relationship of a pathogen reflects that of its host, their evolutionary histories may also be linked (Wyand and Brown 2003). It seems reasonable to assume that a cereal ancestor carried some rust species that diverged along with the radiation of various descendant cereal and grass species. The rust may have specialised on some of the new species, loosing pathogenicity to some sibling species (Heath 1991). In such an evolutionary radiation, speciation in the grass family and rusts is likely to take place simultaneously. In that case we would expect either (1) that plant QTLs determining the feasibility for being a host for a rust pathogen tend to be effective to taxonomically related rusts, and/or (2) that such QTLs are effective to rusts that share the same host species.

Seven rust species used in the present study belong to three different groups of rust fungi of grasses: *P. hordei, P. persistens*/*P. triticina* and *P. graminis* group containing 2, 3 and 2 rust species respectively. Our evidence on such an evolutionary association is inconclusive. Some QTLs are effective to two closely related rust species (e.g. *P. triticina* and *P. persistens*), others to two rusts that are pathogenic to the same host (e.g. *P. triticina* and *P. graminis* f. sp. *tritici*). Others however to two less related rust species on less related host species (e.g. *P. hordei* and *P. persistens*). Hence, our data do not confirm a tendency for QTLs to be effective to rusts that have a close phylogenetic relationship. Our data further illustrate that evolutionary distance and host status to a certain potential pathogen are not associated either. For example, the rusts *P. allii* and *P. holci* are closely related to *P. hordei,* but cannot infect barley, not even the experimental line SusPtrit (Data not presented). Two moderately related rusts, *P. cerinthes-agropyrina* and *P. persistens* both can infect *Elytrigia repens,* but only the latter is also able to infect SusPtrit. It is therefore likely that biological specialisation in biotrophic fungi like rust pathogens of grasses and cereals is influenced by some other factors such as geographical origin of pathogens as reviewed by Wyand and Brown (2003).

### Possible nature of the QTL genes

The extreme susceptibility of SusPtrit suggests that it is deficient for some key gene(s) in signal transduction or defence response to rust fungi. However, this barley line is well able to prevent haustorium formation by rye leaf rust (data not shown) and is also immune to several other rusts (Atienza et al. 2004). Also, it is unlikely that each heterologous rust elicits different transduction pathways or defence genes, especially not when the rusts are more or less related to each other. Although some other scenarios are conceivable, we presume that the genes at the QTLs described here encode for proteins that play a role as specific and quantitative recognition factors that should be specifically negated by effectors delivered by the rust to successfully suppress basal defence. Such a role, but not necessarily their molecular identity, could be comparable to that of HR inducing *R*-genes that interact with specific *AVR* factors produced by the pathogens.

## MATERIAL AND METHODS

### Development of the mapping population

A barley research line that is in the seedling stage fully susceptible to *P. triticina* and to some other heterologous rusts, named SusPtrit, (Atienza et al. 2004) was crossed with the West European cultivar Vada. The latter accession represents “normal” barley which is fully resistant to heterologous rusts (Atienza et al. 2004) and has several quantitative trait loci (QTLs) for partial resistance to *P. hordei* (Qi et al. 1998b). After seven generations of single seed decent, from 200 $F_2$ plants a population of 152 $F_8$-drived Recombinant Inbred Lines (RILs) was obtained.

### DNA extraction and molecular marker analysis

Genomic DNA of the 152 RILs and the parents (SusPtrit and Vada) was isolated from fresh leaves according to the CTAB based protocol of (Steward and Via 1993) adjusted for a 96-well format. The AFLP procedure was performed by two step amplification (Vos et al. 1995) with some minor modifications. In this study 25 *Eco*RI/*Mse*I primer combinations each with three selective nucleotides and three *Pst*I/*Mse*I primer combinations with two selective nucleotides were used to generate the AFLP markers. The scoring of the AFLP products was based on absence or presence of amplification products. Segregating markers showing polymorphism between two parents were scored in the mapping population by using Quantar-Pro software (Keygene, Wageningen, The Netherlands). In addition, the RILs were genotyped for 12 polymorphic SSR markers.

### Linkage analysis and map construction

Linkage analysis was carried out with JoinMap 3.0 (Kyazma B.V. Wageningen, The Netherlands) and Kosambi’s function was used for map distance calculation. Linkage groups were assigned to the respective barley chromosomes by morphological markers, SSR markers and 152 common AFLP markers that had already been mapped (Qi et al. 1998a) in the L94 × Vada mapping population. In case of ambiguity, the freely available software programme

RECORD (Van Os et al. 2005) was used to find the most probable order of the loci on the linkage maps on the basis of recombination events (RECORD software is available online from Laboratory of Plant Breeding, Wageningen University). A subset of 179 markers was extracted for use in a skeleton map for QTL mapping. The selection was based on spacing of the markers at about 5 to 10 cM, and agreement between RECORD and Joinmap results on their relative position.

### Pathogen material

Ten isolates of rust fungi were used in infection studies. *P. triticina* of bread wheat (three isolates), *P. triticina* of durum wheat (labelled for convenience "*P. triticina "duri"*"), *P. persistens* (= *P. agropyrina*), *P. hordei-murini*, *P. hordei-secalini*, and *P. graminis* f. sp. *lolii* were multiplied on their respective host plants (See Atienza et al. 2004). *P. hordei* (isolate 1.2.1) and *P. graminis* f. sp. *tritici* were increased using the susceptible barley line L98 and wheat cultivar Morocco, respectively. Urediniospores were collected, dried for 2-7 days and then stored at -80 °C until the time to be used for inoculation.

### Phenotyping of the mapping population for resistance to heterologous rust fungi

Seedlings of RILs were grown in boxes (37 x 39 cm) along with both parents and susceptible host plants. Twelve days after sowing, completely unfolded primary leaves were placed in horizontal position with the adaxial side facing up, and inoculated with approximately 10 mg of spores per box (deposition of about 600 urediniospores per cm$^2$) using a settling tower (Atienza et al. 2004). After inoculation the plants were incubated overnight in a dew chamber for 10 hours (17-18 ºC, 100% relative humidity and without light) and then transferred to a greenhouse (22 ºC day and 18 ºC night). Twelve days after inoculation the level of infection was quantified by estimating the FVIS and two additional parameters, IF and RIF. The test was performed in three consecutive replications except in case of *P. graminis* f. sp. *tritici* and *P. triticina-"duri"* where phenotyping was carried in two replications.

The quantitative data for each replication and the average of three replications were used as phenotypic values for QTL mapping.

### Phenotyping of the mapping population for resistance to host rust fungi

The RILs were grown as described above and tested for level of partial resistance to *P. hordei* (isolate 1.2.1), as described by Qi et al. (1998b). The latency period (LP) of the rust on each plant was evaluated by estimating the time (in hours) at which 50% of the ultimate number of pustules were visible. The relative latency period on seedlings (RLP) was calculated relative to the LP of L94 plants, as described by Parlevliet (1975). The average over three replications was considered to reflect the level of partial resistance of each RIL. For *P. graminis* f. sp. *tritici* only 5 mg of spores were used per box. *P. graminis* f. sp. *tritici* often formed large pale green halos around the uredinia and adjacent halos frequently merged. Therefore, total leaf area covered by these halos was used as parameter for QTL mapping.

**Table 3.** Rust isolates used in this study

| **Rust**[a] | **Host** | **Collections**[b] | **Location** | **GenBank accession no.** |
|---|---|---|---|---|
| *Puccinia allii* | *Allium porrum* | MS 165/62 | Germany | AY187090[c] |
| *P. allii* | *A. sativum* | YA8799 | Turkey | AF511073[c] |
| *P. bromina* | *Bromus sterilis* | RN-13 | The Netherlands | DQ460719 |
| *P. cerinthes-agropyrina* | *Elytrigia sp.* | RN-9 | Spain | DQ460720 |
| *P. coronata* f. sp. *avenae* | *Avena sativa* | CDL93MN437 | USA | AY114290 |
| *P. coronata* f. sp. *lolii* | *Lolium perenne* | PRC 202 | Czech Republic | DQ460728 |
| **_P. graminis_. f. sp. _lolii_** | *Lolium perenne* | RN-1 | The Netherlands | DQ460726 |
| *P. graminis* f. sp. *tritici* | *Triticum aestivum* | CDL 62ND72C | USA | DQ460725 |
| **_P. graminis_ f. sp. _tritici_** | *Triticum aestivum* | HUN[e] | Hungary | |
| *P. graminis* f. sp. *avenae* | *Avena sativa* | RN-20 | Hungary | DQ460727 |
| *P. holcina* | *Holcus lanatus* | PRC 159 | Czech Republic | DQ512999 |
| *P. holcina* | *Holcus mollis* | RN -5 | The Netherlands | DQ513000 |
| **_P. hordei_ isolate 1.2.1** | *Hordeum vulgare* | RN-17 | The Netherlands | DQ460717 |
| **_P. hordei-murini_** | *H. murinum* | RN-4 | The Netherlands | DQ460718 |
| **_P. hordei-murini_**[f] | *H. murinum* | Aragon | Spain | |
| **_P. hordei-secalini_** | *H. secalinum* | RN-3 | France | DQ460723 |
| *P. persistens* | *Elytrigia repens* | PRC 227 | Ireland | DQ460722 |
| **_P. persistens_** | *Elytrigia repens* | RN-8 | The Netherlands | DQ460721 |
| *P. striiformis* f. sp. *hordei* | *Hordeum vulgare* | HSZ0711 | USA | DQ460729 |
| *P. striiformis* f. sp. *tritici* | *T. aestivum* | HSZ0718 | USA | DQ417401[d] |
| *P. triticina* | *T. aestivum* | CDL00LA87 | USA | AF511083 |
| **_P. triticina_** | *T. aestivum* | Flamingo | The Netherlands | |
| **_P. triticina_** | *T. aestivum* | Swiss | Switzerland | |
| **_P. triticina_** | *T. aestivum* | French | France | |
| **_P. triticina- duri_** | *T. turgidum ssp. durum* | RN-22 | Spain | DQ460724 |

Rust isolates used for QTL analysis are highlighted in **bold**. Remaining isolates were included either to determine phylogenetic distance of rusts in relation to their host or for increasing of stability of parsimony tree obtained from analysis of internal transcribed spacer (ITS) sequence data.

[a] Scientific names of rusts are used as convenient labels and do not reflect necessarily the taxonomic status of the rusts.

[b] Prefix designations and sources for collections: CDL;Cereal Disease Laboratory, USDA ARS; HSZ, L. J. Szabo; RN; Rients Niks; MS, Markus Scholler, Staatliches Museum für Karlsruhe, Karlsruhe, Germany; PRC, J. Marková, Charles University, Prague, Czech Republic; YA, Y. Anikster, Institute for Cereal Crop Improvement, Tel Aviv University, Israel.

[c] Anikster et al. 2004.

[d] C.W. Barnes and L. Szabo, unpublished data

[e] Kindly provided by Dr. K. Manninger from Plant Protection Institute of Hungarian Academy of Science, Budapest, Hungary

[f] Same QTLs were identified for both isolates of *P. hordei-murini*.

## QTL mapping

The software Map QTL version 5.0 (Kyazma B.V. Wageningen, The Netherlands) was used to perform QTL mapping. A permutation test was applied to determine the LOD value for both chromosome-wide and genome-wide significance threshold at level of 5%. By interval mapping putative QTLs were detected on each set of disease data and were verified by application of MQM mapping. In the region of putative QTLs the peak markers with the highest LOD value were selected as co-factors for running Multiple QTL Mapping (MQM) (Jansen and Stam 1994). Only those QTLs that occurred in three replications and average were considered as reliable QTLs. Regions that were implicated in resistance at LOD values 2.0 – 2.7 were considered potentially real only when the same chromosomal region reached significant LOD values with at least one other rust species.

## ITS sequences of rust fungi

DNA was extracted from either dried urediniospores collected from infected host material or from dried infected host leaf tissue as described by Anikster et al. (2004). In some cases, OminiPrep (Genotech, St. Louis, MO, USA) DNA extraction kit was used instead of CTAB DNA extraction method. Nuclear ribosomal internal transcribed spacer (ITS) region and the 5’ end of the large subunit was amplified using PCR and amplification products were cloned (Anikster et al. 2004). Primer pairs used for amplification were ITS1F (Gardes and Bruns 1993) and RUST1 (Kropp et al. 1995). DNA sequencing reactions were performed using a Thermo Sequenase Primer Cycle sequencing kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and analyzed on an automated DNA sequencer (LI-COR, Lincoln, NE, USA). At least three clones were sequenced for each sample and the DNA sequence was assembled and edited with Sequencer (Gene Codes Corp., Ann Arbor, MI, USA). DNA sequences were initially aligned using the program CLUSTAL W (Thompson et al 1994) and then hand edited using the multiple sequence editor in MacVector (version 7.2.3; Accelrys Inc. San Diego, CA, USA). Approximately 1250 bp of DNA was sequenced for each sample including the 3’-end of the nuclear ribosomal 18S subunit, the complete ITS region (ITS1, 5.8S and ITS2) and the 5’-end of the 28S subunit providing an aligned sequence of 1367 characters. Variation in the 5’ end of the 28S subunit was low and therefore not included in the phylogenetic analysis. Of the 664 aligned characters analyzed, 510 were constant, 148 were variable and parsimony-informative, and 6 were variable and parsimony-uninformative. Phylogenetic analysis of the data sets included the complete ITS1, 5.8S and ITS2 region was carried out using Neighbor Joining and heuristic parsimony program (PAUP version 4.04b10, Swofford 2001). Support for the nodes of the trees was determined by analysis of 1,000 bootstrap replicas. Phylogenetic analysis in the present study was performed based on ITS sequence of 20 rust species and forms (Table 3) from which seven species were applied for phenotyping of RILs and QTL mapping.

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## AUTHOR-RECOMMENDED INTERNET RESOURCES

Department of Plant Breeding, Wageningen University, The Netherlands; freely available software RECORD http://www.dpw.wau.nl/pv/index.htm.

# CHAPTER 4

## High diversity of genes for nonhost immunity of barley to heterologous rust fungi

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## ABSTRACT

Inheritance studies on the nonhost immunity of plants would normally require interspecific crosses that suffer from sterility and abnormal segregation. Therefore we developed the barley – *Puccinia* rust model system to study by forward genetics the specificity, number and diversity of genes involved in nonhost immunity. We developed two mapping populations by crossing line SusPtrit, exceptionally susceptible to heterologous rust species, with immune barley cultivars Vada and Cebada Capa. These two mapping populations along with the Oregon Wolfe Barleys population, which showed unexpected segregation for resistance to heterologous rusts, were phenotyped with four heterologous rust fungal species. Positions of QTLs conferring nonhost immunity in the three mapping populations were compared using an integrated consensus map. The results confirmed that nonhost immunity in barley to heterologous rust species is controlled by QTLs with different and overlapping specificities, and by occasional contribution of a *R*-gene for hypersensitivity. In each population different sets of loci were implicated in resistance, with very few genes in common between the populations, suggesting a high diversity of genes conferring nonhost immunity to nonhost pathogens. These loci were significantly associated with QTLs for partial resistance to the pathogen *P. hordei*, and also with defense related genes.

**Keywords:** nonhost immunity, partial resistance, quantitative trait locus (QTL), genetic map, *Hordeum vulgare*

## INTRODUCTION

Plants are exposed to a tremendous number of potential pathogens representing a very wide array of microorganisms. Although plants lack an immune system similar to mammals, susceptibility to a plant pathogen is obviously more exception than rule. Plants have evolved a sophisticated innate immunity network to recognize and react to potential pathogens. The genetic basis of innate immunity in plants, in particular to non-adapted (=nonhost, called here “heterologous”) pathogens, is one of the most intriguing aspects to be elucidated in the field of plant genetics but is very hard to study, since it typically requires interspecific crosses between host and nonhost species. Progeny from such crosses, if feasible at all, normally suffer from sterility and abnormal segregation, hampering identification of individual genetic factors. Immunity network in plants, including basal defense, nonhost resistance and gene-for-gene resistance, hamper potential pathogens to colonize and use a plant as a nutrition source. Suppression of plant defense seems to be a key step in pathogenesis (NOMURA *et al.* 2005; MENDGEN and HAHN 2002). Only pathogens that have evolved the ability to suppress the defense responses of a plant will be able to take up residence (DE WIT 2002). The crucial role of suppression has been demonstrated in particular for rust and powdery mildew pathogens that form long-term biotrophic relationships with their host plants and for plant pathogenic bacteria (MELLERSH and HEATH, 2001; PANSTRUGA 2003; HOLUB and COOPER 2004). One of the exciting discoveries in plant biology during the latest decade was the role and function of bacterial effector proteins to suppress basal defense in plants. The main output of basal defense of plants to bacteria is localized callose deposition in the plant cell wall (GOMEZ-GOMEZ *et al.* 1999; BROWN *et al.* 1998). Callose deposition has been shown to be suppressed by *Pseudomonas syringae* type III effectors in *Arabidopsis* plants (HAUCK *et al.* 2003). Also fungi can suppress host defense by delivery of effectors. Unlike Gram-negative bacteria, no secretion system has been reported for fungal pathogens to inject effectors in plant cells. In haustorium forming fungi like rust and powdery mildew, haustoria may have a role in delivering effectors into the plant intercellular space (CHISHOLM *et al.* 2006). Some of these effectors may act as transcription factors (LAHAYE and BONAS 2001) others may cleave specific cytoplasmic host proteins (SHAO *et al.* 2003; COAKER *et al.* 2005). Recently, in the flax rust fungus *Melampsora lini* about 20 haustorial-secreted proteins have been identified (CATANZARITI *et al.* 2006). It is therefore conceivable that specific host genes or their products are targeted by biotrophic pathogens to achieve suppression of defense. Our knowledge about inheritance and specificity of host genes that are targeted by pathogens to suppress basal defense is negligible, mainly due to lack of good systems to study the genetics of nonhost immunity. We recently proposed the "Barley-*Puccinia* rust fungus" pathosystem to study the inheritance and specificity of plant factors in barley targeted by rust fungi to suppress basal defense in barley (JAFARY *et al.* 2006). Barley (*Hordeum vulgare*) is nearly a nonhost to wheat leaf rust, *Puccinia triticina* and to some other heterologous rust species. Only few, mainly exotic, accessions of barley are at the seedling stage somewhat susceptible to those rust species (ATIENZA *et al.* 2004). In a previous study we mapped resistance genes in a recombinant inbred line (RIL) population obtained from a cross between an immune barley line (Vada) and a research line, named SusPtrit, with exceptional full susceptibility to wheat leaf rust *P. triticina* and some other heterologous rust species and *formae speciales* (ATIENZA *et al.* 2004; JAFARY *et al.* 2006). We showed that nonhost immunity in barley line Vada to heterologous rust species is controlled by sets of QTLs with different and overlapping specificities, and occasional contribution of an *R*-gene for hypersensitivity (JAFARY *et al.* 2006). Since the large majority of barley accessions is (nearly) immune to heterologous rust species (ATIENZA *et al.* 2004) one may wonder whether their immunity is due to the same genes as found in Vada. To address this question, we developed an additional RIL population from a cross between susceptible research line SusPtrit and the South American barley line "Cebada Capa". The latter accession is like Vada and other regular barley cultivars immune to heterologous rusts (ATIENZA *et al.* 2004). In addition, Cebada Capa possesses one major gene (*Rph7*) for race-specific HR resistance and several QTLs for non-hypersensitive partial resistance to *P. hordei* (PARLEVLIET and VAN OMMEREN 1985; QI *et al.* 2000). Surprisingly, we found segregation for resistance to heterologous rust fungi in the commonly used Oregon Wolfe Barleys (OWB) reference mapping population (COSTA *et al.* 2001). We aimed to map QTLs involved in resistance to four heterologous rust species in Cebada Capa x SusPtrit (C x S) and OWB to compare them with the QTLs identified in Vada x SusPtrit (V x S) (JAFARY *et al.* 2006). The availability of three populations segregating for resistance to heterologous rust fungal species allows addressing the question whether barley accessions commonly share resistance genes to such heterologous rust species. Mapping the genes involved in nonhost resistance in C x S and the OWB populations also allows confirmation of the findings reported before in V x S (JAFARY *et al.* 2006) on inheritance and specificity of the genes conferring nonhost immunity. Furthermore, with this extensive data set we were able to test whether the genes involved in nonhost immunity co-locate with loci for partial resistance of barley to the pathogenic barley leaf rust fungus (*P. hordei*) and with defense gene homologue (DGH) loci that possibly play a role in plant defense (MARCEL *et al.* 2006).

## MATERIAL AND METHODS

### Plant material

A set of Recombinant Inbred Lines (RILs) was obtained from a cross between barley research line SusPtrit, which is in the seedling stage fully susceptible to several heterologous rust fungal species (ATIENZA *et al.* 2004; JAFARY *et al.* 2006) and immune barley cultivar Cebada Capa. By single seed descent from 150 $F_2$ plants a population of 113 $F_9$-derived RILs was obtained. The second mapping population in this study was the doubled-haploid population (DH) Oregon Wolfe Barleys (OWB; 92 lines), derived from a cross between parents Dom and Rec (COSTA *et al.* 2001), kindly provided by Dr. Patrick Hayes of the Oregon State University.

### DNA extraction and genotyping of RIL population

Genomic DNA of the 113 RILs and the parents SusPtrit and Cebada Capa was extracted from leaf tissue of two-weeks old seedlings according to the CTAB based protocol of STEWARD and VIA (1993) adjusted for a 96-well format. The mapping population was genotyped using AFLP and SSR markers. The AFLP procedure was performed according to VOS *et al.* (1995) with some minor modifications. The *Eco*RI primer with three selective nucleotides was labeled with fluorescent near-infrared group (IRD-700 or IRD-800). AFLP fragments were separated by denaturing polyacrylamide gel (PAGE %5.5 Ready to use Gel Matrix, KB Plus, Westburg) on a LI-COR 4200 DNA automated sequencer (LI-COR® Biosciences, Lincoln, NE).

The scoring of the AFLP fragments was based on absence or presence of amplification products. For scoring of the AFLP fingerprints we used Quantar-Pro software (Key gene, Wageningen, NL). In the present study 37 *Eco*RI/*Mse*I primer combinations, each with three selective nucleotides, were used to generate the AFLP markers. Most of these primer combinations (25 out of 37) had been used before to generate AFLP markers for construction of the L94 x Vada (QI *et al.* 1998b), V x S (JAFARY *et al.* 2006) and L94 x 116-5 maps (QI *et al.* 2000). In addition, the C x S population was genotyped for a set of 14 SSR markers. The PCR product for each SSR was loaded in PAGE and visualized on a LI-COR 4200 DNA automated sequencer as described before for AFLP markers.

### Marker analysis and genetic map construction

Marker data of the C x S population were analyzed with JoinMap® 3.0 (VAN OOIJEN and VOORRIPS 2001). Map distances were calculated using Kosambi’s function. Linkage groups were assigned to the respective barley chromosomes by SSR markers and AFLP markers that had already been mapped in the L94 × Vada (QI *et al.* 1998b) and the V x S (JAFARY *et al.* 2006) mapping populations. In addition, the most probable order of loci within the linkage groups was estimated using the freely available (http://www.dpw.wau.nl/pv/index.htm) software programme RECORD (VAN OS *et al.* 2005). A set of 159 markers was selected for use in a skeletal map for QTL mapping. The selection was based on spacing of the markers at about 5 to 10 cM, and agreement between RECORD and Joinmap results on their relative position.

The marker data set of the OWBs was downloaded from the barley project web site of Oregon State University (OSU) (http://barleyworld.org/). From 769 markers in the locus genotype file, a subset of 133 markers was used, as occur in the skeletal map “OWBbase” downloaded from OSU Barley Project website.

### Pathogen materials and phenotyping of the C x S and OWB with heterologous rust fungi

Urediniospores of four heterologous rusts: *P. triticina*, *P. persistens* (old name= *P. agropyrina*), *P. hordei-murini (Phm)* and *P. hordei-secalini (Phs)* were multiplied on their appropriate host plants (Table 1). The spores of *P. hordei* isolate 1.2.1 were multiplied on susceptible barley line L98. Spores of each rust fungus were collected separately, dried for a short period (2-7 days) and stored at -80 °C until inoculation. Seedlings of RILs and DHs were grown in boxes (37 x 39 cm) along with their parents and susceptible host plants. Twelve days after sowing, the first seedling leaves were fixed in horizontal position, the adaxial side facing up. The inoculations were carried out with about 600 spores per $cm^2$ per box using a settling tower (ATIENZA *et al.* 2004). The plants were incubated overnight in a dew chamber during 10 hours (17-18 ºC) at 100% relative humidity and darkness and then transferred to a greenhouse compartment at about 22/18ºC (day/night). Twelve days after inoculation the level of infection was quantified by two parameters: Visible Infection Sites per $cm^2$ (VIS, based on flecks and sporulating pustules) and Infection Frequency, (IF, based on sporulating pustules only) (JAFARY *et al.* 2006).

The disease test was performed in three consecutive replications. The quantitative data for each replication and the average of the three replications were used as phenotypic values for QTL mapping. Resistance in OWB to *Phs* was evaluated in two replications, each on three seedlings per DH line and to *P. persistens* in one replication.

**Table 1.** Pathogen material used in this study

| **Rust species/*forma specialis* *** | **Host plant** | **Common name** | **Location** |
|---|---|---|---|
| *P. hordei* isolate 1.2.1 | *Hordeum vulgare* | barley leaf rust | Wageningen, The Netherlands |
| *P. hordei-murini* | *H. murinum* | Wall barley leaf rust | Sariñena, Aragón, Spain |
| *P. hordei-secalini* | *H. secalinum* | Meadow barley leaf rust | Mesquer, Loire Atlantique, France |
| *P. persistens* | *Elytrigia repens* | Wheat grass leaf rust | Rhenen, Utrecht, The Netherlands |
| *P. triticina* isolate Flamingo | *Triticum aestivum* | Wheat leaf rust | The Netherlands |

*Scientific names of rusts are used as convenient labels and do not reflect necessarily the taxonomic status of the rusts

### Phenotyping C x S and OWB with *P. hordei*

The RILs were grown as described above and tested for the level of partial resistance to *P. hordei* (isolate 1.2.1) using 3 mg spores per plant box resulting in a deposition of about 200 urediniospores per $cm^2$ as described by QI *et al.* (1998a). The latency period (LP) of the fungus on each plant was evaluated by estimating the period (in hours) at which 50% of the ultimate number of pustules became visible. The relative latency period of seedlings (RLP) was then calculated relative to the LP of L94 plants, where L94 was set at 100, as described by PARLEVLIET (1975). Three plants per RIL were tested in three consecutive replications and the average over three seedlings was considered to reflect the level of partial resistance for each line and parents. Phenotyping data for *P. hordei* in OWB were obtained from MARCEL *et al.* (2006).

### QTL mapping

QTL analysis in both RIL and DH populations were performed by using Map QTL software (VAN OOIJEN 2004). In order to map QTLs for resistance to each rust species, interval mapping was performed for both IF and VIS traits in all replications and the average separately. The QTL mapping procedure was followed by automatic co-factor selection, MQM and restricted MQM mapping (JANSEN and STAM 1994; VAN OOIJEN 1999). Significance LOD thresholds at level of 5% for each trait were obtained by running a permutation test on data sets. In a previous study, the QTLs for partial resistance to *P. hordei* present in Cebada Capa had been mapped by QI *et al.* (2000). They used line 116-5 as partially resistant line. This line was derived from a cross between L94 and Cebada Capa, followed by elimination of *Rph7* from the background of Cebada Capa by selection against hypersensitive resistance, but selection for high level of partial resistance. The study by QI *et al.* (2000) provides information on the location of the quantitative genes for resistance of Cebada Capa to *P. hordei* and the results were used to compare with those mapped in the present study.

### Comparison of QTL positions using an integrated consensus map

In order to compare the distribution of QTLs for nonhost resistance and to find possible overlapping QTLs mapped in the three different populations, we used a recently developed integrated map of barley (MARCEL *et al.* 2006). This high-density map has been constructed based on six mapping populations, of which three were used in the present (OWB, C x S) or previous study (V x S; JAFARY *et al.* 2006). The consensus map contains 3,258 markers spanning 1,081 centiMorgans (cM) with an average distance between two adjacent loci of 0.33 cM and the map has been divided into 210 BINs of about 5 cM each. In addition, sixty-three defense gene homologous (DGHs) have been located into the BINs (MARCEL *et al.* 2006). MARCEL *et al.* (2006) further placed 19 QTLs contributing to partial resistance of barley to the pathogen *P. hordei* on their consensus map. Therefore, placement of the QTLs for nonhost resistance identified in the three populations on the consensus map allowed us (1) to compare the QTL positions between the three populations, (2) to find a possible association between nonhost resistance and partial resistance to *P. hordei*, and (3) to find a possible association between the QTLs for nonhost resistance with DGHs.

Coincidence of QTLs for nonhost immunity and DGHs on the consensus map was analyzed by counting the BINs that were occupied by QTLs and DGHs. BINs encompassing the corresponding peak marker of a QTL or molecular marker(s) derived from a DGH(s) were considered as occupied BINs. A chi-square test was applied to test the null hypothesis assuming independent distribution of BINs occupied with a QTL and BINs occupied with a DGH(s). In the same way we determined whether loci for quantitative resistance to heterologous rust fungi tended to coincide with loci for partial resistance to *P. hordei*.

## RESULTS

### Construction of the genetic map

The genetic map of the C x S contained 502 markers (AFLP, SSR and one morphological marker). The order and relative positions of 12 SSR markers and 158 common AFLP markers in the present map were consistent with those on previously published maps (RAMSAY *et al.* 20001; LIU *et al.* 1996; QI *et al.* 1998b; JAFARY *et al.* 2006). The extracted skeletal map for QTL analysis comprised 159 markers, spanned 1132 cM with an average interval between two consecutive markers of 7 cM and a linkage group length varying between 199 cM for chromosome 2(2H) and 127 cM for chromosome 4 (4H). The complete data set of the mapping population has recently been submitted to GrainGenes (http://wheat.pw.usda.gov).

### Phenotyping and QTL mapping in C x S population

The mapping population C x S showed a quantitative segregation for the level of resistance to *P. triticina*, *P. persistens*, *Phm* and *Phs* (Figure1B - E). In none of these cases the resistance was based on a macroscopically visible hypersensitive reaction. Visible infection sites that failed to develop into sporulating pustules were pale green as immature colonies. Cebada Capa was almost immune and SusPtrit the most susceptible accession. The continuous and quantitative segregation for the levels of infection to four heterologous rust fungi indicates polygenic inheritance of resistance. The traits IF and VIS gave for all rust fungi very similar mapping results, although for three heterologous rusts (*Phm*, *Phs* and *P. triticina*) VIS resulted in one additional “minor” QTL. We present here the mapping data obtained from VIS. To each heterologous rust species, four or five QTLs were identified (Table 2). Most of these QTLs (six out of ten) were effective to only one rust species, but some association occurred. One QTL on chromosome 3(3H) was effective to all four heterologous rust fungi with identical peak marker. Support intervals of another QTL on chromosome 4(4H) overlapped for those rusts.

There was very strong and positive correlation between quantitative data obtained for the VIS (r=0.92) and the IF (r=0.82) in case of *Phs* and *Phm* and therefore not surprisingly three chromosomal regions were found to affect both *Phs* and *Phm* (Table 2). This may suggest genes with dual effectiveness to both rusts, or close linkage of genes, each with a rust species specific effect. For all heterologous rust fungi, the resistance alleles originated from Cebada Capa. For *P. hordei* 61% of the RILs showed a hypersensitive reaction due to segregation of the *Rph*7 resistance allele of Cebada Capa. The segregation ratio for HR phenotype deviated significantly from the expected 1:1 ratio (*p*=0.05). The remaining 44 RILs varied quantitatively in level of partial resistance (PR) to *P. hordei* between the parental values, indicating that only Cebada Capa contributed alleles for resistance (Figure 1A). This low number of RILs was insufficient to reliably map the QTLs for partial resistance to *P. hordei*. QI *et al.* (2000) mapped three Cebada Capa derived QTLs for partial resistance in L94 x 116-5, of which one with the highest LOD value on chromosome 2 (2H), was confirmed in the present C x S population (Table 2).

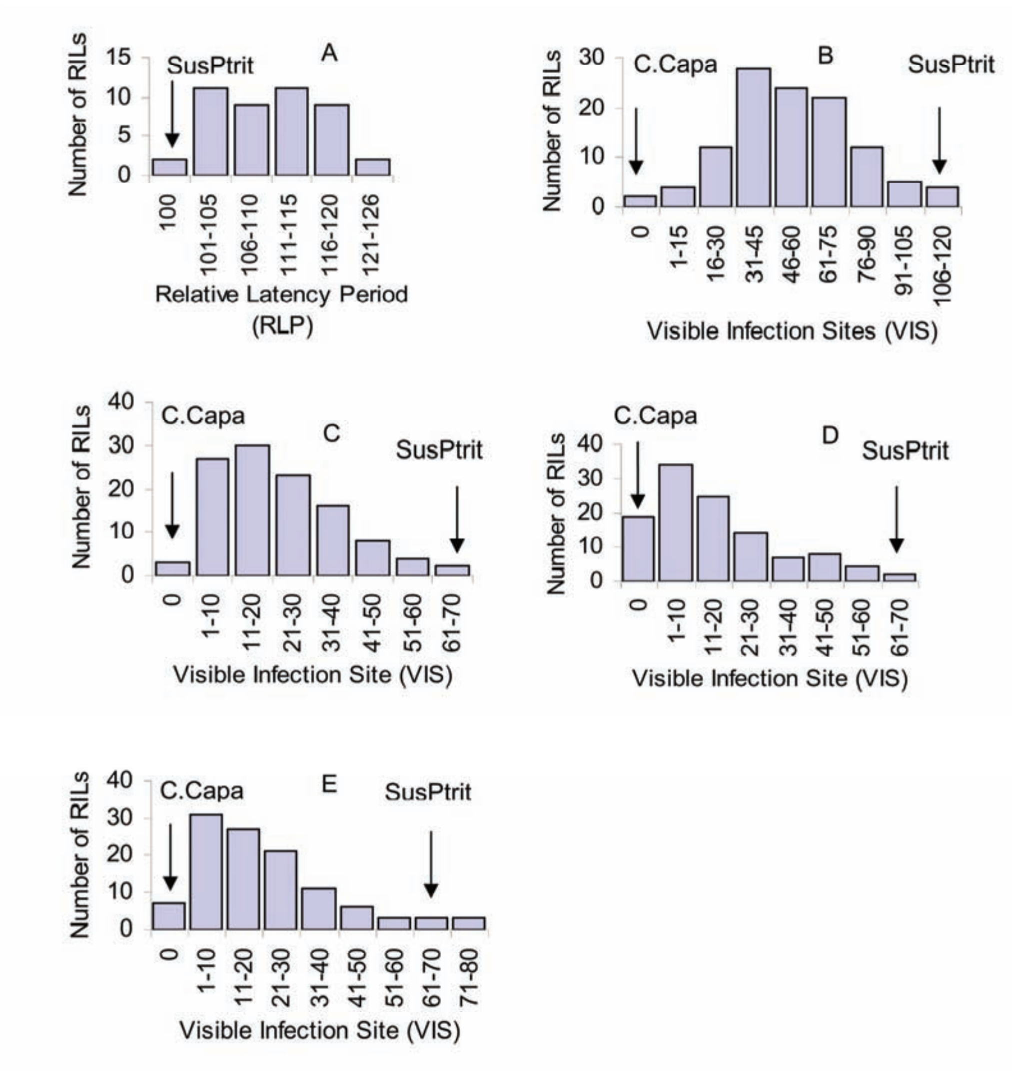


**Figure 1.** Frequency distribution of phenotypes for resistance to five rust species in barley mapping population Cebada Capa x SusPtrit. Values of the two parental lines are shown by arrow. For *Puccinia hordei*, the RLP of Cebada Capa was not measurable due to the hypersensitivity reaction.
A) *P. hordei* B) *P. triticina* C) *P. persistens* D) *P. hordei-murini*
E) *P. hordei-secalini*

### Phenotyping and QTL mapping in OWB population

Resistance to *P. triticina* and *P. persistens* segregated quantitatively in the OWB population, without obvious HR. The frequency distribution of the RILs for the quantitative traits (IF and VIS) was approximately normally distributed (Figure 2A and D). The two parents had a similar intermediate resistance level to *P. triticina*, but differed strongly for *P. persistens*, Rec being immune and Dom being fully susceptible (Figure 2A and D). This indicates a rust specific effect of the underlying genes. Segregation for resistance to *Phs* and *Phm* was both quantitative and qualitative. Fifty-two (57 %) of the DHs exhibited HR to *Phm* and *Phs*. This reaction showed as chlorotic spots, extensive chlorosis, or small, brown necrotic flecks occasionally with small pustules (Figure 3C). All DHs showing HR to *Phm* also showed HR to *Phs*. The major gene for HR reaction originated from parent Rec and was mapped by Joinmap to the short arm of chromosome 5(1H) (56 cM) (Figure 3D). The position of this gene is similar to that of an *R*-gene in the V x S population conferring HR to *Phs* (JAFARY *et al.* 2006). These data suggest involvement of one *R*-gene or of a *R*-gene cluster that is effective to both heterologous rust fungi. The 40 DHs without the hypersensitivity gene showed various levels of infection (0 -78 pustules per cm$^2$) to both rust pathogens, indicating a segregation for additional genes with quantitative effects. QTL mapping for IF indicated, not surprisingly, one large-effect QTL with LOD values 18 and 15 for *Phs* and *Phm*, respectively, on the position of the *R*-gene for hypersensitive resistance (Figure 3A), and one additional QTL that was either specific to *Phs* (chromosome 4(4H)or specific to *Phm* (chromosome 7(5H) ) (Table 2). The quantitative resistance to *Phm* and *Phs* could only be quantified in 40 of the DH lines that did not carry the *R*-gene for hypersensitivity. This low number of lines was probably insufficient to detect all segregating QTLs.

### Comparison of QTLs mapped for resistance in three genetic maps

The QTLs for resistance to the four heterologous rust species and to *P. hordei* mapped in C x S and OWB populations (Table 2) were compared with those detected in the V x S population (JAFARY *et al.* 2006). The map positions of QTLs in that table do not run exactly parallel because of the different length of linkage groups in three different maps. Therefore the integrated consensus map (MARCEL *et al.* 2006) was used to determine the possible overlapping in the support intervals of QTLs mapped in the three populations. We considered QTL regions as the same if their confidence intervals overlapped. Based on the consensus map (Figure 4) 22 chromosomal regions were involved in resistance to at least one heterologous rust species of which the vast majority (16) mapped in only one of the three populations. One QTL for resistance to *P. persistens*, on chromosome 6(6H), segregated in all three populations and five QTLs overlapped in two populations, including the *R*-gene on chromosome 5(1H) (Figure 4). This suggests that the resistance to heterologous rusts of Cebada Capa, Vada and in the OWB population is due to surprisingly different sets of genes. In the C x S population immunity to each rust species is controlled by 4 to 5 QTLs while in OWB both a major gene with strong qualitative effect and minor genes are involved in nonhost immunity.

In the C x S population ten QTLs contributed in resistance to four heterologous rusts of which two were effective to all four heterologous rusts and five were specifically effective to one rust species. In V x S out of 12 QTLs implicated in nonhost immunity, 8 QTLs were significantly effective to only one rust species and none of them was effective to all four rusts (JAFARY *et al.* 2006). In OWB in total 7 QTLs were involved in nonhost immunity of which only one region, corresponding to the position of the *R*-gene on chromosome 5, was effective to two rusts species and the remaining were effective to only one rust species. The QTLs mapped in C x S tended to be less rust species specific than those mapped in V x S and OWB.

**Table 2.** Summary of QTLs conferring resistance to four heterologous rust species and partial resistance to *P. hordei* at the seedling development stage in three barley mapping populations

| **Rust species** | **Cebada Capa X SusPtrit** | | | **Vada X SusPtrit** | | | **Oregon Wolfe** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Chr. | cM$^{a}$ | LOD | Chr. | cM | LOD | Chr. | cM | LOD |
| ***P. triticina*** | 1(7H) | 35-50 | 5.0 | 1(7H) | 85-100 | 8.0 | 2(2H) | 50-70 | 5.0 |
| | 3(3H) | 50-75 | 3.1 | 1(7H) | 125-140 | 7.0 | 2(2H) | 150-165 | 6.5 |
| | 3(3H) | 130-145 | 11.7 | 3(3H) | 110-128 | 3.8 | | | |
| | 4(4H) | 35-50 | 4.2 | 2(2H) | 80-100 | 3.9 | | | |
| | 6(6H) | 70-80 | 3.8 | 6(6H) | 50-65 | 11.0 | | | |
| ***P. hor.-murini*** | 2(2H) | 0-15 | 6.0 | 1(7H) | 125-140 | 5.0 | $^{c}$**5(1H)** | **45-65** | **14.6** |
| | 3(3H) | 130-145 | 6.5 | 3(3H) | 40-60 | 3.1 | 7(5H) | 110-130 | 4.2 |
| | 4(4H) | 40-60 | 6.5 | 5(1H) | 20-35 | 4.0 | | | |
| | 6(6H) | 60-75 | 5.0 | | | | | | |
| | 6(6H) | 113-134 | 5.0 | | | | | | |
| ***P. hor.-secalini*** | 2(2H) | 0-15 | 7.5 | 1(7H) | 65-75 | 4.2 | 4(4H) | 100-120 | 3 |
| | 3(3H) | 130-145 | 3.0 | 5(1H) | 45-60 | 11.0 | $^{c}$**5(1H)** | **45-65** | **18.0** |
| | 4(4H) | 33-53 | 5.8 | 7(5H) | 120-130 | 6.0 | | | |
| | 4(4H) | 95-110 | 2.9 | | | | | | |
| ***P. persistens*** | 1(7H) | 0-20 | 5.0 | 1(7H) | 85-100 | 4.4 | | | |
| | 1(7H) | 65-75 | 4.1 | 1(7H) | 125-140 | 4.2 | | | |
| | 3(3H) | 130-145 | 3.8 | 2(2H) | 120-140 | 5.6 | | | |
| | 6(6H) | 60-75 | 5.8 | 6(6H) | 50-65 | 2.2 | 6(6H) | 50-70 | 4.1 |
| | 4(4H) | 26-53 | 8.0 | 7(5H) | 120-130 | 3.2 | 7(5H) | 170-190 | 11.0 |
| | | | | 3(3H) | 1-20 | 3.5 | | | |
| $^{b}$***P. hordei*** | | | | 1(7H) | 100-110 | 2.7 | 2(2H) | 50-60 | 4.3 |
| | 2(2H) | 100-130 | 12.0 | 2(2H) | 130-140 | 12.1 | 2(2H) | 118-134 | 5.7 |
| | $^{d}$2(2H) | 158-172 | 3.5 | 3(3H) | 100-120 | 3.0 | 3(3H) | 33-54 | 4.8 |
| | $^{d}$6(6H) | 65-70 | 12.8 | | | | 4(4H) | 56-65 | 3.4 |
| | | | | | | | 7(5H) | 156-163 | 11.6 |

$^{a}$Approximate position (cM) of two LOD support intervals based on the results of restricted MQM mapping

$^{b}$Data for QTL mapping for *P. hordei* in the Vada x SusPtrit and OWB population adopted from JAFARY *et al.* (2006) and MARCEL *et al.* (2006) respectively.

$^{c}$Position of *R*-gene for HR to *P. hordei-secalini* and *P. hordei-murini*

$^{d}$ QTLs mapped in L94 x 116-5 by QI *et al.* (2000) which did not appear in the C x S.

### Association of genes for resistance to heterologous rusts with genes for resistance to *P. hordei*

The possible association between nonhost resistance and two types of host resistance (hypersensitive and partial resistance) was investigated in the present study. The major gene for HR reaction to *P. hordei* (*Rph7*) of Cebada Capa was mapped by Joinmap near the top of chromosome 3(3H) (4cM). None of the QTLs for resistance to heterologous rust species coincided with the position of *Rph7*. This indicates that *Rph7* for HR resistance to *P. hordei* does not affect infection by heterologous rusts. In the consensus map (MARCEL *et al.* 2006) QTLs for partial resistance were indicated as they were mapped in L94 x Vada (QI *et al.* 1998a), populations OWB, Steptoe x Morex (both by MARCEL *et al.* 2006), L94 x 116-5 (by QI *et al.* 2000), in V x S (JAFARY *et al.* 2006) and C x S (present work). The consensus map is divided into BINs of 5 cM. The numbers of BINs harboring a peak marker of a QTL for resistance to at least one of the four heterologous rusts mapped in V x S, C x S and OWB, and a peak marker for a QTL for partial resistance to *P. hordei* were counted, to test for possible association between the two traits (Table 3).

One QTL for partial resistance on chromosome 1(7H), *Rphq8*, mapped in several populations, and had different peak markers in each population. It therefore occupied two BINs. Since *R*-genes are likely to have a separate system, superimposed on the non-hypersensitive basal resistance, we did not expect a particular association between the *R*-gene for nonhost resistance on chromosome 5 (56cM) of OWB with the loci for partial resistance on the integrated map. We therefore excluded that locus from calculation. The Chi-square test indicated that there

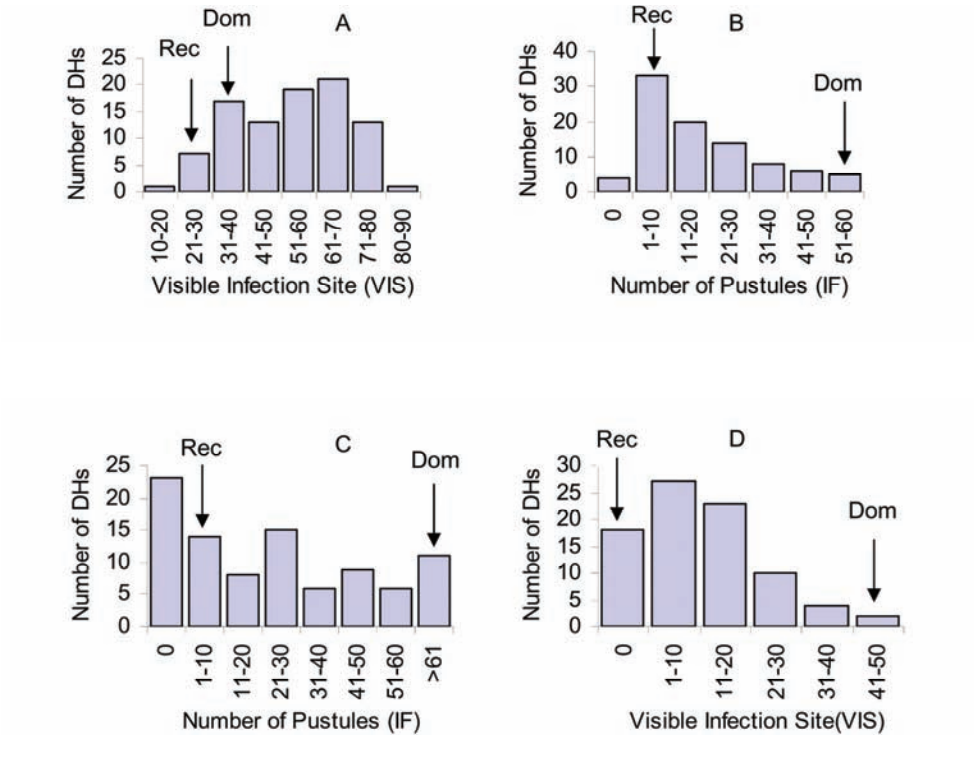


**Figure 2.** Frequency distribution of phenotypes for resistance to four heterologous rust species in OWB mapping population. Values of the two parental lines are shown by arrows.
A) *P. triticina* B) *P. hordei-murini* C) *P. hordei-secalini* D) *P. persistens*

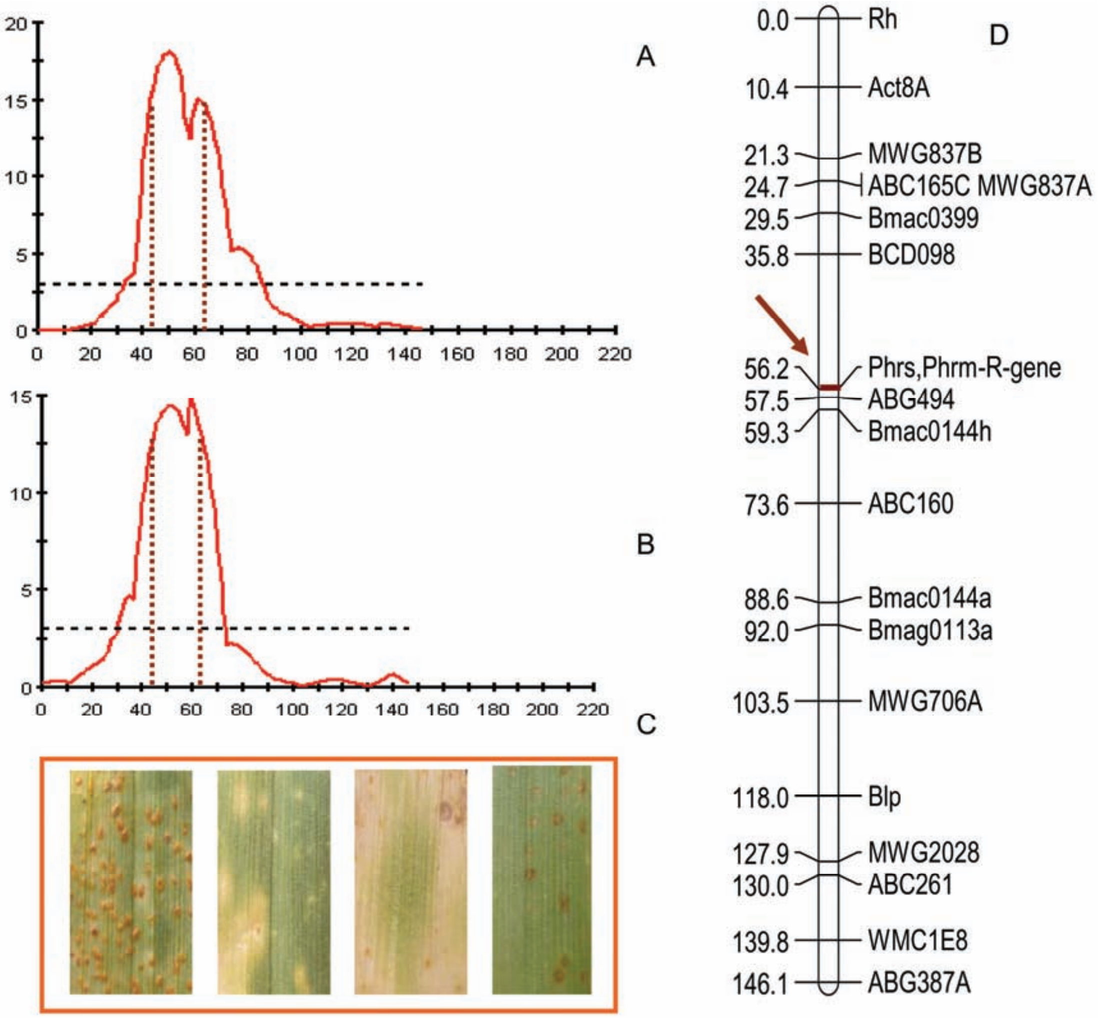
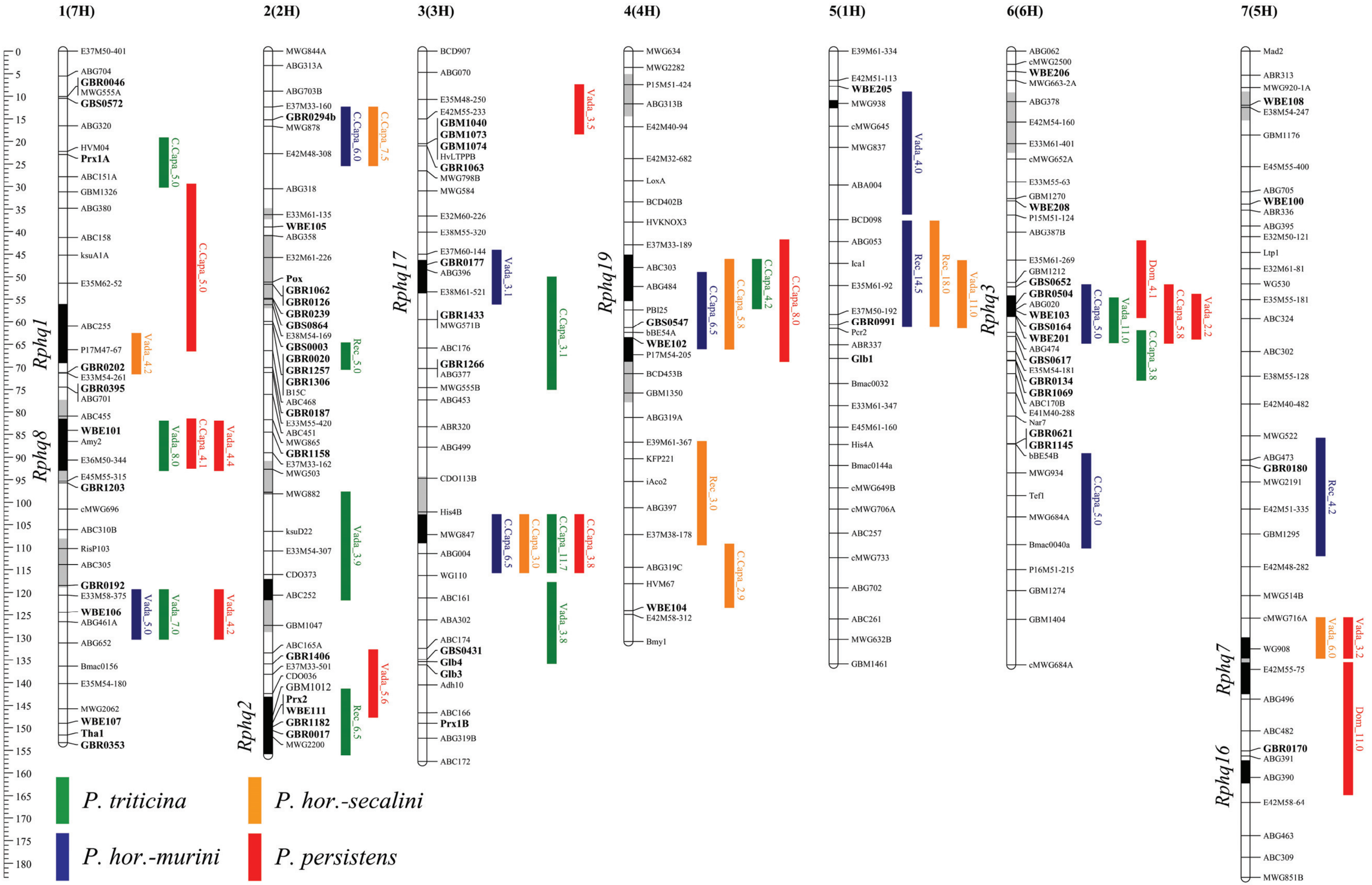


**Figure 3.** Mapping of an *R-gene* for hypersensitive resistance (HR) to *P. hordei-secalini* and *P. hordei-murini* in OWB mapping population.
A and B) LOD profiles of QTL (based on interval mapping) on chromosome 5(1H) with the highest effect to *P. hordei-secalini* (A) and *P. hordei-murini* (B). Horizontal and vertical dotted lines show LOD threshold value and support interval of the QTL, respectively.
C) The HR reaction of OWB (chlorotic spots, extensive chlorosis and small necrotic flecks) to compare with susceptible reaction (left) of OWB infected with *P. hordei-secalini.*
D) Position of *R*-gene conferring HR resistance to both *P. hordei-secalini* and *P. hordei-murini* mapped using Joinmap software on chromosome 5(1H) (56.2 cM) in the support interval of the QTLs with the highest effect.

**Figure 4.** (Next page**)**. Locations of QTLs for nonhost immunity, mapped in three individual barley linkage maps to four heterologous rust species, on a BIN map extracted from a high-density consensus map of barley (MARCEL *et al.* 2006). Length of QTL bars corresponds approximately to the two LOD support intervals (from peak marker) based on the results of rMQM. The parental line contributing the allele for resistance and the LOD value obtained by rMQM are indicated on the right side of the QTL bars. Within chromosome bars, regions for which an effect for partial resistance to barley leaf rust was previously detected (MARCEL *et al.* 2006) are indicated in black if overlapping with the confidence intervals of QTL(s) for nonhost immunity presented in this study or in grey if not overlapping. The name of the QTL for partial resistance (*Rphq-*) is indicated when its peak marker(s) co-located in the same BIN as peak marker(s) of QTL(s) for nonhost immunity. The 63 loci in **bold** are DGH based markers. The ruler on the left end side of the figure indicates the distance in centiMorgans (according to Kosambi) from the top of each chromosome.


1(7H)
2(2H)
3(3H)
4(4H)
5(1H)
6(6H)
7(5H)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
130
135
140
145
150
155
160
165
170
175
180
E37M50-401
ABG704
GBR0046
MWG555A
GBS0572
ABG320
HVM04
Prx1A
ABC151A
GBM1326
ABG380
ABC158
ksuA1A
E35M62-52
ABC255
P17M47-67
GBR0202
E33M54-261
GBR0395
ABG701
ABC455
WBE101
Amy2
E36M50-344
E45M55-315
GBR1203
cMWG696
ABC310B
RisP103
ABC305
GBR0192
E33M58-375
WBE106
ABG461A
ABG652
Bmac0156
E35M54-180
MWG2062
WBE107
Tha1
GBR0353
Rphq1
Rphq8
C.Capa_5.0
C.Capa_5.0
Vada_4.2
Vada_8.0
C.Capa_4.1
Vada_4.4
Vada_5.0
Vada_7.0
Vada_4.2
MWG844A
ABG313A
ABG703B
E37M33-160
GBR0294b
MWG878
E42M48-308
ABG318
E33M61-135
WBE105
ABG358
E32M61-226
Pox
GBR1062
GBR0126
GBR0239
GBS0864
E38M54-169
GBS0003
GBR0020
GBR1257
GBR1306
B15C
ABC468
GBR0187
E33M55-420
ABC451
MWG865
GBR1158
E37M33-162
MWG503
MWG882
ksuD22
E33M54-307
CDO373
ABC252
GBM1047
ABC165A
GBR1406
E37M33-501
CDO036
GBM1012
Prx2
WBE111
GBR1182
GBR0017
MWG2200
Rphq2
C.Capa_6.0
C.Capa_7.5
Rec_5.0
Vada_3.9
Vada_5.6
Rec_6.5
BCD907
ABG070
E35M48-250
E42M55-233
GBM1040
GBM1073
GBM1074
HvLTPPB
GBR1063
MWG798B
MWG584
E32M60-226
E38M55-320
E37M60-144
GBR0177
ABG396
E38M61-521
GBR1433
MWG571B
ABC176
GBR1266
ABG377
MWG555B
ABG453
ABR320
ABG499
CDO113B
His4B
MWG847
ABG004
WG110
ABC161
ABA302
ABC174
GBS0431
Glb4
Glb3
Adh10
ABC166
Prx1B
ABG319B
ABC172
Rphq17
Vada_3.1
C.Capa_3.1
Vada_3.5
C.Capa_6.5
C.Capa_3.0
C.Capa_11.7
C.Capa_3.8
Vada_3.8
MWG634
MWG2282
P15M51-424
ABG313B
E42M40-94
E42M32-682
LoxA
BCD402B
HVKNOX3
E37M33-189
ABC303
ABG484
PBI25
GBS0547
bBE54A
WBE102
P17M54-205
BCD453B
GBM1350
ABG319A
E39M61-367
KFP221
iAco2
ABG397
E37M38-178
ABG319C
HVM67
WBE104
E42M58-312
Bmy1
Rphq19
C.Capa_6.5
C.Capa_5.8
C.Capa_4.2
C.Capa_8.0
Rec_3.0
C.Capa_2.9
E39M61-334
E42M51-113
WBE205
MWG938
cMWG645
MWG837
ABA004
BCD098
ABG053
Ica1
E35M61-92
E37M50-192
GBR0991
Pcr2
ABR337
Glb1
Bmac0032
E33M61-347
E45M61-160
His4A
Bmac0144a
cMWG649B
cMWG706A
ABC257
cMWG733
ABG702
ABC261
MWG632B
GBM1461
Vada_4.0
Rec_14.5
Rec_18.0
Vada_11.0
ABG062
cMWG2500
WBE206
MWG663-2A
ABG378
E42M54-160
E33M61-401
cMWG652A
E33M55-63
GBM1270
WBE208
P15M51-124
ABG387B
E35M61-269
GBM1212
GBS0652
GBR0504
ABG020
WBE103
GBS0164
WBE201
ABG474
GBS0617
E35M54-181
GBR0134
GBR1069
ABC170B
E41M40-288
Nar7
GBR0621
GBR1145
bBE54B
MWG934
Tef1
MWG684A
Bmac0040a
P16M51-215
GBM1274
GBM1404
cMWG684A
Rphq3
C.Capa_5.0
Vada_11.0
Dom_4.1
C.Capa_3.8
C.Capa_5.8
Vada_2.2
C.Capa_5.0
Mad2
ABR313
MWG920-1A
WBE108
E38M54-247
GBM1176
E45M55-400
ABG705
WBE100
ABR336
ABG395
E32M50-121
Ltp1
E32M61-81
WG530
E35M55-181
ABC324
ABC302
E38M55-128
E42M40-482
MWG522
ABG473
GBR0180
MWG2191
E42M51-335
GBM1295
E42M48-282
MWG514B
cMWG716A
WG908
E42M55-75
ABG496
ABC482
GBR0170
ABG391
ABG390
E42M58-64
ABG463
ABC309
MWG851B
Rphq7
Rphq16
Rec_4.2
Vada_6.0
Vada_3.2
Dom_11.0
P. triticina
P. hor.-secalini
P. hor.-murini
P. persistens

was a strong significant association between QTLs for heterologous rusts with QTLs for partial resistance, indicating that genes for both types of resistance encode a similar function in basal defense or basic compatibility (Table 3).

In total nine BINs harbored a peak marker both for nonhost resistance and a peak marker for partial resistance. Under the assumption of independent distribution of those QTLs the expected number of BINs would be 3. Of those nine BINs, in six cases the resistance alleles were contributed by the same and in two cases by different barley lines indicating that mostly pleiotropy and to a lesser extent alternative alleles explain dual effectiveness of QTLs involved in resistance to host and nonhost pathogens.

### Association of QTLs for resistance to heterologous rusts with Defense Gene Homologue-derived marker loci

The BIN system of the barley consensus map was also used to test for a possible association between the distribution of loci for nonhost resistance and the distribution of 63 previously mapped DGHs (MARCEL *et al.* 2006). The peak markers of loci for nonhost resistance occupied 29 BINs while the DGH derived markers occupied 43 BINs. We only focused on the basal, non-hypersensitivity class of resistance genes and therefore the *R*-gene for HR to *Phs* and *Phm* in OWB was excluded. Thirteen BINs were co-occupied by QTL peak marker(s) and by DGH derived marker(s). Uunder the assumption of independent distribution the expected number would have been 5.9 (Table 4). We tested by chi-square test the null hypothesis assuming an independent distribution of BINs occupied by nonhost QTL peak marker(s) and BINs occupied by DGH marker(s). The null hypothesis was rejected with a high probability (Table 4) suggesting that there is an association between distribution of the QTLs and of DGHs over the consensus map of barley and some of DGHs are possibly responsible for quantified traits for nonhost immunity.

The DGHs identified as candidate genes to explain nonhost resistance in this study represent 12 genes or gene families (Table 5). Some of them were previously reported as candidate genes to explain partial resistance to barley leaf rust (MARCEL *et al.* 2006) while some other genes were not previously identified: chitinase 2 (3 hits), BAX inhibitor 1 (2 hits), phenylalanine ammonia-lyase (2 hits), fibrillin-like protein (2 hit), *Rar1* (1 hit), pathogenesis-related protein 1.2 (1 hit) and nitrate-induced protein (1 hit). It may imply that some of those DGHs possibly contribute to nonhost immunity.

**Table 3.** Chi-square test on the probability of independent distribution of QTLs conferring resistance to heterologous rusts (QTLnh) and QTLs conferring partial resistance to barley leaf rust (QTLpr) over BINs on the consensus genetic map of barley

| Class[a] |  | Observed results (O) | Expected results (E) |  | $(O-E)^2$ / E |
|---|---|---|---|---|---|
| **QTLnh** | **QTLpr** |  |  |  |  |
| 0 | 0 | 169 | (181*189)/210 | = 162.9 | 0.22 |
| 0 | 1 | 12 | (181*21)/210 | = 18.1 | 2.05 |
| 1 | 0 | 20 | (29*189)/210 | = 26.1 | 1.42 |
| 1 | 1 | 9 | (29*21)/210 | = 2.9 | 12.83 |
|  |  |  |  |  | **$\chi^2$ = 16.52**[b] |

a class 0 indicates BINs unoccupied by QTLnh and/or QTLpr and a class 1 indicates occupied by a QTLnh and/or by a QTLpr

[b] with a number of degrees of freedom (df) = 3 the null hypothesis is rejected with probability $p < 0.005$

**Table 4.** Chi-square test on the probability of independent distribution of QTLs conferring resistance to heterologous rusts (QTLnh) and DGHs over BINs on the consensus genetic map of barley

| Class[a] |  | Observed results (O) | Expected results (E) |  | $(O-E)^2$ / E |
|---|---|---|---|---|---|
| **QTLnh** | **DGH** |  |  |  |  |
| 0 | 0 | 151 | (181*168)/210 | = 144.8 | 0.34 |
| 0 | 1 | 30 | (181*42)/210 | = 36.2 | 1.34 |
| 1 | 0 | 16 | (29*168)/210 | = 23.2 | 2.16 |
| 1 | 1 | 13 | (29*42)/210 | = 5.8 | 8.39 |
|  |  |  |  |  | **$\chi^2$ = 12.23**[b] |

[a] class 0 indicates BINs unoccupied by QTLnh and/or DGH and a class 1 indicates BINs occupied by a QTLnh and/or by one to several DGH(s)

[b] with a number of degrees of freedom (df) = 3 the null hypothesis is rejected with a probability $0.005 < p < 0.01$

## DISCUSSION

### Segregation for near-nonhost resistance in barley

A plant species is called host when all or the majority of accessions are susceptible to the pathogen. Full nonhost resistance can be defined as immunity displayed by an entire plant species against all genotypes of a plant pathogen (HEATH 2000). Drawing a discrete line between host and nonhost status is not always straightforward since some plant species – pathogen species combinations suggest marginal host or near-nonhost status, when only few accessions of a plant species are at most moderately susceptible to a heterologous pathogen (NIKS 1987). It has been shown, for example, that barley is a full host to *P. hordei*, near-nonhost to some heterologous rust species and full nonhost to some other rust species like *P. recondita* f. sp. *recondita* of rye (ATIENZA *et al.* 2004; ZHANG *et al.* 1994; HOOGKAMP *et al.* 1998). We found that wild (*Hordeum spontaneum*), ancient and exotic cultivated barleys, landraces and accessions of barley with naked seeds have more chance to be somewhat susceptible to heterologous rust fungi than modern cultivars (ATIENZA *et al.* 2004). Since the parents of the OWB population have been generated by convergent crosses of exotic barley accessions (COSTA *et al.* 2001), it was not entirely unexpected that this population segregated for resistance to heterologous rust fungal species. It is still a challenge to understand why some ancient cultivated and some wild barley accessions possess susceptibility genes and what is the co-evolutionary implication of carrying such genes by those accessions. We think that inheritance studies of near-nonhost resistance are relevant to understand the mechanisms and the genetics behind full nonhost immunity and to gain insight into the co-evolutionary aspects of nonhost immunity in plants.

### Role of *R*-genes in nonhost resistance

Our data show that the *Rph7* gene for hypersensitive resistance to *P. hordei* carried by Cebada Capa does not confer resistance to the other rust species tested here. This confirms earlier evidence that this gene is not effective to *P. triticina* (NEU *et al.* 2003; NIKS and RUBIALES 1994). NIKS and RUBIALES (1994) however reported that in L94 background the *Rph7* gene conferred complete resistance to *Phm* (IT0 on the 0 – 9 scale). They used an isolate of *Phm* collected in the Netherlands, whereas our isolate was collected in Aragón, Spain. We checked both isolates on the near-isogenic line L94-*Rph7* and confirmed that the Dutch isolate is avirulent to *Rph7*, while the Spanish isolate was moderately virulent. Further investigations are required to verify this possible isolate specificity of resistance in barley to these two isolates of *Phm*. In the OWB population we mapped one *R*-gene on chromosome 5(1H) for HR reaction to both *Phs* and *Phm* and not to *P. hordei*. *Phs* and *Phm* are not closely related rust fungi: the former belonging to the *P. triticina* group and the latter to the *P. hordei* group (JAFARY *et al.* 2006). Their respective host species are both in the genus *Hordeum*, which may explain the dual effectiveness of this *R*-gene. Interestingly, in the V x S population we found one *R*-gene at about the same position, but that was effective to *Phs* and not to *Phm* nor to *P. hordei*. The locus in OWB may carry an allele of the *R*-gene that is effective to both *Phs* and *Phm*, whereas the allele of Vada is only effective to *Phs*. Alternatively, the locus may be a cluster of homologous genes, of which one is effective to *Phs* (in Vada as well as OWB), but another only present in OWB to *Phm*. It is intriguing that cultivated barley, including a modern cultivar like ‘Vada’, carries an effective *R*-gene against a rust species (*Phs*) that is not pathogenic to cultivated barley, and hence is very unlikely to be selected for by breeders. Our data suggest that occasionally *R*-genes with qualitative effects may contribute to nonhost immunity in barley to heterologous rust fungi.

**Table 5.** Identification of candidate barley ESTs to explain the QTLs for resistance to heterologous rust species mapped in four barley mapping populations by looking at co-location of defence gene homologues with QTL peak markers in the 210 BINs of the high-density barley consensus map of MARCEL *et al.* (2006).

| **BIN Nr.**[a] | **Resistance locus** | **DGH name**[l] | **Distanc (cM)**[c] | **Tentative Annotation**[d] | **Gene symbol**[e] | **Acc. Nr.**[f] | ***E* value**[g] |
|---|---|---|---|---|---|---|---|
| 2H_02.2 | *P. hor.-murini*_(SuxCC; 6.0) | GBR0294b | 2.83 | Phenylalanine ammonia-lyase (Fragment) | *Pal* | Z49146 | 4.7E-188 |
| 2H_02.2 | *P. hor.-secalini*_(SuxCC; 7.5) | GBR0294b | 2.83 | Phenylalanine ammonia-lyase (Fragment) | *Pal* | Z49146 | 4.7E-188 |
| 2H_08.1_k8 | *P. triticina*_(OWB; 5.0) | GBR0187 | 1.53 | *Rar1* {*Hordeum vulgare*} | *Rar1* | AF192261 | 5.6E-11 |
| 2H_15.2 | *P. persistens*_(SuxV; 5.6) | Prx2 | 1.19 | Peroxidase precursor | *Prx2* | X62438 | 3.5E-119 |
| 2H_15.2 | *P. persistens*_(SuxV; 5.6) | WBE111 | 1.19 | Peroxidase precursor | *Prx2* | X62438 | 1.5E-57 |
| 2H_15.2 | *P. persistens*_(SuxV; 5.6) | GBR1182 | 2.17 | similar to Peroxidase precursor | *Prx2* | X62438 | 7.8E-60 |
| 2H_15.2 | *P. persistens*_(SuxV; 5.6) | GBR0017 | 2.97 | similar to Peroxidase precursor | *Prx2* | X62438 | 1.5E-57 |
| 2H_15.2 | *P. triticina*_(OWB; 6.5) | Prx2 | 0.64 | Peroxidase precursor | *Prx2* | X62438 | 3.5E-119 |
| 2H_15.2 | *P. triticina*_(OWB; 6.5) | WBE111 | 0.64 | similar to Peroxidase precursor | *Prx2* | X62438 | 1.5E-57 |
| 2H_15.2 | *P. triticina*_(OWB; 6.5) | GBR1182 | 1.62 | similar to Peroxidase precursor | *Prx2* | X62438 | 7.8E-60 |
| 2H_15.2 | *P. triticina*_(OWB; 6.5) | GBR0017 | 2.42 | similar to Peroxidase precursor | *Prx2* | X62438 | 1.5E-57 |
| 4H_06.2 | *P. persistens*_(SuxCC; 8.0) | GBS0547 | 1.56 | homologue to Thaumatin-like protein TLP4 | *pWir232* | X58394 | 1.3E-50 |
| 4H_07.1_k7 | *P. hor.-murini*_(SuxCC; 6.5) | WBE102 | 2.41 | similar to Nitrate-induced NOI protein | *Rin4* | NM_113411 | 2.9E-05 |
| 5H_13.2/13.3[h] | *P. persistens*_(OWB; 11.0) | GBR0170 | 1.25 | homologue to Thaumatin-like protein TLP7 | *pWir232* | X58394 | 3.2E-37 |
| 6H_06.1 | *P. persistens*_(SuxV; 2.2) | GBS0652 | 1.55 | similar to Fibrillin-like protein | *At4g22240* | BT020480 | 1.8E-20 |
| 6H_06.1 | *P. persistens*_(SuxV; 2.2) | GBR0504 | 2.26 | homologue to BAX inhibitor 1 | *pBi-1* | AJ290421 | 1.7E-103 |
| 6H_06.1 | *P. persistens*_(OWB; 4.1) | GBS0652 | 4.79 | similar to Fibrillin-like protein | *At4g22240* | BT020480 | 1.8E-20 |
| 6H_06.1 | *P. persistens*_(OWB; 4.1) | GBR504 | 0.98 | homologue to BAX inhibitor 1 | *pBi-1* | AJ290421 | 1.7E-103 |
| 6H_06.2 | *P. hor.-murini*_(SuxCC; 5.0) | WBE103 | 1.31 | homologue to Glycoprotein | *Sod* | W21624 | 3.7E-22 |
| 6H_06.2 | *P. persistens*_(SuxCC; 5.8) | WBE103 | 1.31 | homologue to Glycoprotein | *Sod* | W21624 | 3.7E-22 |
| 6H_06.2 | *P. hor.-murini*_(SuxCC; 5.0) | GBS0164 | 0.15 | homologue to Glycoprotein | *Sod* | W21624 | 3.7E-22 |
| 6H_06.2 | *P. persistens*_(SuxCC; 5.8) | GBS0164 | 0.15 | homologue to Glycoprotein | *Sod* | W21624 | 3.7E-22 |
| 6H_06.2 | *P. hor.-murini*_(SuxCC; 5.0) | WBE201 | 0.12 | weakly similar to Probable serine/threonine-protei kinase pelle | *HvNR-F6* | BU670764 | 6.3E-92 |
| 6H_06.2 | *P. persistens*_(SuxCC; 5.8) | WBE201 | 0.12 | weakly similar to Probable serine/threonine-protei kinase pelle | *HvNR-F6* | BU670764 | 6.3E-92 |
| 6H_06.2 | *P. triticina*_(SuxV; 11.0) | WBE103 | 2.22 | homologue to Glycoprotein | *Sod* | W21624 | 3.7E-22 |

**Table 5.** continued

| BIN Nr.[a] | Resistance locus | DGH name[b] | Distance (cM)[c] | Tentative Annotation[d] | Gene symbol[e] | Acc. Nr.[f] | *E* value[g] |
|---|---|---|---|---|---|---|---|
| 6H_06.2 | *P. triticina*_(SuxV; 11.0) | WBE201 | 1.03 | weakly similar to Probable serine/threonine-protei kinase pelle | *HvNR-F6* | BU670764 | 6.3E-92 |
| 6H_07.2 | *P. triticina*_(SuxCC; 3.8) | GBR0134 | 0.46 | homologue to Peroxidase BP 1 precursor | *Prx2* | X62438 | 1.4E-29 |
| 6H_07.2 | *P. triticina*_(SuxCC; 3.8) | GBR1069 | 0.59 | weakly similar to Peroxidase precursor (Fragment) | *Prx2* | X62438 | 8.7E-25 |
| 7H_02.2 | *P. triticina*_(SuxCC; 5.0) | Prx1A | 0.26 | homologue to Peroxidase BP 1 precursor | *Prx2* | X62438 | 1.4E-29 |
| 7H_06.2 | *P. hor.-secalini*_(SuxV; 4.2) | GBR0202 | 3.66 | similar to Pathogenesis-related protein 1. precursor | *Bsi1* | Z48728 | 5.4E-94 |
| 7H_07.2 | *P. persistens*_(SuxCC; 4.1) | WBE101 | 2.58 | no significant similarity found | *HvNR-F1* | BU670759 | 1.2E-27 |
| 7H_07.2 | *P. persistens*_(SuxV; 4.4) | WBE101 | 2.12 | no significant similarity found | *HvNR-F1* | BU670759 | 1.2E-27 |
| 7H_11.2 | *P. triticina*_(SuxV; 7.0) | WBE106 | 0.00 | homologue to Chitinase 2 | *Cht1b* | X15349 | 4.0E-81 |
| 7H_11.2 | *P. hor.-murini*_(SuxV; 5.0) | WBE106 | 0.00 | homologue to Chitinase 2 | *Cht1b* | X15349 | 4.0E-81 |
| 7H_11.2 | *P. persistens*_(SuxV; 4.2) | WBE106 | 0.00 | homologue to Chitinase 2 | *Cht1b* | X15349 | 4.0E-81 |

[a] BIN number as assigned on the barley consensus map of MARCEL *et al.* (2006)

[b] name of the Defense Gene Homologue (DGH) mapped in the same BIN as the corresponding QTL

[c] distance (in cM) between a QTL peak marker and a DGH marker co-localizing in the same BIN on the barley consensus map of MARCEL *et al.* (2006)

[d] tentative annotation of the barley ESTs corresponding to the DGHs identified (in TIGR Barley Gene Index)

[e] symbol of the defense/resistance gene used for tBLASTx in the TIGR Barley Gene Index

[f] GenBank accession number of the defense/resistance gene

[g] *E* value obtained for barley ESTs by tBLASTx analysis with defense/resistance genes in the TIGR Barley Gene Index

[h] *P. persistens*_(OWB; 11.0) peak marker and GBR0170 do not co-locate in the same BIN but were placed next to each other in two consecutive BINs on the barley consensus map of MARCEL *et al.* (2006)

## Specificity of QTLs involved in nonhost immunity and partial resistance

One *R*-gene for nonhost resistance in OWB and three QTLs in C x S were effective to both *Phm* and *Phs.* These data show that there is an association in genetics of resistance to *Phm* and *Phs* in both OWB and the C x S populations and suggest that the relationship between hosts (both *Hordeum*) is more important than the relationship between rust pathogens. This may imply that the host plants determine to a greater degree the quality of effectors that a pathogenic rust fungus produces than the taxonomic position of the rust. However, our data from the V x S did not support this hypothesis since in that population three QTLs confer resistance to only *Phm* while combination of one major gene and two QTLs are involved in resistance to *Phs* and none of the QTLs of Vada are effective to both rust fungi (JAFARY *et al.* 2006). We checked the QTLs with dual effects in the consensus map (Figure 4) and found that two combinations, *Phs/Phm* and *P. triticina/P. persistens*, occurred more frequently than the others. In the former combination two host plants are phylogenetically closely related and in the latter combination two rusts pathogens are phylogenetically closely related (JAFARY *et al.* 2006). In all three populations some QTLs showed effectiveness to more than one rust fungus which can either be due to close linkage of genes or to pleiotropy. However, in all three populations rust species specificity is the predominant characteristic of QTLs contributing to nonhost immunity in barley. The significant association between loci for partial resistance to *P. hordei* and the QTLs for resistance to heterologous rusts (Table 3) confirms earlier evidence that these two traits are associated with each other (NIKS 1983a; ZHANG *et al.* 1994; HOOGKAMP *et al.* 1998). Indeed, both are based on reduced haustorium formation by the rust, without substantial hypersensitivity (NIKS 1983 a and b). Therefore we presume that genes governing partial resistance to *P. hordei* play similar roles in basal resistance as genes for resistance to heterologous rusts.

## Diversity of loci carrying nonhost resistance

In Vada QTLs for heterologous rusts tended to be located most commonly on chromosomes 1(7H), 3(3H), 5(1H) and the least on chromosome 4(4H). The QTLs in Cebada Capa, however, were located most on chromosome 4(4H), and none of them was mapped on chromosome 5(1H). This emphasized that the QTLs in Vada are mostly different from those in Cebada Capa. It is remarkable that QTLs mapped for nonhost resistance are very diverse in the three different mapping populations. This reminds the differential expression of resistance QTLs to host pathogens which has been reported by BILGIC *et al.* (2005). They found that quantitative resistance alleles in barley to Spot blotch, caused by *Cochliobolus sativus*, may appear in different map positions in three different populations while all populations share the same resistant parent. They could rule out the possible effects of epistasis between resistance loci and QTL x environment interactions but they could not elaborate a plausible hypothesis to this apparent inconsistency. In our study, we presume the susceptibility alleles in SusPtrit to be the peculiar alleles. We expected those susceptibility alleles to appear in any cross between SusPtrit with any immune barley. The differential expression of QTLs might be due to unknown causes, as in the work of BILGIC *et al.* (2005). Alternatively, immune barleys may also contain some susceptibility alleles on certain QTLs, and therefore will not segregate for the same QTLs if crossed with SusPtrit. This possibility is consistent with the observation that crossing exotic barley lines with slight susceptibility to heterologous rusts resulted always in transgression towards increased susceptibility, leading to the extreme research line SusPtrit (ATIENZA *et al.* 2004). Our data suggest that, for example, Vada and Cebada Capa do not share QTLs for resistance to *Phm* and *Phs* (Figure 4). This suggests that crossing both immune accessions should result in at least some susceptible transgressive offspring.

Our evidence that each barley accession has a different combination of loci carrying resistance alleles to heterologous rust fungi parallels previous findings on partial resistance of barley to *P. hordei*. QI *et al.* (2000) showed that in the cross L94 x 116-5 a different set of QTLs for partial resistance segregated than in population L94 x Vada, with only one out of five QTLs coinciding in these two mapping populations. More recently MARCEL *et al.* (2006) compared positions of QTLs for partial resistance in five barley mapping populations and showed that each crossing combination segregated for different sets of QTLs, with only few QTLs shared by any pair of cultivars. These data suggest that there is an abundance of loci carrying alleles for partial resistance which is very similar to the abundance of QTLs governing resistance to heterologous rust fungi.

## Role of QTL genes in nonhost immunity

Nonhost resistance is a durable and complete type of resistance in plants and may encompass passive and active defense responses (NÜRNBERGER *et al.* 2004). Innate immunity includes the active defense response in plants which is initiated by perception of Pathogen-Associated Molecular Patterns (PAMP) (FELIX *et al.* 1999; NÜRNBERGER *et al.* 2004). Perception of PAMPs by extra-cellular receptor-like kinases (RLK) activates PAMP-triggered immunity (PTI) which requires signals through MAP kinase cascades (CHISHOLM *et al.* 2006). Immunity of Cebada Capa, Vada and other barley accessions to different heterologous rust fungi tested is not likely to be caused by preformed barriers. It is more likely to be due to genes that are involved in compatibility or resistance as we discussed in our previous study (JAFARY *et al.* 2006).

Rust fungi studied here, similar to other microbes, contain PAMPs, which have a critical function in the life style of the organism (NÜRNBERGER *et al.* 2004). PTI therefore is probably activated in all barley accessions, whether immune like Cebada Capa or susceptible like SusPtrit, following to perception of PAMPs from rust fungi. This perception should result in immunity, unless rust fungi deliver appropriate effector proteins that target multiple host proteins to suppress basal immune responses of the plant. The QTLs mapped here are likely to be specific host factor genes that are targeted by rust species to suppress basal defense. For successful pathogenesis, therefore, rust fungi need to recognize and negate these genes to be able to suppress basal defense. The role of some of the candidate genes that co-located with QTLs for nonhost resistance (Table 5) is in agreement with our hypothesis. For example the candidate gene co-located with a QTL for nonhost resistance to *P. persistens* in three populations is a homologue of BAX inhibitor 1. These proteins (BI-1) have been characterized as suppressors of programmed cell death in mammals and plants (KAWAI *et al.*1999; SANCHEZ *et al.* 2000). In barley BI-1 is a suppressor of nonspecific basal resistance and is also up-regulated during interaction with the heterologous wheat pathogen *Blumeria graminis* f. sp. *tritici* (EICHMANN *et al.* 2004). It is the challenge to clone some of the plant genes underlying the QTL effects in order to understand what plant factors determine the success or failure of suppression of basal defense of plant cells, and hence the host status of plants.

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# CHAPTER 5

# The near-nonhost resistance of barley to *Puccinia hordei-bulbosi*: mechanisms and genetics of basal resistance

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## ABSTRACT

It is one of the intriguing questions in plant biology which factors determine whether a plant species is a host or a non-host to a particular organism. We developed the barley–*Puccinia* rust model system to study the inheritance and mechanisms of nonhost immunity in barley to heterologous rust fungi. We screened 105 barley accessions in the seedling stage for resistance to *Puccinia hordei-bulbosi*, the leaf rust fungus of *Hordeum bulbosum*. Our data indicated that only 16% of barley accessions (mainly exotic barleys, landraces and wild barleys) are in the seedling stage somewhat susceptible to *Phb* implying that barley is a near-nonhost to this rust species. Resistance to *Phb* inherited quantitatively in three mapping populations: Vada x SusPtrit (VxS; Vada is immune), Cebada Capa x SusPtrit (CxS; Cebada Capa is immune) and Oregon Wolf Barleys (OWB; Dom x Rec, Dom is immune). QTL analysis based on macroscopically visible infection sites showed that in each mapping population a different set of loci is involved in resistance to *Phb* and only one coincident QTL was found in VxS and CxS populations. QTL analysis in the VxS population using microscopic traits, including colony size, early abortion without necrosis and the percentage of infection units associated with necrotic plant cell(s), showed that each microscopic trait is under control of a different set of genes implying that near nonhost resistance to *Phb* is a multi-component and also multigenic trait. Our data from screening of barley germplasm for resistance to *Phb* and results from QTL mapping did not confirm a possible association between phylogenetic distance of rust fungi and host status or effectiveness of QTLs of barley to a certain potential pathogen.

**Keywords**: Nonhost resistance. *Hordeum vulgare,* Heterologous rust, *Puccinia hordei,* Histology.

## INTRODUCTION

Many plant pathogenic organisms can infect only a limited number of plant species, which are called hosts. It is one of the intriguing questions which factors determine whether a plant species is a host or a non-host to a given pathogenic organism and which genes are involved. This question is especially interesting where host and nonhost species are taxonomically closely related to each other, have very similar leaf morphology and probably also similar biochemical and nutritive composition. A good case in point is the pathogenic rust fungi and their host plants. Rust fungi represent a very ancient group of specialized plant pathogens with extreme host specificity (Anikster and  Wahl 1979). Adaptation of rust fungi to one or a few host species is thought to be a consequence of the co-evolution of rust pathogens with their host plant species (Heath 1991; Thordal-Christensen 2003). Evidence for co-evolution of this group of micro-organisms with their host plant specieshas been reviewed by Anikster and Wahl (1979). Older rust genera, for example, frequently have a narrow host range while the younger genera show broader host ranges as reported a long time ago for two old *Phragmidium* and *Gymnosporangium* genera (Gäumann 1952). Rust pathogens of cereals and grasses are the most extensively studied rust fungi because of their economic importance and world wide distribution. These rust fungi are very specialized to their host plants. Each rust fungal species or *forma specialis* typically can infect only one or a few grass or cereal species, and each gramineous species is exploited by only few rust species. Each gramineous species is therefore “nonhost” for the majority of potentially pathogenic rust fungi of either closely or far related plant species. Barley (*Hordeum vulgare* L.) is fully resistant to *Puccinia recondita*, the rye leaf rust pathogen, and many other rust pathogens of grasses, but some barley lines are in the seedling stage moderately susceptible to *P. triticina*, *P. hordei-murini*, *P. hordei-secalini* and some other heterologous rust fungi (Niks 1996; Atienza et al. 2004). Barley can therefore be considered as an “intermediate host species” (=near-nonhost) for these rust fungi (Niks 1987). Atienza et al. (2004) made use of this near-nonhost status of barley to develop an extreme research line named ‘’SusPtrit’’ in which susceptibility genes to *P. triticina* were accumulated. This line, which showed susceptibility to some other heterologous rust fungi as well, was crossed with the normal barley lines Vada and Cebada Capa, which are (near) immune to all heterologous rust, to study the inheritance and specificity of genes involved in near-nonhost immunity in barley (Jafary et al 2006a and 2006b). In our previous study (Atienza et al. 2004) we found that barley accessions with some level of susceptibility to one heterologous rust tended to be also somewhat susceptible to one or more other heterologous rusts. Similarly, about 22 % of genes for resistance to heterologous rusts mapped in the Vada x SusPtrit (VxS) population were effective to more than one rust species (Jafary et al. 2006a). In that study we used seven rust species belonging to three phylogenetic groups of rust fungi of grasses: *P. hordei, P. persistens*/*P. triticina* and *P. graminis*, each group containing 2, 3 and 2 rust species respectively. We wondered whether association of resistance to different rust taxa was due to phylogenetic relation of the rusts or to phylogenetic relation of their respective host plants, or not due to either. Interesting rust species in this context were *P. hordei-secalini (Phs)*, which is related to the *P. triticina* group but pathogenic to *Hordeum secalinum*, and *P. hordei-murini* (*Phm)*, belonging to the *P. hordei* group and pathogenic to *Hordeum murinum*. From the *P. hordei* group there was only one heterologous rust species available (*Phm*). Recently, we obtained the *P. hordei-bulbosi* (*Phb*), a pathogen of *Hordeum bulbosum*, to which our research line SusPtrit appeared to be susceptible. This rust is probably a heterologous rust, closely related to *P. hordei* because the haploid stage of both *P. hordei* and *Phb* is formed on *Ornithogalum* and the sporophytic generation of *Phb* is morphologically indistinguishable from that of *P. hordei* (Anikster et al. 1971). The objectives of the present study were (1) to quantify the host status of barley to *P. hordei-bulbosi* and to determine whether susceptibility to *Phb* is associated with susceptibility to other rust species; (2) to compare the genetics of resistance to *Phb* in three mapping populations to determine whether genes for resistance to *Phb* tend to map to the same position as those for any of the previously studied heterologous rusts or to the pathogenic *P. hordei*; (3) to quantify the histological components of resistance in the VxS population, to determine whether different components of resistance are under control of different genes, and whether the resistance involves a hypersensitive reaction.

## MATERIAL AND METHODS

### Plant and pathogen material

A collection of 105 spring barley accessions from different geographic origin (Atienza et al. 2004) was used in the present study for host range quantification. The set comprised of 5 wild barley (*H. vulgare* ssp. *spontaneum*) accessions, 24 landraces from Asia, Africa, Europe, South America, and USA, four research lines and the remaining accessions were modern and old cultivars from various continents.

Two recombinant inbred line (RIL) populations and one doubled haploid (DH) populations were used for phenotyping. The RIL populations have been developed at Wageningen University (Wageningen, the Netherlands), and consist of Vada × SusPtrit (VxS, 152 lines) and Cebada Capa × SusPtrit (CxS, 113 lines) developed by Jafary et al. (2006a and 2006b). The DH population Oregon Wolfe Barleys, derived from a cross between parents *Dom* and *Rec* (OWB, 92 lines; Costa et al. 2001), has been developed in North America and is a reference mapping population. Teliospores of *P. hordei-bulbosi* (*Phb*) were collected at Ariel (Israel) and used for inoculation of the secondary host plant (*Ornithogalum eigii*). Aeciospores collected from the leaves of the secondary host were used to inoculate *H. bulbosum* seedlings and to produce urediniospores (Anikster and Manisterski, personal communication). These spores were kindly provided to us by Prof Anikster. Several *H. bulbosum* accessions obtained from the Centrum voor Genetische Bronnen, Nederland, Wageningen, (http://www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources/Collections) were tested for susceptibility to *Phb*. Relatively few susceptible accessions were found, and these were used for spore multiplication. Urediniospores were collected from the inoculated leaves by a cyclone spore collector, kept in an exsiccator for 2-4 days and stored in a freezer at -80 ºC until use for inoculation.

### Inoculation and evaluation for resistance

Seeds of 105 barley accessions and RILs/DHs of three mapping populations were sown in 37 x 39 cm boxes, each box containing about 14 lines (including host species and L94 or parents of each cross). Each barley accession was represented by at least three seedlings. When the primary leaves were full-grown (12 days after sowing), the seedlings were fixed in horizontal position and inoculated with about 10 mg of spores per box (resulting in a deposition of about 600 urediniospores per $cm^2$) using a settling tower (Jafary et al. 2006a). After inoculation, plants were incubated in a dew chamber during 10 hours (17-18 ºC) at 100% relative humidity and then transferred to a greenhouse compartment at about 22/18ºC (day/night).

Susceptibility of the barley accessions to *Phb* was quantified by counting the number of pustules and flecks on the leaves, 12 days after inoculation. The scored values for each of the 105 accessions was converted to susceptibility score (0-5 from immune to fully susceptible), following Atienza et al. (2004) to summarize the results. Susceptibility of RILs/ DHs of the three mapping populations was evaluated by counting the frequency of visible infection sites (VIS; the number of both flecks and pustules per $cm^2$) and infection frequency (IF; the number of pustules per $cm^2$), following Jafary (2006a). The quantitative data for three seedlings and the average were used as phenotypic values for QTL mapping.

### Histological analysis

The VxS population was subjected to histological study. Seedlings of 86 RILs and parents were grown in boxes and inoculated with 5 mg of spores (about 300 spores per cm$^2$). For each RIL two seedlings were inoculated of which one seedling was sampled for histological studies 42 hours after inoculation and the second seedling was used for evaluation of VIS and IF 12 days after inoculation as described earlier. Collected leaf samples for histology were immediately fixed and cleared by boiling for about two min in a water bath in lactophenol-ethanol (1:2 v/v). The staining was with Uvitex 2B (Ciba-Geigy) as described by Rubiales and Niks (1996) and observations were made with a fluorescence microscope (Zeiss Axiophot, exciter filter BP 395-440, chromatic beam splitter FT 460 nm and barrier filter LP 420) at 400 x magnification.

The leaf segments were screened for 45 to 55 sporelings and classified for stage of development. Infection units with up to six haustorial mother cells were considered early aborted (EA, see Niks, 1982), colonies with more haustorial mother cells and more hyphae were considered established. We measured the diameter of the established colonies by eye-piece micrometer, measuring along the long axis of the colony, which is mostly parallel to the long axis of the leaf. Per infection unit we noted whether the fungus was associated with one or more autofluorescent plant cells that are interpreted as plant cell necrosis (Niks and Kuiper 1983).

### Linkage maps and QTL analysis

The molecular marker data for the base maps of VxS (179 markers) and CxS (159 markers) were obtained from our previous studies (Jafary et al. 2006a and 2006b). The marker data set of the OWBs was downloaded from the barley project web site of Oregon State University (OSU) (http://barleyworld.org/). From 769 markers in the locus genotype file, a subset of 133 markers was used as base map “OWBbase” downloaded from the OSU Barley Project website. QTL analysis in the three mapping populations was performed on IF and VIS traits. For VxS, three parameters of infection at the microscopical level were also used as phenotypic traits for QTL analysis, using Map QTL software version 5 (Van Ooijen 2004): % of early abortion without necrosis (EA-N), % of infection units associated with at least one necrotic plant cell (%IU+N) and colony size (CS). Interval mapping, automatic co-factor selection and MQM mapping (Jansen and Stam 1994; Van Ooijen 1999) were applied and the significance LOD thresholds at level of 5% for all traits were obtained by running a permutation test on data sets. LOD profiles for all macroscopic and microscopic traits in VxS were graphically displayed using MapChart version 2.2 (Voorrips 2006).

A recently developed integrated consensus map (Marcel et al. 2006 ) was used to compare the position of QTLs mapped for resistance to *Phb* in the three different populations. This high-density map has been constructed based on six mapping populations, including three populations used in this study (OWB, CxS and VxS) and contains 3,258 markers spanning 1,081 centiMorgans (cM) divided into 210 BINs of about 5 cM each (Marcel et al. 2006).

## RESULTS

### Host status of barley to *Phb*

Barley germplasm showed a low degree of susceptibility to *P. hordei-bulbosi*. The majority of barley accessions were immune and only 16% of the barley accessions were at least somewhat susceptible (score values 2 and higher) to the rust fungus (Table1). This degree is lower than that for many other heterologous rust fungi. High susceptibility with more than 100 pustules per leaf occurred in only 5% of the accessions (Table1). Trigo Biasa, a landrace from Indonesia and one of the parents of SusPtrit (Atienza et al. 2004), was clearly the most susceptible accession with about 700 pustules per leaf. Susceptible *H. bulbosum* seedlings, which have narrower leaves than barley, had on average 520 pustules per leaf. Wild barley and landraces were more susceptible to *Phb* than modern barley lines as observed for other heterologous rust fungi (Atienza et al. 2004). All accessions that allowed some reproduction by *Phb* (scale 2 or higher) were also somewhat susceptible to at least one other heterologous rust.

The relatively few accessions that were susceptible included one modern USA cultivar (Lacey) and three older European cultivars. Susceptibility scores (as number of pustules) of barley accessions to *Phb* showed the highest correlation with data for *P. graminis* f.sp. *lolii* (r=0.83), *P. hordei-murini* Cordoba (r=0.69) and *P. triticina* Flamingo(r=0.59). The highest correlation between data from *P. graminis* f. sp. *lolii* and *Phb* was due to the high number of immune barley accessions in common for both rust fungi (Table 1). Remarkably, the susceptible accessions to *P. triticina* were also frequently susceptible to *Phb* except eight

Japanese and 4 Ethiopian accessions which were somewhat susceptible to *P. triticina* but not to *Phb.* Interestingly, L94, which is extremely susceptible to all tested isolates of *P. hordei*, and also relatively susceptible to some heterologous rust species including *Phm*, and even to rusts that are not closely related to *P. hordei*, such as *Phs* and *P. triticina*, was completely resistant to *Phb.*

**Table 1** Percentage of barley accessions per susceptibility scores 0 – 5 for *P. hordei-bulbosi* to compare with 6 other heterologous rust species and *Puccinia hordei* at seedling stage

| | **Susceptibility score**[a] | | | | | |
|---|---|---|---|---|---|---|
| **Pathogen**[*] | **0** | **1** | **2** | **3** | **4** | **5** |
| ***P. hordei-bulbosi*** | 83 | 1 | 3 | 8 | 4 | 1 |
| ***P. graminis* f. sp. *lolii*** | 72 | 0 | 14 | 11 | 3 | 0 |
| ***P. hordei-murini* Córdoba** | 31 | 11 | 44 | 8 | 6 | 0 |
| ***P. hordei-secalini*** | 30 | 37 | 9 | 16 | 7 | 1 |
| ***P. triticina* Flamingo** | 55 | 10 | 19 | 13 | 3 | 0 |
| ***P. persistens*** | 39 | 7 | 9 | 37 | 8 | 0 |
| ***P. recondita* f. sp. *recondita*** | 100 | 0 | 0 | 0 | 0 | 0 |
| ***P. hordei* (1.2.1)**[b] | 3 | 0 | 0 | 5 | 66 | 26 |

[*] Data for 6 heterologous rust species and *P. hordei* adopted from Atienza et al. (2004)
[a] Susceptibility score (per leaf):
0: Immune or near immune (less than 3 pustules and few flecks)
1: Less than 3 pustules and medium or many flecks
2: 3-10 pustules
3: 10-100 pustules
4: More than 100 pustules
5: More than 500 pustules
[b] Amount of inoculum was three times less than for heterologous rusts

## Histological analysis

The morphology of the infection structures of *Phb* was very similar to that of *P. hordei* (Niks 1986) and *Phm*, and distinct from that of *P. triticina*, *P. persistens* and *Phs*. The morphology of infection structures is characteristic for the various taxonomic groups of rust fungi on cereals and grasses (Niks 1986). Also the ITS sequence suggested a close phylogenetic relationship between *Phb*, *P. hordei* and *Phm* (Szabo, personal communication). The infection units of *Phb* were well able to find and penetrate stomata of the barley seedling leaves, without obvious effects of the plant genotype. We could distinguish early aborted infection units (Figure1 A,B) with two to about four hyphae and up to six haustorial mother cells, and established infection units that had formed more and branched hyphae and more haustorial mother cells (Fig 1D and E). A proportion of the established colonies had only about seven to about 12 haustorial mother cells and/or few hyphae (Fig 1C). Since haustorial mother cells often occur in clusters, they are not so easy to count. Therefore, discrimination of early aborted and established colonies was less straightforward and more subjective than in barley – *P. hordei* (Niks 1982 and 1986). Only about 15% of the early aborted infection units was associated with plant cell autofluorescence (EA+N, Fig 1B), and in all the RILs more EA units were without than with necrosis. On SusPtrit the proportion of early aborted infection units without plant cell necrosis (EA-N Fig 1A) was substantial (20 %), but this line had about the largest average size of established colonies (Table 2). On Vada both components of infection were indicative for resistance: high early abortion and small average size of established colonies. The RILs varied considerably for those components (Table 2).

**Table 2**. Summary of the components of infection of 86 barley RILs by *Puccinia hordei-bulbosi* and the two parental lines, SusPtrit and Vada on seedling leaves sampled five days after inoculation.

| | **% EA-N**[a] | **% EA+N**[b] | **Average longest diameter of established colonies (µm)** | **% infection units associated with fluorescent plant cells** |
|---|---|---|---|---|
| **SusPtrit** | 20 | 1 | 283 | 31 |
| **Vada** | 47 | 13 | 154 | 22 |
| **Range for 86 RILs** | 0 – 60 | 0 – 20 | 126 – 281 | 0 - 75 |

[a] Early aborted infection units not associated with auto fluorescent (necrotic) plant cells

[b] Early aborted infection units associated with auto fluorescent (necrotic) plant cells

The proportion of infection units associated with one or more autofluorescent plant cells ranged from 0 % to 75 %. This figure includes the relatively few EA+N infection units. However, this necrosis occurred frequently as only one cell in relatively large established colonies (Fig 1D), and was not particularly strong in Vada or in RILs with a similar level of resistance as Vada. This suggests that the necrosis was not a relevant factor explaining the resistance.

### Phenotypic analysis and QTL mapping

The three mapping population showed a quantitative segregation in the level of resistance to *Phb*. In none of the three populations the resistance was based on a macroscopically visible hypersensitivity. Frequency distributions of phenotypes for VIS in the three mapping populations are shown in Figure 2. In all three mapping populations the majority of lines were immune and only a small fraction of RILs/DHs showed some level of susceptibility (Figure 2). Parental lines in all three mapping populations were at the two extremes of the range; Cebada Capa, Vada and Dom were immune, SusPtrit and Rec were the most susceptible accessions. This absence of transgressive segregation suggests that resistance alleles originated only from one of the parents.

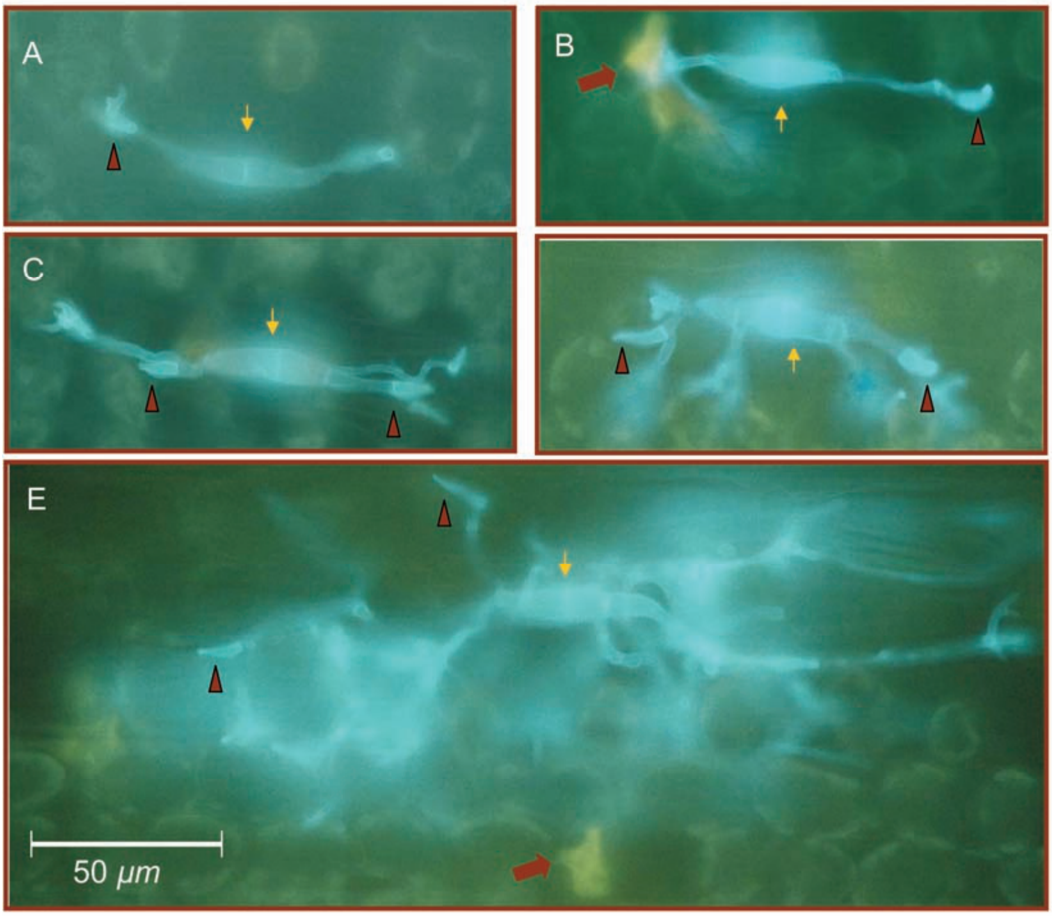

**Figure 1.** Histology of infection to *P. hordei-bulbosi* in the VxS population.
Substomatal vesicles are shown with arrow. Some haustorial mother cells are indicated with arrow-head. Autofluorescent plant cells are shown with the block arrow
A) Early abortion without necrosis (EA-N) B) Early abortion with necrosis (EA+N)
C) Intermediate established colony with only few haustorial mother cells
D) Established colony with small colony size
E) Established colony with large colony size

In the all three mapping populations two macroscopic traits (IF and VIS) were highly correlated (r=0.94, 0.85 and 0.84 for VxS, CxS and OWB populations respectively). Different levels of susceptibility in OWB to *Phb* are shown in Figure 3. Some of the DHs including the resistant parent Dom (Figure 3A) occasionally showed also many small pale flecks which did not occur in all three tested seedlings and therefore were not taken for counting. In the susceptible DHs including the susceptible parent Rec the majority of flecks turned into the pustules 12 days after inoculation, some pustules being surrounded by chlorotic halos (Figure 3 F).

The QTLs mapped for two macroscopic traits (VIS and IF) in three mapping populations and also for three microscopic traits in the VxS population are summarised in Table 3 and Figure 4. In the VxS population three QTLs mapped for macroscopically visible infection traits (VIS and IF) with identical flanking markers, but the LOD values were often higher for VIS than for IF (Figure 4). In this population in total nine QTLs mapped for five different traits of which one QTL for colony size (CS) was found to affect VIS /IF as well. This co-incident QTL showed the highest LOD value for both VIS/IF and for CS (Table 3 and Figure 4). Another QTL for CS co-located with a QTL for EA-N. There was a negative correlation (r= -0.59) between CS and EA-N implying that that there is an association in genetics of both components for resistance. However, the correlation between CS and % of

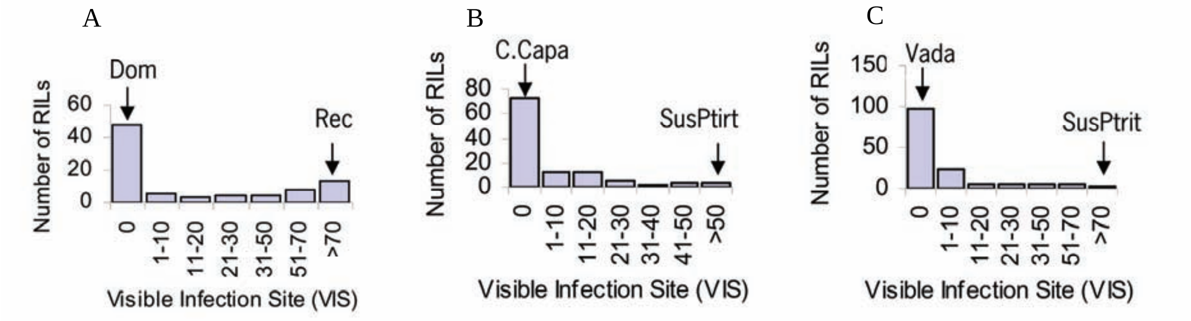

**Figure 2.** Frequency distribution of phenotype (VIS) for resistance to *P. hordei-bulbosi* in three different populations. Values of the two parental lines are shown by arrows.
A) Oregon Wolfe Barleys B) Cebada Capa x SusPtrit C) Vada x SusPtrit

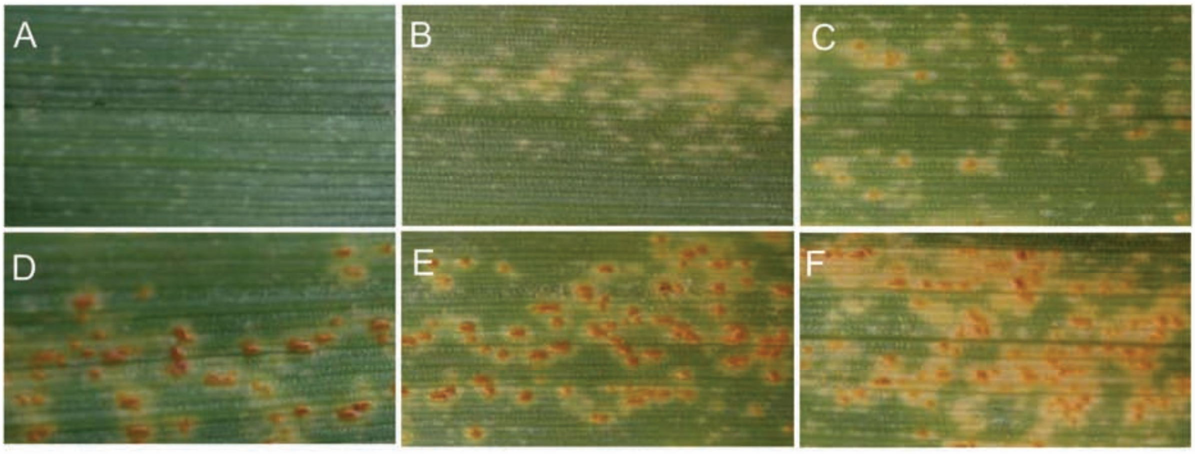

**Figure 3.** Segregation of OWB mapping population for nonhost resistance to *P. hordei-bulbosi* Resistant parent (A), quantitative segregation for resistance (B, C, D, E) and susceptible parent (F).

RILs with larger CS tend to associate more with necrosis than RILs with smaller CS. None of two QTLs for %IU+N overlapped with QTLs for any of the microscopic and macroscopic traits. The alleles for the high %IU+N and EA-N were contributed by both SusPtrit and Vada. In CxS two QTLs, both with rather high effect on IF and VIS, mapped on chromosome1 (7H) of which one, in 123-133 cM, overlapped with a QTL mapped in the VxS population (Table 3). In OWB three QTLs mapped for resistance of which only one affected both IF and VIS and the other two either IF or VIS. Two additional chromosomal regions for both VIS and IF traits had LOD values just below the threshold value (Table 3). None of the QTLs in OWB overlapped with those mapped in either VxS or CxS. One QTL for resistance to *Phb* with the highest LOD value in the VxS and another in the CxS (for both VIS and IF traits) overlapped with a QTL for *P. triticina* (Table 3). Out of the nine QTLs mapped for different components of resistance to *Phb* in VxS five QTLs coincided with QTLs to *P. triticina*, *P. persistens*, *Phs* and *Phm* (Table 3). Only the latter rust belongs to the *P. hordei* group.

**Table 3.** Summary of QTLs contributing to nonhost immunity to *P. hordei-bulbosi* in three barley mapping populations

| Population | Chr. | cM[a] | LOD | Threshold[b] | traits[c] | Bin Number[d] | Co-location [e] |
|---|---|---|---|---|---|---|---|
| VxS | **1(7H) [f]** | **118-136** | **7.3** | **2.6** | **VIS/IF** | **7H-11.2** | *Ptrt/Phm/Pper* |
| | 5(1H) | 45-62 | 2.9 | 2.6 | VIS/IF | 1H-06.2 | *Phs* |
| | 6(6H) | 103-122 | 3.1 | 2.6 | VIS/IF | 6H-11.2 | |
| | **1(7H)** | **118-136** | **12.8** | **3.0** | **CS** | **7H-11.2** | |
| | **2(2H)** | **97-113** | **5.0** | **3.0** | **CS** | **2H-11.2** | |
| | 2(2H) | 135-150 | 4.1 | 3.0 | CS | 2H-14.2 | *Ptrt* |
| | 7(5H) | 133-148 | 3.8 | 3.0 | CS | 5H-12.2 | *Pper/Phs* |
| | **2(2H)** | **97-113** | **3.0** | **2.8** | **EA-N** | **2H-11.2** | |
| | 7(5H) | 26-39 | 3.2 | 2.8 | EA-N | 5H-03.1 | |
| | 4(4H) | 95-112 | 3.0 | 3.0 | %IU+N | 4H-10.2 | |
| | 6(6H) | 50-67 | 3.2 | 3.0 | %IU+N | 6H-05.2 | *Ptrt/Pper* |
| CxS | 1(7H) | 38-60 | 8.2 | 2.7 | VIS/IF | 7H-05.1 | *Ptrt* |
| | 1(7H) | 123-133 | 8.0 | 2.7 | VIS/IF | 7H-12.1 | |
| OWB | 1(7H) | 73-82 | 2.1 | 2.3 | VIS/IF | 7H-07.2 | |
| | 3(3H) | 73-88 | 2.3 | 2.3 | IF | 3H-08.1 | |
| | 5(1H) | 132-139 | 2.1 | 2.3 | VIS/IF | 1H-14.2 | |
| | 6(6H) | 97-102 | 4.5 | 2.3 | VIS/IF | 6H-11.1 | |
| | 7(5H) | 85-92 | 3.2 | 2.3 | VIS | 5H-09.2 | *Phm* |

[a] Approximate position (cM) of two LOD support intervals based on the results of MQM mapping on an integrated consensus map (Marcel et al.2006).

[b] Threshold values are obtained from permutation test for each data set.

[c] For VIS/IF traits, the LOD values are only shown for VIS.

[d] BIN number reflects the position of peak marker of QTL on the consensus map (Marcel et al. 2006)

[e] Co-location between QTL for *Phb* and QTLs mapped for other heterologous rusts species (Jafary et al., 2006b).

[f] The overlapping QTLs in VxS population between traits are highlighted in **bold.**

## DISCUSSION

### Host status of barley and its evolutionary implications

Our data show that mainly ancient cultivated and wild barley accessions are susceptible to *Phb*. These results parallel our previous findings where we quantified the host range of barley using 19 rust isolates and *formae speciales* and showed that old barley lines, landraces and wild barley accessions have more chance to be susceptible to heterologous rust species than modern barley cultivars (Atienza et al. 2004) . The low level of susceptibility of barley germplasm (Table 1) implies that barley is a near-nonhost to *Phb* like it is to some other rust pathogens, including *P. triticina, P. persistens, Phs* and *Phm*.

Biotrophic plant pathogens, and rust fungi in particular, have a very specific and narrow host range. Basically, changes in the host range of plant pathogens are infrequent, which is an important indication for the stability of nonhost resistance (Heath 2000; Kamoun 2001). However, during evolution, some pathogens have evolved to breach nonhost defence mechanisms through the acquisition of virulence factors which enabled them to suppress plant defence mechanisms and to reproduce on a particular plant species (Neu et al. 2003; Abramovitch and Martin 2004; Alfano and Collmer 2004). Such plants are considered hosts that were rendered susceptible to colonization, resulting in a homologous plant–microbe interaction and basic compatibility. If suppression of host defence results in susceptibility of the given plant accessions then one may expect that the basal defence of a plant species that is phylogenetically related to the host plant of a particular pathogen has more chance to be suppressed rather than a far-related plant species. *H. bulbosum* is more closely related to *H. vulgare/H. spontaneum* than any other *Hordeum* species (in our case *H. murinum* and *H. secalinum*). Actually, *H. bulbosum* is the only *Hordeum* that can produce true hybrids with *H. vulgare* (Pickering et al. 1995). For two other heterologous rust species, *Phm* and *Phs*, we have shown an association in genetics of resistance in both OWB and the CxS populations suggesting that the relationship between hosts (both are pathogens of a *Hordeum* species) is more important than the relationship between rust pathogens (Jafary et al., 2006b). However, our data from the VxS did not support that hypothesis, since in that population none of the QTLs of Vada are effective to both rust fungi (Jafary et al. 2006a).

The data from the present study also did not confirm a tendency of barley to be more susceptible to heterologous rust fungi which are pathogenic to *Hordeum* species than to rusts that have hosts belonging to other genera. Remarkably, L94 has complete resistance to *Phb*, although it is susceptible to all

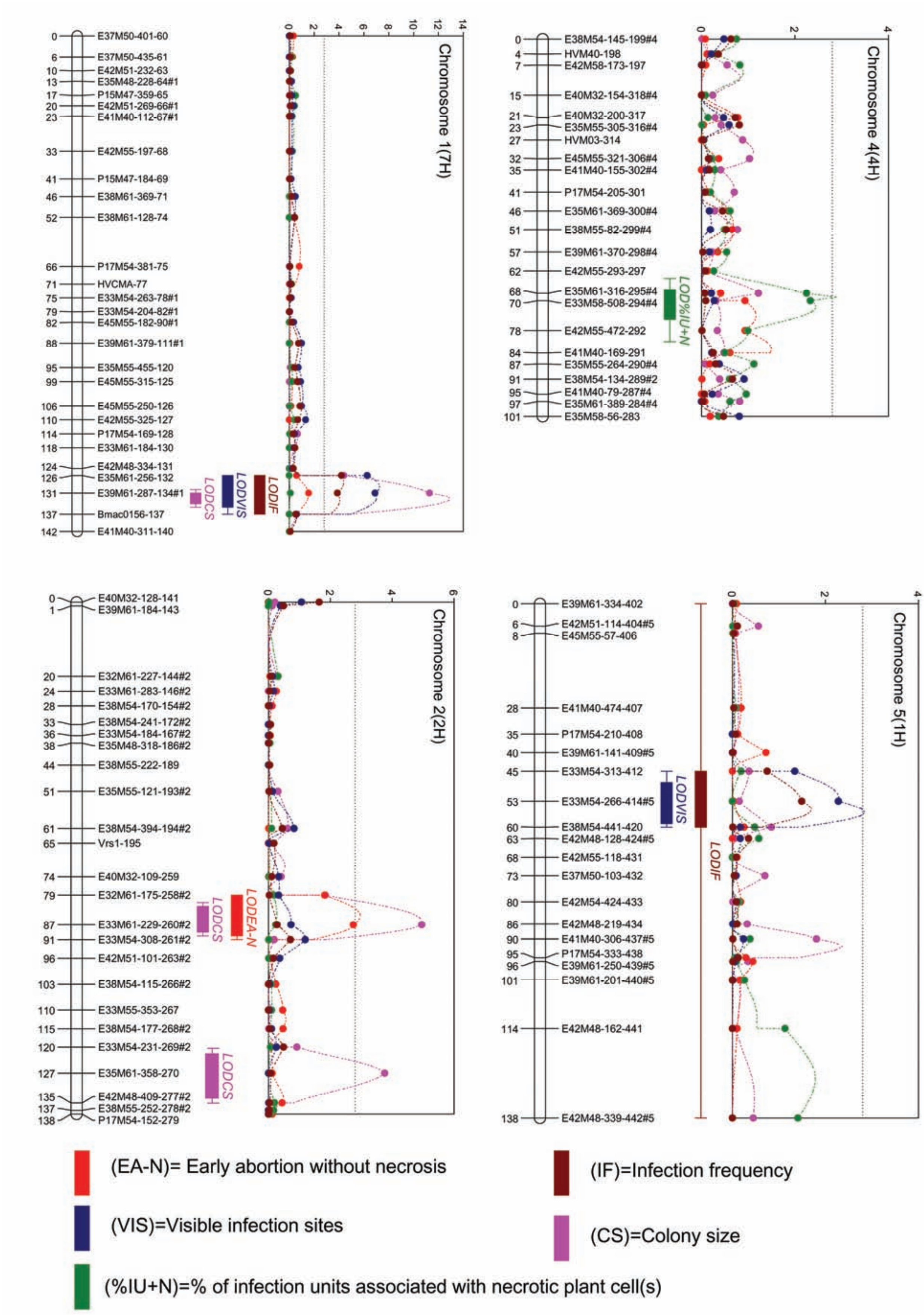

**Figure 4.** LOD profiles of all detected QTLs for different macroscopic and microscopic traits in the VxS mapping population.

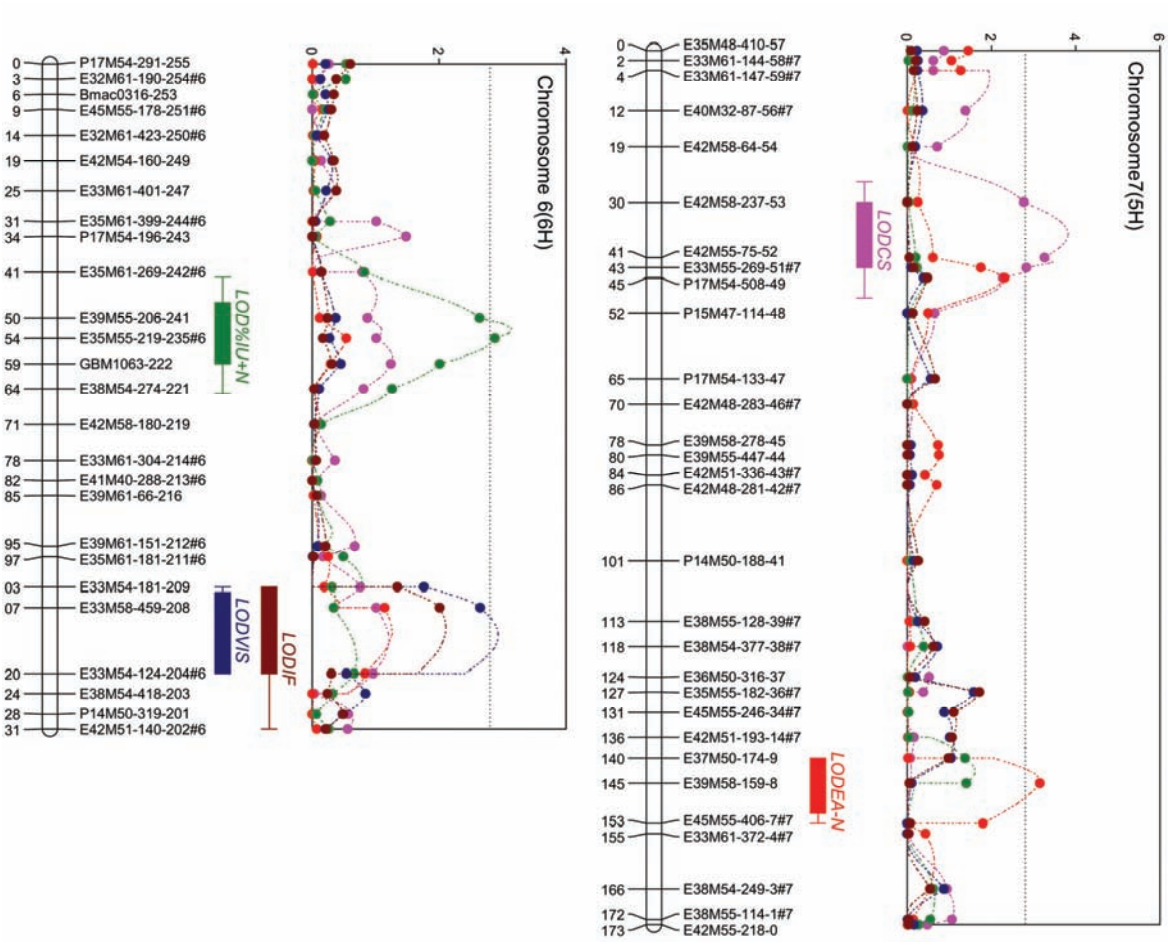

**Figure 4.** Continued

previously tested isolates of *P. hordei*, to *Phm* and even to *P. triticina*, which is pathogenic to members of the *Triticum* genus. One of the objectives of the present study was to find whether there is an association between resistance in barley to different rust taxa that are phylogenetically related. *Phb* is an informative rust in this respect, since the morphology of the infection structures of *Phb* and the ITS sequence (Szabo, personal communication) confirm the close relationship of *Phb* to *Phm* and *P. hordei*.

Our data from screening of barley germplasm inoculated with *Phb* showed that interestingly susceptibility to *Phb* is more correlated with susceptibility to less-related rust species like *P. graminis* f.sp. *lolii* than with susceptibility to *Phm* which is closely related to *Phb*. We also found that barley is more resistant to *Phb* than to some other rust fungal species like *P. persistens* and *P. triticina* (Table 1), which are not closely related to *P. hordei* (based on their ITS sequence). Our results from QTL mapping also did not confirm a possible association between phylogenetic distance of rust fungi and host status of barley to a certain potential pathogen. In case of association we would expect that plant QTLs contributing to resistance to *Phb* tend to co-locate predominantly with QTLs for the taxonomically related rust taxon *P. hordei-murini*. But our results (Table 3) show that QTLs mapped in barley to *Phb* tend to overlap more frequently with QTLs for resistance to less-related rust species, like *P. triticina* and *P. persistens*. This indicates that neither taxonomic relationship between rusts nor taxonomic relationship between hosts is very relevant to predict the level of host status of barley.

### Specificity and diversity of QTL for resistance to *Phb*

In the present study we showed that resistance in barley to *Phb* is governed by QTLs of which some are effective to several other heterologous rusts species and some specifically effective to *Phb.* This confirms our previous finding that nonhost immunity in barley to different heterologous rust fungi is controlled by sets of resistance genes with different and overlapping specificities (Jafary et al. 2006a).

Only one QTL in chromosome 1(7H) overlapped in both VxS and CxS populations (Table3). This finding is in agreement with our previous results where we showed that a high diversity of genes is involved in nonhost immunity to four heterologous rust species (Jafary et al. 2006b). The diversity of loci involved in basal non-hypersensitive resistance is an interesting aspect which has been demonstrated earlier in barley for partial resistance to *P. hordei* (Qi et al. 2000) and for quantitative resistance to Spot blotch, caused by *Cochliobolus sativus* (Bilgic et al. 2005).

Our findings that QTLs with effect to *Phb* are not frequently the same as those effective to closely related rust species, such as *Phm*, suggest that for any rust species a different set of genes is involved to lead to nonhost immunity. Our data suggest that there is an abundance of loci in barley for resistance to *Phb* like there is for resistance to other heterologous rusts species (Jafary et al. 2006b).

### Mechanisms of near-nonhost resistance in barley to *Phb*

A combination of macroscopic and microscopic traits was examined in the present study. Colony size was closely associated with both macroscopic traits (VIS and IF) since a QTL with the highest effect to colony size overlapped with a QTL for the VIS/IF (Figure 4). None of the QTLs for EA-N coincided with VIS or IF. This suggests that low VIS and IF is more due to poor growth and development of established infection units (infection units as depicted in Fig 1 C, D), than due to poor establishment of infection units (EA, as in Fig 1 A). This contrasts with the finding that in barley lines with high basal (partial) resistance to the homologous *P. hordei* EA played a more important role in determining IF than "late abortion", i.e. abortion after the infection units had established (Niks 1982). The QTLs for microscopic parameters of infection suggest that EA-N and CS are under separate genetic control. In the susceptible parent SusPtrit the proportion of EA-N was about 20 % but this line had the largest average size of established colonies (Table 2). The finding that some QTLs are affecting EA but not colony size, and other QTLs are responsible for colony size but not EA is remarkable and implies that EA-N and colony size are not under control of the same genes.

These results are surprising, since observations on basal resistance to *P. hordei* (Niks 1986) suggested that blocking of haustorium formation is responsible for slow colony growth (and hence, long latency period) and high EA-N (and hence, low infection frequency). Percentage of infection units associated with necrotic plant cells (%IU+ N) had no QTL in common with any other microscopic and macroscopic trait (Table 3). This necrosis occurred mainly as one or two autofluorescent cells in relatively large established colonies (Fig 1D) and it did not often encompass all plant cells in the colony area, suggesting that the resistance of Vada to *Phb* rust is essentially based on a non-hypersensitive mechanism. This kind of necrosis was not particularly strong on Vada and the most resistant RILs, suggesting that the necrosis was not a relevant factor explaining the resistance. Our findings together suggest that nonhost resistance is a multi-component trait and each component of resistance is controlled by a specific set of genes. Multigenic inheritance of nonhost resistance is likely to be the most relevant factor explaining the durability of this type of resistance in nature.

## ACKNOWLEDGMENTS

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# CHAPTER 6

## General Discussion

## Introduction

Our knowledge about the genetics of nonhost immunity is negligible mainly due to lack of proper model pathosystems. In the past, nonhost immunity to fungal parasites has been mainly investigated using mutagenesis or examined by cytological and pharmacological studies. The aim of the research described in this thesis was to develop a new model system for ongoing studies to understand the genetics, mechanisms and evolutionary aspects of nonhost immunity in plants to fungal pathogens. In the previous Chapters we showed that some barley accessions are in the seedling stage somewhat susceptible to heterologous rust fungi. This provided a unique chance to accumulate genes for susceptibility in a research line. This line was used successfully to map the plant factors implicated in nonhost immunity to rust fungi from several taxa and enabled us to study the specificity and diversity of genes involved in nonhost immunity. In this chapter we will discuss the host status of barley and its evolutionary implications in nonhost immunity and will illustrate the specificity, diversity, and possible role and nature of QTLs mapped for resistance to heterologous rust fungi. Application of our model pathosystem for future studies to investigate the different aspects of nonhost immunity will also be addressed in this chapter.

## Host status of barley and its evolutionary implications in nonhost immunity

The ability of fungal species or races to infect and reproduce on a particular host, or a group of host plants, is regarded as an example of biological specialization (Wyand and Brown 2003). This phenomenon is thought to result from a co-evolutionary process between biothrophic fungi and their host plant species. In Chapters 2 and 5 we showed that barley is not a regular host for heterologous rust pathogens tested, although some accessions, in particular wild and old cultivated barleys, allowed heterologous rusts to reproduce to some extent (Chapter 2). We discussed in Chapter 3 and 4 that quantitative genes which confer immunity to heterologous rusts probably encode quantitative plant factors. These plant factors should be specifically neutralized by the rust to successfully suppress host defence. We showed that these plant factors are very specific (Chapter 3 and 5). It is amazing, if this “neutralization” is so specific, how a pathogen can at the same time negate the plant factors in two very distinct plant species. For example *Oidium neolycopersici* can infect tomato (Solanaceae) and *Arabidopsis* (Crucifers) (Xiao et al. 2003). Host-jumping is presumed to be an evolutionary process that occasionally occurs in some biotrophic plant pathogens such as powdery mildews (Takamatsu et al. 2000) and Oomycetes (Voglmayr 2003). More recently, Kiss and his colleagues reported that a powdery mildew fungus (*Golovinomyces sp.*) can jump from *Arabidopsis* (Cruciferae) even to a far related plant species *Torenia fournieri* (Scrophulariaceae) (personal communication). It is interesting that this host-jumping occurred in a pathogen species that has been shown already to jump relatively easily to new plant species as part of its evolutionary strategy. It is likely therefore that some rust fungi during co-evolution acquire virulence factors to overcome or suppress host defences. For extending a host range with an additional plant species, the pathogen should be able to suppress defence in that plant species (Nomura et al. 2005) but it is still a challenge to understand how the virulence systems of pathogens can overcome the innate immunity system of plant species that are taxonomically far-related to their host plants.

The model pathosystem presented in this work can help to shed light on the nonhost immunity using inheritance studies. Our studies have revealed several intriguing and puzzling questions that still remain to be solved including (i) how come that the only fully susceptible *H. vulgare* accession to *P. hordei-bulbosi* (and susceptible to many other heterologous rusts) is an Indonesian landrace? (ii) how is it possible that modern cultivars like Vada contain an *R*-gene to a heterologous rust *P. hordei-secalini,* for which breeders are unlikely to have selected for? (iii) susceptibility to different heterologous rusts tends to be associated, like in the accessions as Trigo Biasa and L94 that are susceptible to several heterologous rust pathogens (Chapter 2 and Chapter 5). Also selection for SusPtrit and SusPmur resulted in high susceptibility not only to their target rusts, but also to additional heterologous rusts, suggesting rust species non-specific factors. How to explain then that the majority of resistance genes each are effective to only one heterologous rust, even for closely related rusts like *P. hordei-murini (Phm), P. hordei-bulbosi (Phb)* and *P. hordei* (Chapter 3 and 5)?

## The role and nature of QTLs mapped in barley for nonhost immunity

In barley – rust and –mildew interactions, the plant cell is the place where either a compatible or an incompatible interaction occurs. Pathogens in a compatible interaction have evolved virulence systems to overcome host defence but it is not clear which plant factors are involved and how they act. Until now mutagenesis, transformation and silencing of genes are used as tools to reveal key-genes involved in nonhost immunity, but they may play their role in the transduction pathway and defence, rather than that they explain the success or failure of suppression of the plant defence by the potential pathogen. Plant pathogenic bacteria e.g. *Pseudomonas syringae* contain the type III protein secretion system (TTSS) by which they secrete and probably translocate a large number of virulence effector proteins into the host cell (Hauck et al. 2003; Kim et al. 2005). It has recently been shown that the suppression of basal defence in plants is a major virulence function of TTSS effectors in bacteria (Nomura et al. 2005). Plant pathogenic bacteria employ diverse strategies to undermine plant defences and target components of plant immunity including HR-based programmed cell death (PCD), cell wall-based defences, jasmonic acid (JA) signalling, and the expression of defence genes as reviewed by Abramovitch and Martin (2004).

The role and function of effectors in plant pathogenic fungi to suppress host defence is not as well described as in bacteria. Most of the cloned effectors of fungal pathogens are small proteins with unknown function containing a signal for secretion into the apoplast but it is not clear how these elicitors can enter into the plant cell and contribute to fungal pathology (Chisholm et al. 2006). The suppressor from a pea pathogen, *Mycosphaerella pinodes*, inhibits both the ATPase activity and the polyphosphoinositide metabolism in plasma membranes of pea, resulting in suppression of the signal transduction pathway (Shiraishi et al. 1997). It has long been hypothesized that plant pathogenic fungi, particularly rust and powdery mildew fungi that form long-term biotrophic relationships with their hosts need to suppress host defence reactions (Mellersh and Heath 2001; Holub and Cooper 2004; Panstruga 2003) but no secretion system similar to that of bacteria was found in fungi.

Haustoria in rust fungi are special infections structures which differentiate from haustorium mother cells, through penetration of the plant cell and invagination of the plant plasmalemma and play a role in acquisition of nutrients from the host. The haustorium is thought to have some other roles as well: it is supposed to influence the plant cell metabolism and to suppress defence responses (Voegele and Mendgen 2003), and may induce rearrangements of the host cell cytoskeleton and plant nuclear migration (Kobayashi et al. 1994; Heath 1997), and may deliver effectors into the plant cytoplasm in order to suppress a basal defence reaction through interaction with certain pathogenicity targets. Evidence for such a transport of effectors from the haustorium into the plant cytoplasm was obtained recently by Catanzariti et al.( 2006). They analysed the haustorially secreted proteins from the flax rust fungus, *Melampsora lini*, and characterized 21 secreted proteins. Remarkably, two secreted effector proteins (AvrP4 and AvrM), were able to induce programmed cell death when expressed inside the plant cells suggesting their translocation into plant cells during infection. Effectors are thought to realize suppression of the nonhost immunity that would be triggered by the PAMPs (see General Introduction), but several of the pathogenicity targets act also as a cognate *R*-gene product or are guarded by a *R*-gene product. Therefore, when the effector binds to the pathogenicity target, an HR reaction may be initiated (Figure1).

In Chapter 3 we speculated that the genes at the QTLs for nonhost immunity are likely to encode proteins that act as pathogenicity targets for the suppression of basal defence. It is also possible that the QTLs represent different components of a common basal pathway responding to non-specific elicitors that is suppressed during successful infection. Then the rust species specificity can be considered as a result of differences in the ability of rust effectors to influence particular components of that pathway. The hypothetical pathogenicity targets the QTL genes code for may (1) be resistance factors to contribute to the perception system, or (2) be compatibility factors, contributing to the success of infection attempts. Under the first assumption, loss of function of these genes will result in susceptibility and under the second assumption loss of function will result in resistance. In either case, the proposed function as pathogenicity target for QTLs is in agreement with the "Minor-gene for minor-gene" hypothesis proposed by Parlevliet and Zadoks (1977) and implies that there is not a general suppressor of defence and plant pathogens may have extremely divers effectors. Probably protein – protein recognition is involved in defence against heterologous rusts, either upstream at the perception of the PAMPs or at various stages along the signal transduction and defence pathway. It is however a great challenge to find out which plant factors determine whether defence is "easy" or "hard" to be suppressed by an attacking rust fungus.

### Genetics of plant immunity

The best characterized type of immunity in plants is based on gene-for-gene interaction in which specific resistance genes (*R*-genes) of a host species confer resistance to specific genotypes of a pathogen. Race specific immunity has a very narrow spectrum of action and mainly a short-term usefulness under agricultural conditions. Nonhost immunity however is common, broad spectrum and a durable type of immunity in plants (Heath 2000), but its genetics is poorly understood. The main characteristics of nonhost immunity pointed out in this research are as follows:

**Gene specificity for nonhost immunity:** The common nature and durability of nonhost immunity might imply that a set of conserved genes with wide action spectrum are involved in immunity of a plant species to all heterologous pathogens. The results described in Chapter 3, however, show that to each heterologous rust fungus a different set of genes is involved in bringing about nonhost immunity. Many of the QTLs mapped are rust -species specific and probably represent specific pathogenicity targets that should to be neutralized by any rust pathogen. However, assuming such specific plant factors that require specific effectors in the pathogen, it is remarkable that some biotrophic pathogens such as *Oidium neolycopersici* can have combinations of totally non-related host plants like tomato and *Arabidopsis* (Xiao et al. 2003).

Our data indicate that in contrast to thier high rust species specificity, the resistance QTLs are generally effective to several isolates per rust taxon. Identical QTLs were mapped for different isolates of *P. triticina* in the VxS population (Chapter 3) although one minor QTL was found to be effective only to the Flamingo isolate and not to others. This predominantly isolate-non-specific effect of QTLs needs to be corroborated in the near future.

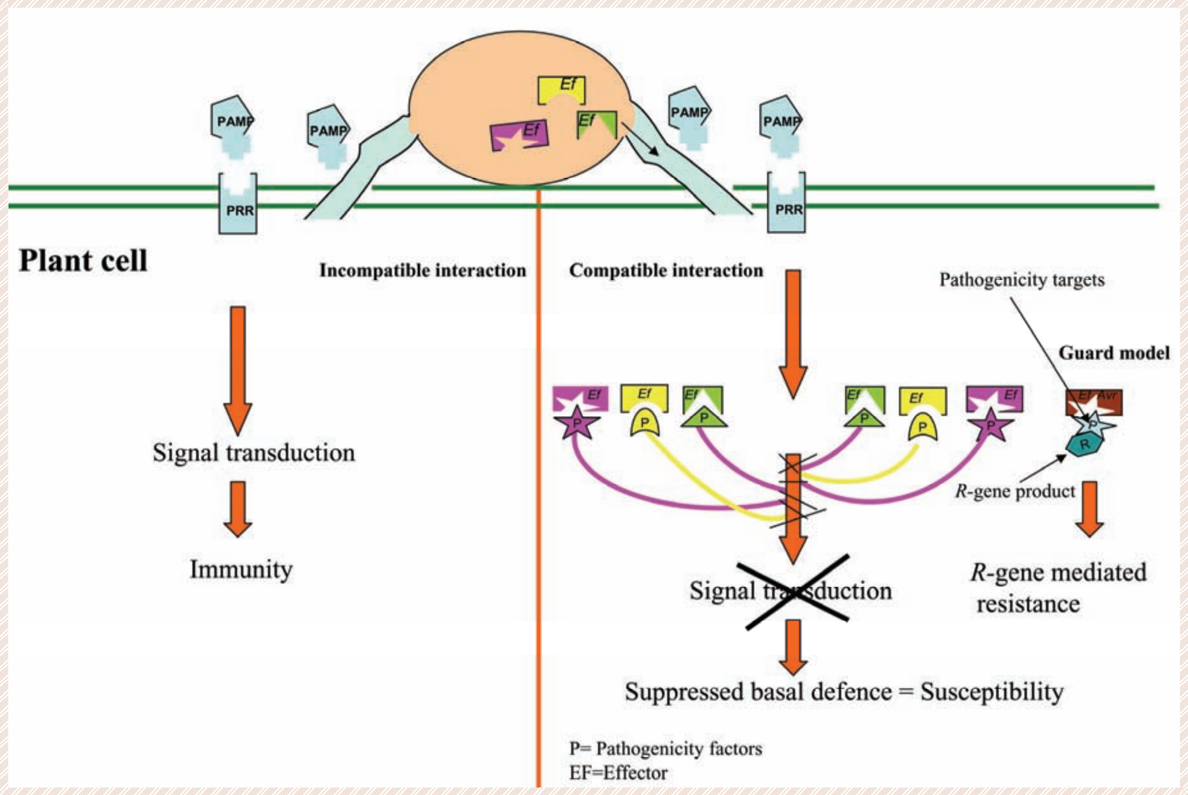

**Figure 1.** The Model describing the nature of QTLs mapped for nonhost immunity in this research. Rust fungi in both compatible (right) and incompatible (left) interaction contain PAMPs which trigger PAMPs-triggered immunity. This perception can result in immunity (left) unless rust fungi deliver appropriate effector proteins into the plant cell (right). These effector proteins target multiple pathogenicity target proteins to suppress basal immune responses of the plant. The QTLs mapped here are likely to be specific host factor genes that are targeted by rust species to suppress basal defence. The guard model in the right is depicted as one *R*-gene product that takes care of one effector protein that acts as Avr protein of a rust pathogen and may result in HR.

**Great diversity of genes for nonhost immunity:** The relatively easy accumulation of susceptibility genes (Chapter 2) is evidence of diversity of genes for nonhost immunity in barley germplasm. This high diversity was confirmed by the finding that each mapping population showed segregation for a different set of QTLs to the same rust species (Chapters 4 and 5). This diversity of genes may also occur in other plant species. Mellersh and Heath

(2003) found that the soybean rust *Uromyces vignae* could form some haustoria in some *Arabidopsis* accessions, and not in others. An interesting possibility would be to intercross such *Arabidopsis* accessions to accumulate genes for susceptibility to this heterologous rust, as we did in barley (Chapter 2).

**Evolutionary perspective of nonhost immunity:** The high diversity of QTLs in barley and the high specificity of QTLs are interesting features to develop theories on the co-evolution between plant and pathogen, to which our study does not provide answers so far. Our work, however, seems to contradict the theory that pathogen speciation (=formation of species) followed speciation in the host plant. An associated speciation would lead to the expectation that the plant QTLs determining the feasibility for being a host for a rust pathogen tend to be effective to taxonomically related rust species and/or to rusts that are pathogenic to related host species. Eight rust species used in this thesis belonged to three different groups of rust fungi of grasses: *P. hordei*, *P. persistens*/*P. triticina* and *P. graminis* groups containing 3, 3 and 2 rust taxa respectively. We did not find evidence that phylogenetic distance of rust fungi and/or of their host species were relevant factors determining the spectrum of effectiveness QTLs for near-nonhost resistance in barley. For *P. hordei-murini* and *P. hordei-secalini* we showed an association in genetics of resistance in both the OWB and the CxS populations suggesting that the relationship between hosts (both are pathogens of a *Hordeum* species) is more important than the relationship between rust pathogens (Chapter 4). However, in the VxS population we could not find evidence to support that hypothesis (Chapter 3). The nonhost status of barley and the effectivity spectrum of the QTLs effective to *P. hordei-bulbosi*, (Chapter 5) did not indicate relevance of phylogenetic relationship between heterologous rusts or of their host species either.

**Mechanisms of nonhost immunity:** There were indications that hypersensitivity resistance may contribute to the resistance to heterologous rust fungi. In some barley germplasm accessions many visible flecks but hardly any pustule were found after inoculation with heterologous rust species (Chapter 2) suggesting a relatively successful haustorium formation, but followed by HR. In Chapter 3 and 4 we found an *R*-gene effective to *P. hordei-secalini* with similarities in its phenotype (HR reaction) and its genetics (monogenic inheritance) with the *R*-genes, named *Rph*, in barley to *P. hordei* that cause a posthaustorial hypersensitive response. It is not clear whether that *R*-gene against a nonhost pathogen (*P. hordei-secalini*) acts in similar way as *Rph* genes against *P. hordei*. Christopher-Kozjan and Heath (2003) showed that biotrophic fungi trigger different cell death execution processes in host and nonhost cells during the hypersensitive response. The extent to which the gene-for-gene model applies to nonhost immunity is an interesting topic to be investigated.

Resistance to heterologous rusts species was mainly based on non-hypersensitive mechanisms (without visible HR at least at the macroscopic level). Study of the histology of infection in the VxS to *P. hordei-bulbosi* showed that plant cell necrosis was a minor phenomenon, mainly occurring as necrosis of one or two plant cells in relatively large colonies. Therefore, the resistance of Vada to *P. hordei-bulbosi* was not due to hypersensitivity. Study of the histology of infection in the VxS to *P. hordei-bulbosi* showed that for each component of nonhost resistance a different set of genes is implicated. These findings that some QTLs are affecting early abortion but not colony size, and other QTLs are responsible for colony size but not for early abortion were remarkable and imply that early abortion and colony size are not under the same genetic control. These findings suggest that nonhost resistance is a multi-component trait and each component of resistance is controlled by a specific set of genes. Multigenic inheritance of nonhost resistance is likely to explain the durability of this type of resistance in nature.

**Association between nonhost immunity and host resistance:** Since resistance to heterologous rusts is based on a different mechanism than post-haustorial hypersensitive resistance, but a similar mechanism as non-hypersensitive partial resistance (see Chapter 5), and the genes tend to map (see Chapter 4) to loci known to carry also QTLs for partial resistance to *P. hordei*, we presume that QTLs for resistance to heterologous rusts have a similar evolutionary origin as genes for partial resistance. Hence, understanding any aspect of partial resistance to the homologous pathogen *P. hordei* is relevant to understand nonhost immunity and *vice versa*. Our data indicate that mostly pleiotropy and to a lesser extent alternative alleles explain dual effectiveness of QTLs involved in resistance to host and nonhost pathogens (Chapter 4). Cloning of QTLs will provide more information on the genetics and mechanistic requirements for both partial resistance and nonhost immunity, and will also reveal to what extent molecular mechanisms and elements are shared among these two types of resistance.

## Perspective and future use of the barley-*Puccinia* model system

Nonhost resistance has been a topic of interest to plant pathologists for a long time, however, during recent years a combination of forward and reverse genetic approaches has contributed substantially to our current understanding of the genetics and molecular requirements of nonhost immunity in plants. Recent advances in the genetic dissection of nonhost immunity in *A. thaliana* include discovery of PAMP triggered immunity (PTI) and effector triggered immunity systems (ETI) in *Arabidopsis* against *P. syringae*, and the type III secretion system in bacteria. These findings have helped us in better understanding of the mechanisms underlying nonhost immunity in plants against bacterial pathogens (Abramovitch and Martin 2004; Hauck et al. 2003; Kim et al. 2005; Nomura et al. 2005; Chisholm et al. 2006; Jones and Takemoto 2004). However, it is doubtful whether those findings explain the natural differences in host status between plant species. Therefore the model pathosystem proposed in this work can give us the hope of dissecting the genetics and mechanisms of nonhost resistance using different genomic technologies as suggested below.

**Fine mapping and cloning of genes for nonhost immunity:** In our research we mapped QTLs which can be subjected to further analysis by fine mapping and possibly map based cloning. Fine mapping needs construction of special populations with large numbers of recombination in the region of an identified QTL. Three groups of QTL are interesting; (i) QTLs that are non-specific and show dual or multiple effectiveness to more than one rust species (ii) QTLs that are specific to one heterologous pathogen iii) QTLs that are involved in both partial and nonhost resistance. It is important that candidate QTLs for cloning should explain a high proportion of the phenotypic variation and are located in high recombination areas (Künzel et al. 2000). Cloning of QTLs with above specifications will contribute in our understanding about the nature and biological function of specific and less specific QTLs, pleiotropy versus genetic linkage of genes involved in nonhost immunity and partial resistance to *P. hordei* and molecular mechanism shared between nonhost and partial resistance. The selected QTLs can be target for fine mapping, physical mapping and isolation, and finally for proof of biological function via transformation.

**Candidate gene analysis:** Map-based positional cloning is mainly used for genes that inherit according to Mendelian segregation and can be problematic for QTLs due to the confounding effects of environment and other QTLs on phenotype. Map based cloning may also be affected by uneven distribution of recombination frequencies over the genome (Künzel et al. 2000). Some genomic regions may even lack recombination over a long physical distance and are therefore technically unsuitable for map-based cloning. Using a candidate gene approach can be an alternative way to combine QTL analysis with the expressed gene sequence data, to associate sequence polymorphisms with phenotypic variation. In Chapter 4, coincidence of QTLs for nonhost immunity and defence gene homologues (DGHs) on the consensus map was analysed by counting the BINs that were occupied by QTLs and DGHs (Chapter 4, Table 5). The significant association between distribution of the QTLs and DGHs over the consensus map of barley suggest that some DGHs are possibly responsible for nonhost immunity. Examples of such DGHs are receptor like kinase and BAX inhibitor 1, which co-located with some QTLs mapped for nonhost immunity (Chapter 4). Because of their known function, those DGHs can be considered plausible candidates to play a role in perception of plant pathogens or in suppression of nonspecific basal resistance.

**Gene expression studies:** Gene expression in the barley-*Puccinia* pathosystem can provide information about the plant defence genes that are activated in nonhost interactions. In each barley-rust combination that we tested, typically 4-5 QTLs contributed significantly to immunity. Whether or not each QTL gene is involved in induction of different sets of defence genes is not clear. QTLs with one and QTLs with combined specificities to heterologous rusts species can be introduced into the susceptible SusPtrit background using marker-assisted backcross introgression. The near-isogenic lines can be used to investigate the downstream genes that are regulated by those QTLs during nonhost interaction.

## Concluding remarks

Our investigations show that the barley-*Puccinia* system is an ideal and unique model to identify the factors that determine natural variation for (near) nonhost resistance to specialized pathogens like rusts. There are still many aspects that could be studied more comprehensively, like the role of *R*-genes, verification of QTLs in near-isogenic lines and the DNA sequence of particular QTLs for pre-haustorial resistance. Therefore this model has a lot to offer in the years to come.

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# APPENDIX

**Summary in English**

**Summary in Dutch**

**Acknowledgments**

**About the author**

**List of publications**

**Education certificate of the EPS**

**Summary in Farsi**

**Title page in Farsi**

**Colour figures**

## SUMMARY

The inheritance of nonhost immunity of plants to potential pathogens is notoriously difficult to study since it requires, by definition, interspecific crosses between a susceptible host plant species and a resistant nonhost plant species. The barley–*Puccinia* rust model system, however, allowed us to determine the specificity, number and diversity of genes involved in nonhost immunity. We developed an experimental line, SusPtrit, with exceptional susceptibility to various taxa of heterologous rust fungi, i.e. rust fungi to which the large majority of barley genotypes are immune. We crossed SusPtrit with regular immune barley lines to study the inheritance of (near)nonhost immunity. We found a quantitative inheritance for this trait, requiring a QTL (*quantitative trait loci*) analysis. In contrast to what might be expected, nonhost resistance of barley to various heterologous rust species is not due to a few key-genes with general effectiveness to rust fungi, but to various genes, each with a considerable level of rust species specificity. **Chapter one** gives an introduction about innate immunity, and the molecular and genetic basis of nonhost immunity in plants and describes the scope of this thesis. In **Chapter two**, by screening of 109 barley accessions in the seedling stage, we showed that barley is a complete nonhost against most of the heterologous rust fungi studied, while it showed an intermediate (near-nonhost) status against *P. triticina*, *P. persistens*, *P. hordei-murini*, *P. hordei-secalini*, *P. graminis* f. sp. *lolii* and *P. coronata* ff. spp. *avenae* and *holci*. None of the barley accessions, however, was fully susceptible to any of the heterologous rust pathogens. Differential interaction between barley accessions and heterologous rust fungi was found, suggesting the existence of rust species specific resistance. Some barley accessions tended to be susceptible to several heterologous rusts suggesting that some resistance genes in barley are effective to more than one heterologous rust fungal species. By intercrossing and selection, we accumulated genes for susceptibility against *P. triticina* and *P. hordei-murini* in two genotypes called SusPtrit and SusPmur, respectively. At the seedling stage these accessions were as susceptible as the host species to these rusts. They also showed high susceptibility to other heterologous rusts. In **Chapter three** we describe the construction of a molecular marker map from a cross between the experimental barley line SusPtrit and the cv Vada that is immune to heterologous rusts, like any normal modern cultivar. The recombinant inbred line (RIL) population derived from Vada x SusPtrit (152 lines $F_8$) was exposed to eight rust isolates belonging to five heterologous and two homologous (host) species of rust fungi. Results showed that to all rusts resistance segregated quantitatively. By QTL analysis 18 chromosomal regions were implicated, of which eleven

carried genes that were effective to only one rust species and seven to more than one rust species, implying genetic linkage or pleiotropy. One *R*-gene for hypersensitive resistance to *P. hordei-secalini* was mapped. Sequencing of the internal transcribed spacers (ITS) allowed a phylogenetic analysis of the rusts, and did not confirm a tendency of non-specific QTLs to be effective to rust species that have a close phylogenetic relationship. We suggest that the QTLs involved in nonhost immunity encode specific and quantitative recognition factors that are specifically negated by the rust to successfully suppress innate immunity. In **Chapter four** we describe the great diversity of genes involved in nonhost immunity. We developed an additional RIL population from a cross between the susceptible research line SusPtrit and the resistant South American barley cultivar Cebada Capa. The latter accession is like Vada and other regular barley cultivars immune to heterologous rusts. Surprisingly, we found segregation for susceptibility to heterologous rust fungi in the commonly used Oregon Wolfe Barleys (OWB) reference mapping population. We mapped QTLs involved in resistance to four heterologous rust species in the Cebada Capa x SusPtrit (C x S) and OWB to compare them with the QTLs identified in the Vada x SusPtrit population (chapter 3). Positions of QTLs conferring nonhost immunity in the three mapping populations were compared using an integrated consensus map. The results confirmed that nonhost immunity in barley to heterologous rust species is controlled by QTLs with different and overlapping specificities, and by occasional contribution of an *R*-gene for hypersensitivity. In each population different sets of loci were implicated in resistance, with very few genes in common between the populations, suggesting a high diversity of loci carrying genes for nonhost immunity to heterologous rust fungi. These loci were significantly associated with QTLs for partial resistance to the pathogen *P. hordei*, and also with defence gene homologues (DGH). In **Chapter five** we screened of barley accessions in the seedling stage for resistance to *P. hordei-bulbosi*, the leaf rust fungus of *Hordeum bulbosum*. We showed that some barley accessions (mainly exotic barleys, landraces and wild barleys) are in the seedling stage somewhat susceptible to *Phb* implying that barley is a near- nonhost for this rust species. We studied the genetic basis of resistance to *Phb* using three different segregating populations: Vada x SusPtrit (VxS; Vada is immune), Cebada Capa x SusPtrit (CxS; Cebada Capa is immune) and Oregon Wolf Barleys (OWB; Dom x Rec, Dom is immune). QTL analysis based on macroscopic visible infection sites showed that in each mapping population a different set of genes is involved in resistance to *Phb* and only one QTL overlapped in VxS and CxS populations. QTL analysis in the VxS population using microscopic traits, including colony size, early abortion without necrosis and the percentage of infection units associated with necrotic plant cell(s), showed that each microscopic trait is under control of a different set of genes implying that near nonhost resistance to *Phb* is multi-component trait, each component regulated by different genes. Plant cell necrosis occurred relatively often encompassed only one or two plant cells in relatively large colonies. Therefore, the resistance of Vada to this heterologous rust was essentially based on a non-hypersensitive mechanism. In **Chapter 6** the main results described in this thesis are discussed in a broader perspective. In this chapter the near-nonhost status in barley to some heterologous rust fungi and its evolutionary implication in nonhost immunity is discussed. The nature and the possible role of QTLs mapped for nonhost immunity in suppression of plant defence responses are highlighted. This chapter also addresses the perspective and future use of the barley-*Puccinia* model system to investigate aspects of nonhost resistance to specialised plant pathogens.

## SAMENVATTING

De genetische basis van niet-waard resistentie van planten tegen plantpathogenen is erg lastig te onderzoeken omdat er, per definitie interspecifieke kruisingen, tussen een vatbare waardsoort en een resistente niet-waard soort, voor nodig zijn, . Het gerst – *Puccinia* roestmodelsysteem maakte het mogelijk de specificiteit, het aantal en de diversiteit te bepalen van genen die betrokken zijn bij niet-waard resistentie. Eerst ontwikkelden we een gerstlijn, SusPtrit, met een uitzonderlijke vatbaarheid voor verscheidene soorten en vormen van heterologe roestschimmels. De overgrote meerderheid van gerstgenotypen zijn volledig resistent tegen deze roestschimmels. We kruisten SusPtrit met gewone, resistente, gerstlijnen om de overerving te bestuderen van de (bijna) niet-waard resistentie. De resistentie berustte op een kwantitatieve overerving, waarvoor een QTL (*quantitatieve trait loci*) analyse geëigend is. In tegenstelling tot wat verwacht zou kunnen worden, berustte de niet-waard resistentie van gerst tegen de verschillende roestschimmelsoorten niet op enkele sleutelgenen die effectief waren tegen alle heterologe roesten, maar op verschillende genen met meestal een hoge mate van roestsoort-specificiteit. In **hoofdstuk één** introduceren we de erfelijke resistentie (“innate immunity”), en de moleculaire en genetische basis van niet-waard resistentie in planten. In dat hoofdstuk presenteren we ook het bestek van dit proefschrift. In **hoofdstuk twee** worden 109 gerstlijnen in het zaailingstadium getoetst op resistentie tegen roestschimmels. Daaruit blijkt dat gerst een volledige niet-waard status heeft voor de meeste heterologe roestschimmels, maar een onvolledige (“bijna”) niet-waardstatus heeft voor de roestschimmels *P. triticina*, *P. persistens*, *P. hordei-murini*, *P. hordei-secalini*, *P. graminis* f. sp. *lolii* en *P. coronata* ff. spp. *avenae* en *holci*. Geen van de gerstlijnen was echter volledig vatbaar voor één of meer van deze heterologe roestschimmels. Uit het voorkomen van differentiële interactie tussen gerstlijnen en heterologe roestschimmels bleek dat de resistentie erg roestsoort-specifiek kon zijn. Enkele gerstlijnen waren enigszins vatbaar voor verschillende heterologe roestsoorten, wat suggereert dat er ook resistentiegenen voorkomen die effectief zijn tegen meerdere roestschimmelsoorten. Door kruisen en selecteren brachten we genen voor vatbaarheid tegen *P. triticina* en *P. hordei-murini* samen in twee gerstlijnen, respectievelijk SusPtrit en SusPmur. In het zaailingstadium zijn deze beide lijnen even vatbaar als de normale waardsoort voor deze roestschimmels. De beide lijnen bleken ook erg vatbaar voor een aantal andere heterologe roestschimmelsoorten. In **hoofdstuk drie** beschrijven we de genetische kaart die gebaseerd is op een kruising tussen de onderzoekslijn SusPtrit en het ras Vada. Het ras Vada is volledig resistent tegen heterologe roesten, zoals elk modern gerstras. Een recombinant inteelt lijn populatie (RIL) populatie afkomstig van de kruising Vada x SusPtrit (152 lijnen in $F_8$) werd geïnoculeerd met acht roestisolaten die tot vijf heterologe en twee homologe (d.w.z. gerst is waardsoort) roestschimmelsoorten behoren. De resistentie tegen al deze roestisolaten bleek kwantitatief over te erven. Een QTL analyse identificeerde 18 chromosoomsegmenten met daarop genen die een rol speelden in de resistentie. Elf hiervan waren effectief tegen slechts één roestschimmelsoort, en zeven segmenten bleken een rol te spelen in resistentie tegen twee of meer roestschimmelsoorten. Dat laatste wijst op een gen met een minder roestspecifiek effect, of op koppeling van twee of meer roestspecifieke genen. Eén *R*-gen voor overgevoeligheidsresistentie tegen *P. hordei-secalini* werd ontdekt.

Analyse van de basenvolgorde van het ITS (internal transcribed spacers) DNA gaf een beeld van de fylogenetische verwantschap van de roesten. Er bleek geen tendens te zijn dat QTLs met effectiviteit tegen meer dan één roestschimmelsoort vooral effectief waren tegen soorten die nauw verwant waren. We opperen de hypothese dat de bij niet-waard resistentie betrokken QTLs specifieke en kwantitatieve herkenningsfactoren coderen, die specifiek door de roest moeten worden aangepakt om met succes de niet-waard resistentiereactie te onderdrukken. In **hoofdstuk vier** beschrijven we de grote diversiteit aan genen die betrokken zijn bij niet-waard resistentie. We ontwikkelden een tweede RIL populatie uit een kruising tussen de vatbare lijn SusPtrit en het resistente Zuidamerikaanse gerstras 'Cebada Capa'. Laatstgenoemde is evenals Vada en de meeste andere gerstrassen volledig resistent tegen heterologe roestschimmelsoorten. Tot onze verrassing vonden we ook een uitsplitsing voor vatbaarheid voor heterologe roestschimmelsoorten in de standaard mapping populatie Oregon Wolfe Barleys (OWB). In beide mapping populaties, Cebada Capa x SusPtrit (C x S) en OWB, brachten we de QTLs in kaart die betrokken zijn bij resistentie tegen vier heterologe roestschimmelsoorten. We vergeleken de QTLs in beide populaties met de QTLs die we gevonden hadden in de Vada x SusPtrit populatie (hoofdstuk 3). Voor de vergelijking van de posities van de QTLs gebruikten we een geïntegreerde consensuskaart. De resultaten bevestigden dat niet-waard resistentie in gerst tegen heterologe roestschimmelsoorten bepaald wordt door QTLs met verschillende en overlappende specificiteit. Ook een *R*-gen voor overgevoeligheidsresistentie droeg in sommige gevallen bij aan de niet-waard resistentie. In elke populatie waren verschillende combinaties van loci betrokken bij de resistentie, met erg weinig loci gemeenschappelijk. Dit suggereert een grote diversiteit in gerst van loci met genen voor niet-waard resistentie tegen heterologe roestschimmelsoorten. Deze loci waren significant geassocieerd met posities van QTLs voor partiële resistentie tegen het gerstpathogeen *P. hordei* en ook met loci waar met afweer gerelateerde genen (defence gene homologues, DGH) gelegen zijn. In **hoofdstuk vijf** presenteren we een zaailingtoets van gerstcollectienummers op resistentie tegen *P. hordei-bulbosi*, de bruine roest van *Hordeum bulbosum*. We toonden aan dat sommige gerstlijnen (vooral exotische gerstrassen, landrassen en wilde gerstlijnen) in het zaailingstadium enigszins vatbaar zijn voor *Phb*. Dit impliceert dat gerst een “bijna niet-waard” is voor deze roestsoort. We bestudeerden de genetische basis van de resistentie tegen *Phb* in de drie splitsende populaties die we eerder ook al gebruikten (hoofdstukken 3 en 4): Vada x SusPtrit (VxS; Vada is volledig resistent), Cebada Capa x SusPtrit (CxS; Cebada Capa is volledig resistent) en Oregon Wolf Barleys (OWB; Dom x Rec, Dom is volledig resistent). Een QTL analyse gebaseerd op het aantal macroscopisch zichtbare infecties wees uit dat in elke mapping populatie een andere set genen de resistentie tegen *Phb* bepaalde: slechts één QTL werd zowel in de VxS als in de CxS populatie gevonden. Een QTL analyse in de VxS populatie die gebaseerd was op microscopisch waargenomen infectiecomponenten, te weten koloniegrootte, vroege abortie zonder plantcelnecrose en percentage infectie-eenheden dat geassocieerd is met plantcelnecrose, resulteerde in de ontdekking dat elke infectiecomponent bepaald werd door een verschillende set genen. Dit impliceert dat de bijna niet-waard resistentie tegen *Phb* uit verschillende componenten bestaat, die elk gereguleerd worden door verschillende genen. Plantcelnecrose werd vooral gevonden als één of twee autofluorescerende cellen in relatief grote roestkolonies. Daarom mag geconcludeerd worden dat de resistentie van ‘Vada’ tegen deze heterologe roest in essentie niet op overgevoeligheid gebaseerd is. In **hoofdstuk 6** worden de belangrijkste in dit proefschrift beschreven resultaten behandeld en in een breder perspectief geplaatst. In dat hoofdstuk bespreken we de “bijna-niet waard” status van gerst tegen sommige heterologe roestschimmelsoorten en de evolutionaire implicatie voor niet-waard resistentie. We belichten de aard en mogelijke rol van de genen gelegen op de QTLs voor niet-waard resistentie in de onderdrukking van resistentie. Het hoofdstuk behandelt ook de vooruitzichten die toepassing van het gerst-*Puccinia* modelsysteem biedt om fundamentele aspecten van niet-waard resistentie tegen gespecialiseerde plant pathogenen te onderzoeken.

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Finally, I come to thank my family that without their support I would not be able to complete my thesis. To my parents, especially to my father, thank you very much for your support and patience. I know that you had a very hard time, fighting with your illness and waiting impatiently to see your children and grandchildren, but I praise God that you finally made it. To my wife, dear Nastaran, the road to this achievement was long and laborious and I had to spend long hours in my office to be on schedule. I am totally aware that it caused you a lot of difficulties but you never protested. I am extremely thankful for your understanding, patience and accompanying me during this journey. To my lovely daughter Negin, I am glad that you could make some friends in your school from different countries, you learned Dutch language very well and you mixed up with Dutch culture easily. I hope this rather short-time experience will have a long term positive impact in your personal life. To my lovely son, Yashar, you are going to leave your country of birth soon but my desire is that you get a chance to come back someday to do your PhD in the Netherlands. You may do better than papa!

## About the author

Hossein Jafary was born in 1970 in Saeinghaleh, Zanjan, Iran. After completing high school in biological sciences in 1988, he began his studies at the University of Tabriz, (East Azerbaijan) and obtained a BSc degree (with distinguished degree) in the filed of Plant Protection in 1991. After finishing his bachelor studies he worked for two years at the Zanjan Agricultural Organization (in the north west of Iran) for completion of the required military-civil service. In 1995 he successfully passed the Iranian national MSc exam as the first top student and got scholarship from Ministry of Science, Research and Technology (MSRT) to continue his studies in the field of Plant Pathology at the Tarbiat Modarres University (Tehran, Iran). He received his MSc (with distinguished degree) in the field of Plant Pathology in 1997, and got employed as an academic staff of Plant Pest and Disease Research Institute in Zanjan Agricultural Research Center. In 2002, he was awarded a scholarship from the Ministry of Science, Research and Technology, financed by the Agricultural Research and Education Organization (AREO) of Iran, allowing him to do a PhD abroad. In Dec. 2002, he started his PhD program at Wageningen University and Research Center, Laboratory of Plant Breeding. This dissertation presents the results of his PhD study on developing a barley-*Puccinia* rusts model system to investigate the genetics, evolution and mechanisms of nonhost immunity in plants.

## LIST OF PUBLICATIONS:

### Full papers:

1-Atienza*, S. G., **Jafary, H***., and Niks, R. E. 2004. Accumulation of genes for susceptibility to rust fungi for which barley is nearly a nonhost results in two barley lines with extreme multiple susceptibility. Planta 220:71-79.
**both authors equally collaborated to the work*

2-**Jafary, H**., Szabo, L., and Niks, R. E. 2006. Innate nonhost immunity in barley to different heterologous rust fungi is controlled by sets of resistance genes with different and overlapping specificities. Molecular Plant-Microbe Interaction 19:1270-1279

3-**Jafary, H**., Albertazzi, G., Marcel T. C., and Niks R. E. 2006. High diversity of genes for nonhost immunity of barley to heterologous rust fungi. Submitted.

4-**Jafary, H**., and Niks, R. E. 2006. The near-nonhost resistance of barley to *Puccinia hordei-bulbosi*: mechanisms and genetics of basal resistance. To be submitted.

5-Marcel TC, Varshney RK, Barbieri M., **Jafary H.**, de Kock M. J. D., Graner A., and Niks R. E. 2006. A high-density consensus map of barley to compare the distribution of QTLs for partial resistance to *Puccinia hordei* and of defence gene homologues. Accepted by Theoretical and Applied Genetics.

### Abstracts:

1- **Jafary, H.,** and Niks, R. E, 2004. Mapping of quantitative genes in barley determining the resistance to the heterologous wheat leaf rust fungus (*Puccinia triticina*). Proceedings of the 11th International Cereal Rusts and Powdery Mildews Conference, Norwich, England, 22-27 August 2004, abstract 1.4, Cereal Rusts and Powdery Mildews Bulletin.

2- **Jafary, H.,** Albertazi, G., and Niks, R. E. 2006. Diversity of loci carrying genes for resistance of barley to heterologous rust species. Proceedings of the non-specific and specific innate and acquired plant resistance Symposium. Page 66. Plant Protection Institute of the Hungarian Academy of Sciences, Budapest, Hungary.

4- **Jafary, H.,** and Niks, R. E, 2006. Nonhost resistance in barley to rust fungi is based on highly specific resistance genes. Proceeding of 16th Plant and Animal Genome Conference, Page 187, San Diego, USA.

3- Marcel, T. C, Varshney, R. K., Barbieri, M., **Jafary, H.**, de Kock, M. J. D., Graner, A., and Niks, R. E. 2006. A high-density consensus map of barley to analyse the distribution of QTLs and candidate genes for partial resistance to *Puccinia hordei*. Proceeding of 16th Plant and Animal Genome Conference, Page177, San Diego, USA.

## Education Statement of the Graduate School

## Experimental Plant Sciences

**Issued to:** **Hossein Jafary**
**Date:** **8 December 2006**
**Group:** **Plant Breeding, Wageningen University**

| **1) Start-up phase** | *date* |
|---|---|
| ► **First presentation of your project** | |
| Genetics of Nonhost resistance in barley | Feb 22, 2003 |
| ► **Writing or rewriting a project proposal** | Dec 2002-Jun 2003 |
| ► **Writing a review or book chapter** | |
| ► **MSc courses** | |
| Genetic analysis, Tools and Concepts | Nov-Dec 2004 |
| Breeding for resistance | Apr 2003 |
| ► **Laboratory use of isotopes** | |
| *Subtotal Start-up Phase* | *13.5 credits** |

| **2) Scientific Exposure** | *date* |
|---|---|
| ► **EPS PhD student days** | |
| PhD student day, Amsterdam | Jun 03, 2004 |
| PhD student day, Nijmegen | Jun 02, 2005 |
| PhD student day, Wageningen | Sep 19, 2006 |
| ► **EPS theme symposia** | |
| EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', Wageningen University | Sep 17, 2004 |
| EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', Leiden University | Jun 23, 2005 |
| ► **NWO Lunteren days and other National Platforms** | |
| ALW meeting Lunteren Plant science | Apr 07-08, 2003 |
| ALW meeting Lunteren Plant science | Apr 05-06, 2004 |
| ALW meeting Lunteren Plant science | Apr 04-05, 2005 |
| ► **Seminars (series), workshops and symposia** | |
| WCS Day, 2003 | Jan 30, 2003 |
| ► **Seminar plus** | |
| ► **International symposia and congresses** | |
| 11 the Cereal rust and powdery mildew conference, Norwich UK | Aug 23-28, 2004 |
| 16 th Plant and animal genome conference San Diego, USA | Jan 14-18, 2006 |
| Non specific and specific innate and acquired plant resistance, Budapest, Hungary | Aug 31-Sep 03, 2006 |
| ► **Presentations** | |
| Poster presentation, ALW meeting, Lunteren | Apr 07-08, 2003 |
| Poster presentation(1) Plant and Animal Genome conference, San Diego, USA, | Jan 14-18, 2006 |
| Poster presentation(2) Plant and Animal Genome conference, San Diego, USA, | Jan 14-18, 2006 |
| Oral presentations, Cereal rust and powdery mildew conference, Norwich, UK | Aug 23-28, 2004 |
| Poster presentation, Non specific and specific innate and acquired plant resistance, Budapest, Hungary | Aug 31-Sep 3, 2006 |
| Oral presentation, EPS Theme 2 symposium, Leiden University, The Netherlands | Jun 23, 2005 |
| ► **IAB interview** | Jun 03, 2005 |
| ► **Excursions** | |
| Summer excursion for cereal diseases to Belgium and France | Jun 28, 2004 |
| Excursion to Rijk Zwaan breeding company, The Netherlands | Jun 2003 |
| Excursion to Nunhems seed company, The Netherlands | May 23 , 2006 |
| *Subtotal Scientific Exposure* | *14.1 credits** |

| **3) In-Depth Studies** | *date* |
|---|---|
| ► **EPS courses or other PhD courses** | |
| Functional Genomics: theory and hands-on data analysis, Utrecht University | Aug 25-28, 2003 |
| Signaling in plant development and defence; towards system biology, Wageningen University | Jun 19-21, 2006 |
| ► **Journal club** | |
| Literature study group Plant Breeding | 2002-2006 |
| ► **Individual research training** | |
| Linkage analysis and QTL mapping | Jan 2005 |
| Molecular markers in plant breeding | May 26-Jun 06, 2003 |
| *Subtotal In-Depth Studies* | *9.0 credits** |

| **4) Personal development** | *date* |
|---|---|
| ► **Skill training courses** | |
| Techniques for writing and presenting a scientific paper, Wageningen | Jun 29-Jul 02, 2004 |
| ► **Organisation of PhD students day, course or conference** | |
| ► **Membership of Board, Committee or PhD council** | |
| *Subtotal Personal Development* | *1.2 credits** |

| | |
|---|---|
| **TOTAL NUMBER OF CREDIT POINTS*** | **37.8** |

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

**در فصل چهارم** تنوع در ژنهای موثر در ایمنی غیر میزبانی مورد بررسی قرار گرفته است. یک توده نقشه یابی دیگر شامل 113 لاین(RILs, F9 ) با استفاده از تلاقی بین لاین حساس آزمایشگاهی SusPtrit و رقم ایمن Cebada Capa ایجاد گردید. ژن های مقاومت نسبت به چهار گونه زنگ غیر میزبان در توده مذکور و نیز در توده نقشه یابی Oregon Wolf Barleys (OWB) که به طور استثنایی در حساسیت به زنگ های غیر میزبان تفرق نشان دادند، نقشه یابی گردید. محل قرار گرفتن ژن های مربوط به ایمنی غیر میزبانی بر روی کروموزم های جو با استفاده از یک نقشه ژنتیکی جامع تلفیقی[1] مقایسه گردید. نتایج نشان داد که ایمنی غیر میزبانی به قارچهای عامل زنگ در هر یک از سه رقم جو توسط گروهی از ژن ها با اثر کمی کنترل می شود که تنها تعداد معدودی از این ژن ها بین ارقام مختلف جومشترک میباشند. همبستگی معنی داری در خصوص محل قرار گرفتن ژن های مقاومت به قارچ های غیر میزبان با محل ژن های مقاومت نسبی به *P. hordei* (عامل بیماری زنگ قهوه ای جو) مشاهده گردید که حاکی از ارتباط ژنتیکی بین این دو نوع مقاومت میباشد. مکان های ژنی مقاومت غیر میزبانی با همولوگ های ژن های دفاعی گیاهان نیزبه طور معنی داری هم پوشانی نشان دادند.

**در فصل پنجم** دامنه میزبانی جونسبت به قارچ *P. hordei-bulbosi* بررسی و نشان داده شد که تعداد معدودی از ارقام جو تا حدودی به قارچ مذکور حساس می با شند. ژن های مقاومت به این قارچ در سه توده نقشه یابی (فصل 3 و 4) مقایسه گردید. علاوه بر این مکانیسم مقاومت با استفاده از روش های بافت شناسی[3] مطالعه گردید. نتایج حاصل نشان داد که ژن های مقاومت غیر میزبانی به قارچ مذکور در هر توده متفاوت از توده دیگر می باشد. مقاومت بر اساس مکانیسم هایی غیراز وا کنش فوق حساسیت بوده و اجزای مقاومت در ابعاد میکروسکپی توسط ژن های مختلفی کنترل می شود که این می تواند توجیهی بر پایداری مقاومت غیر میزبانی در گیاهان با شد.

**در فصل ششم** نتایج حاصل از این رساله در ابعاد وسیع تر بررسی شده است. در این فصل وضعیت میزبانی جو نسبت به قارچ های مختلف عامل زنگ و ارتباط تکاملی آن با ایمنی غیر میزبانی بررسی گردیده است. همچنین نقش مکان های ژنی نقشه یابی شده در ایمنی غیر میزبانی در فر آیند خنثی سازی سیستم های دفاعی گیاه بحث و اهمیت پاتوسیستم مورد بررسی به عنوان مدلی جدید در مطالعه مقاومت غیر میزبانی تاکید شده است.

با تشکر از دوست گرامی آقای علیرضا سیفی در ویراستاری چکیده فارسی.

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[1] Integrated consensus map

[2] Defence gene homologues (DGH)

[3] Histology

## خلاصه:

مطالعه اساس ژنتیکی ایمنی غیر میزبانی گیاهان نسبت به عوامل بیماریزا به دلیل نیاز به تلاقی های بین گونه ای بسیار دشوار می باشد. در این تحقیق با استفاده ازپاتوسیستم جو- زنگهای جنس *Puccinia* مدلی ارایه شده است که بر اساس آن بتوان تعداد، تنوع و اختصاصی بودن ژن های موثر در ایمنی غیر میزبانی را تعیین نمود. برای این منظور یک رقم جو آزما یشگاهی بنام SusPtrit تولید و با ارقام معمولی جو که نسبت به زنگ های غیر میزبان ایمن می باشند تلاقی داده شد. توارث کمی صفت ایمنی غیر میزبانی نسبت به قارچ های عامل زنگ بر خلاف انتظا ر به واسطه چند ژن کلیدی با طیف اثر وسیع نبوده و برای هر زنگ عامل بیماری گروهی از ژن های اختصاصی نقشه یابی گردیدند. در **فصل اول** مقدمه ای در باره اساس ملکولی و ژنتیکی ایمنی غیر میزبانی در گیاهان ارایه و اهداف این تحقیق به طور خلاصه تشریح شده است .

**در فصل دوم** دامنه میزبانی جو در مرحله گیاهچه ای نسبت به 19 گونه و فرم مخصوص از قارچ های عامل زنگ مورد بررسی قرار گرفته است. نتایج بررسی نشان داد که جو یک گیاه غیر میزبان کامل برای بیشتر قارچ های مورد بررسی است در حالی که تعداد دیگری از زنگ ها مانند *P. persistens* ، *P. hordei-murini* و *P. hordei-secalini* توانستند در سطح محدود در روی جو بیماری ایجاد نمایند. تعدادی از ارقام جو هم زمان نسبت به بیش از یک گونه از زنگ ها حساسیت نشان دادند، با این حال حساسیت بالا نسبت به هیچکدام از زنگ های غیر میزبان در بین ارقام مورد بررسی مشاهده نگردید. با تلاقی ارقام جو که تا حدودی حساس به قارچ های *P. triticina* و*P. hordei-murini* بودند و با استفاده از روش تجمیع ژن های حساسیت، دو رقم آزمایشگاهی به نام های SusPtrit و SusPmur به ترتیب با حساسیت استثنایی به قارچ های مذکور ایجاد گردید. این دو رقم علاوه بر زنگ های هدف به تعداد دیگری از زنگ ها هم حساسیت نشان دادند.

**در فصل سوم** با تلاقی بین رقم حساس آزمایشگاهی SusPtrit ورقم ایمن وادا (Vada) یک توده نقشه یابی[1] شامل 152 لاین (Recombinant Inbred Lines=RILs, F8) ایجاد گردید. نقشه ژنتیکی[2] جوبا استفاده از نشانگرهای ملکولی[3] (SSR , AFLP) تهیه و توده مذکور به وسیله 8 جدایه قارچی متعلق به پنج گونه از زنگ های غیر میزبان و دو گونه میزبان تلقیح گردید. با استفاده از آنالیز مکان های ژنی کنترل کننده صفات کمی[4] تعداد 18منطقه کروموزومی موثر در مقاومت نقشه یابی گردید که از بین آنها 11 منطقه کروموزومی تنها در مقاومت به یک گونه و 7 منطقه کروموزومی دیگر در مقاومت به بیش از یک گونه از زنگ ها نقش داشتند. یک ژن اصلی با ویژگی مقاومت کیفی مسئول واکنش فوق حساسیت[5] نسبت به قارچ *P. hordei-secalini* نیز نقشه یابی گردید. مطالعه فیلوژنی قارچ های مورد بررسی با استفاده ترادف ITS (Internal Transcribed Spacers) نشان دادکه مکان های ژنی غیر اختصاصی متمایل به تاثیر بر روی قارچ هایی با قرابت فیلو ژنی بالا نیستند.

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[1] Mapping population
[2] Genetic map
[3] Molecular markers
[4] Quantitative Trait Loci
[5] Hypersensitive reaction

# پاتوسیستم جو- زنگهای جنس *Puccinia*: مدلی برای مطالعه اساس ژنتیکی، تکامل و سازوکارهای ایمنی غیر میزبانی در گیاهان

**حسین جعفری**

**آذر 1385**

**رساله دکترای تخصصی**

**دانشگاه واخنینگن**

**هلند**

This research was financially supported by the Agricultural Research and Education Organization (AREO) of I. R. of Iran.

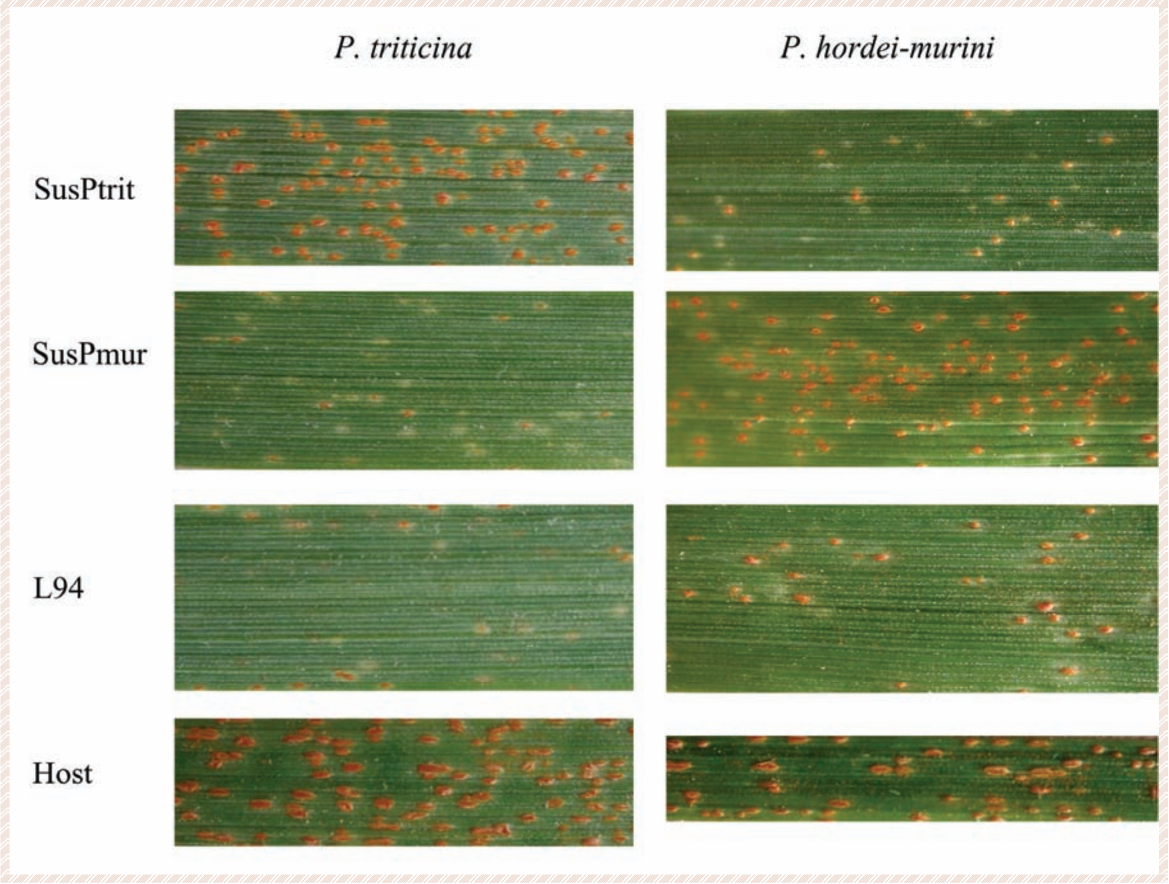
Layout and design: by the author.

**Front page:** Spikes of experimental barley line ‘SusPtrit’ with high number of susceptibility genes to rust fungi spread over the genome.

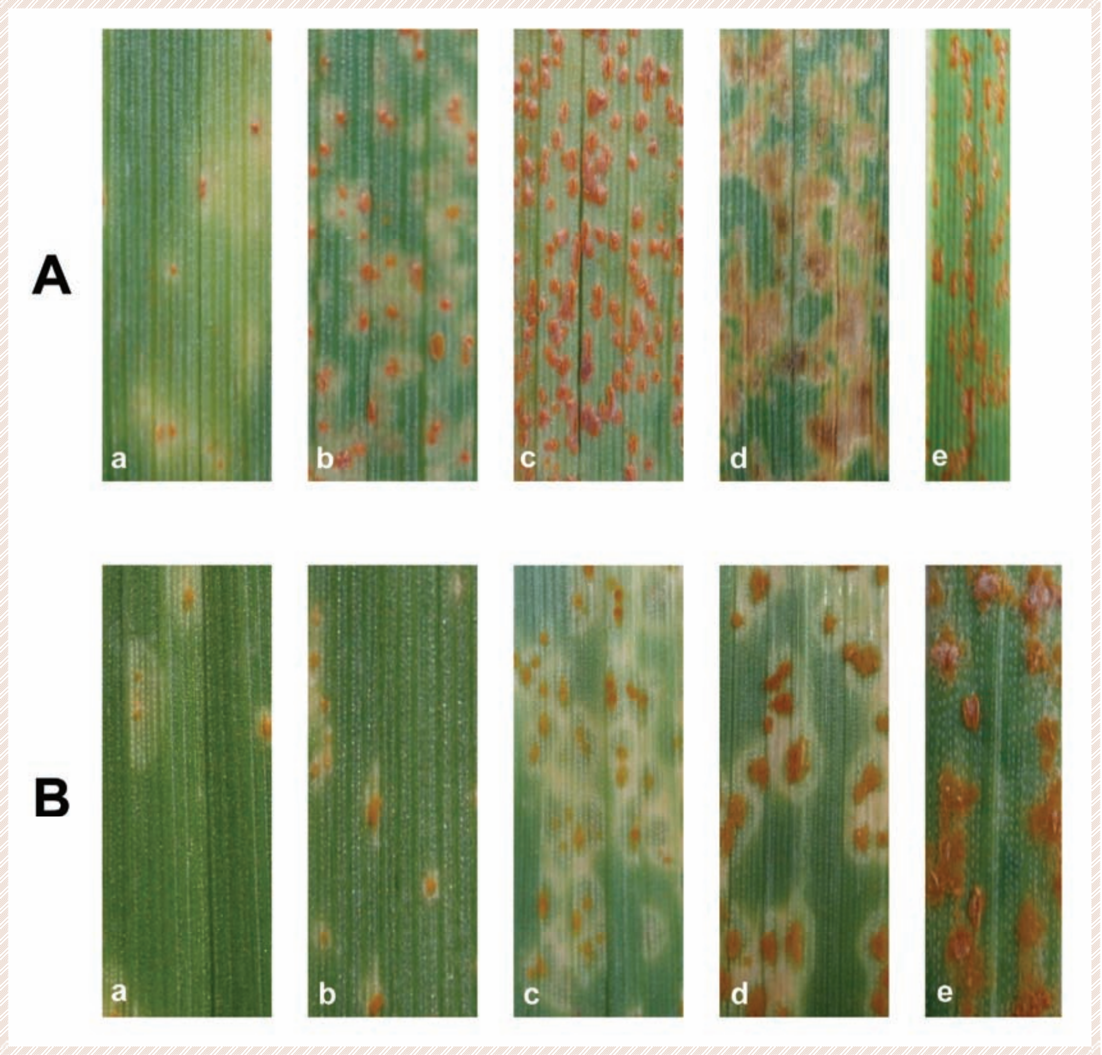
**Back page:** Diversity of loci carrying genes for nonhost immunity in barley to heterologous rust pathogens (Top) and Parsimony tree from the analysis of nuclear ribosomal internal transcribed spacer (ITS) sequence data of rust fungi studied in this research (Bottom).

The Training and Supervision Plan was completed at the Graduate School “Experimental Plant Sciences”

# Colour figures, chapter 2 and 3

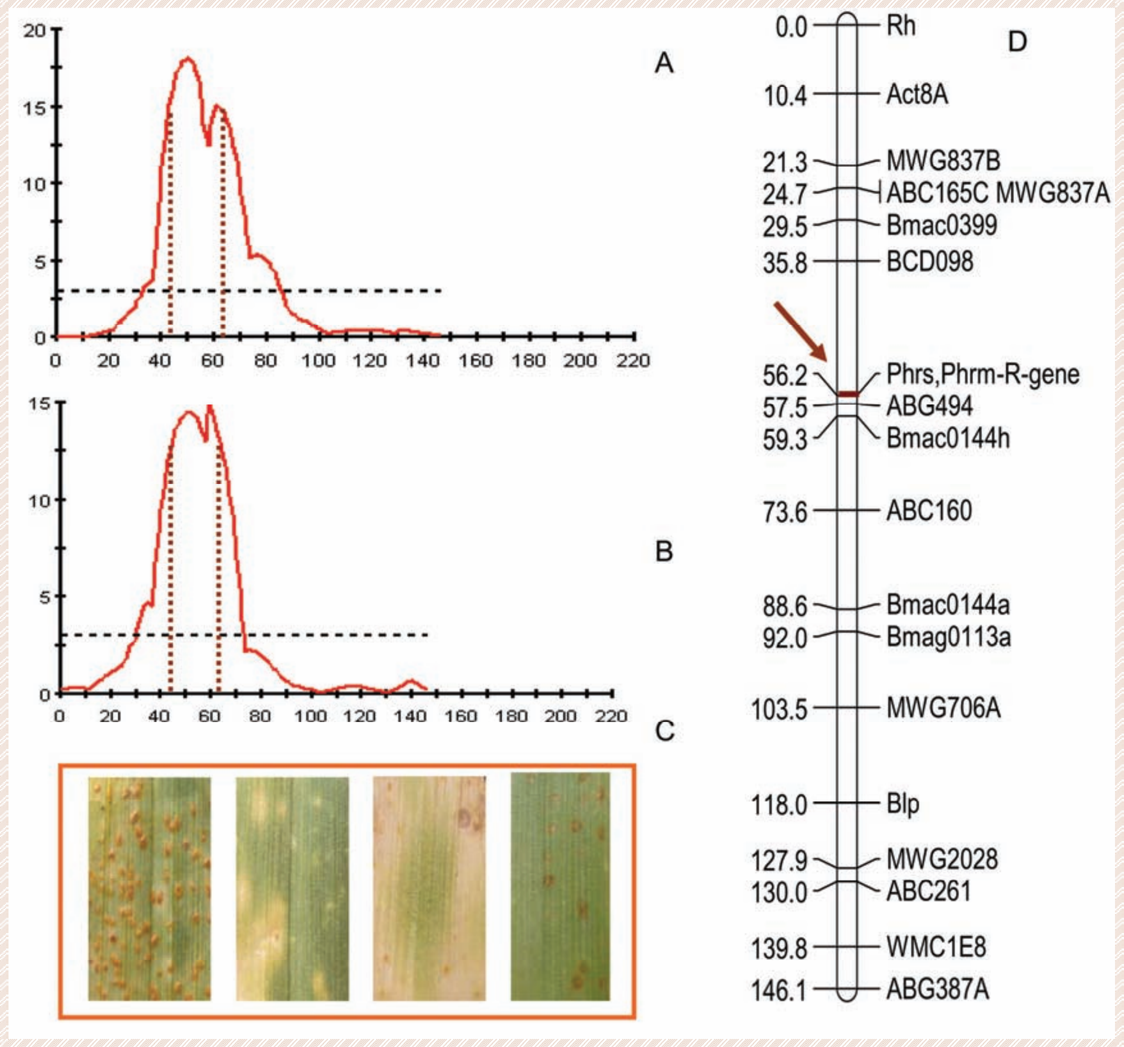


Chapter 2, Page 35, Figure 1



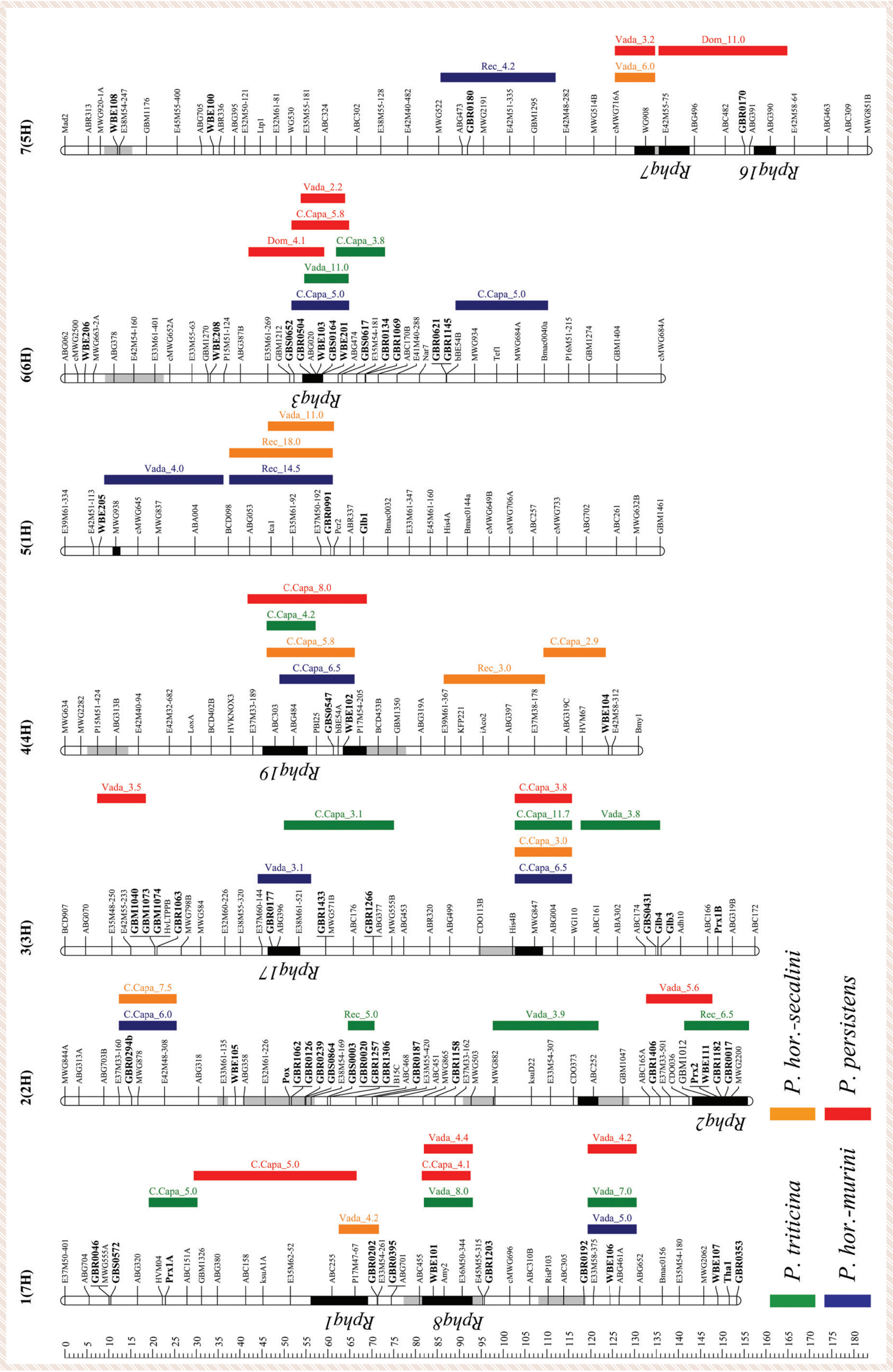
Chapter 3, Page 49,  Figure 2

# Colour figures, chapter 4



Chapter 4, Page 80, Figure 3

Chapter 4, Page 81, Figure 4 (Next page)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Rphq1
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Rphq17
Rphq19
Rphq3
Rphq7
Rphq16
C.Capa_5.0
C.Capa_4.1
Vada_4.4
Vada_4.2
Vada_8.0
Vada_7.0
Vada_4.2
Vada_5.0
C.Capa_7.5
C.Capa_6.0
Rec_5.0
Vada_3.9
Vada_5.6
Rec_6.5
Vada_3.5
C.Capa_3.1
Vada_3.1
C.Capa_3.8
C.Capa_11.7
Vada_3.8
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P. triticina
P. hor.-secalini
P. hor.-murini
P. persistens
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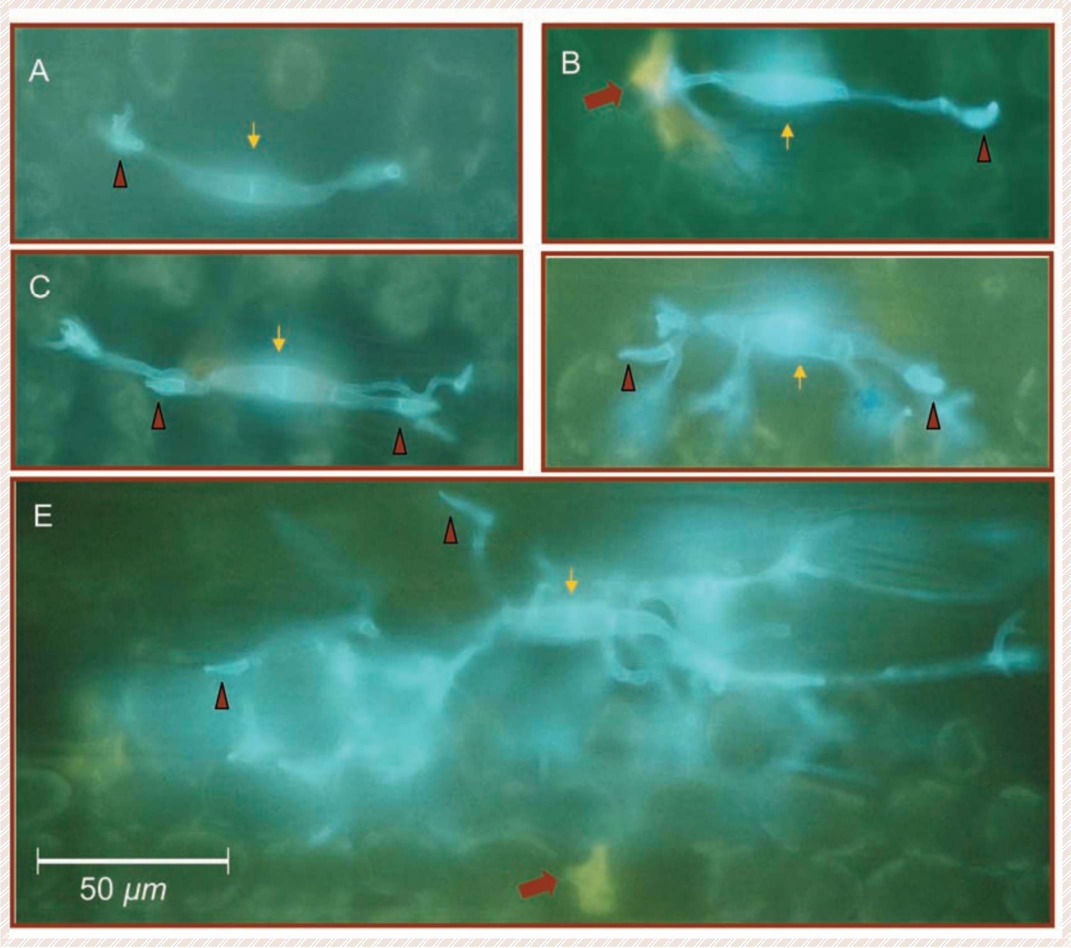
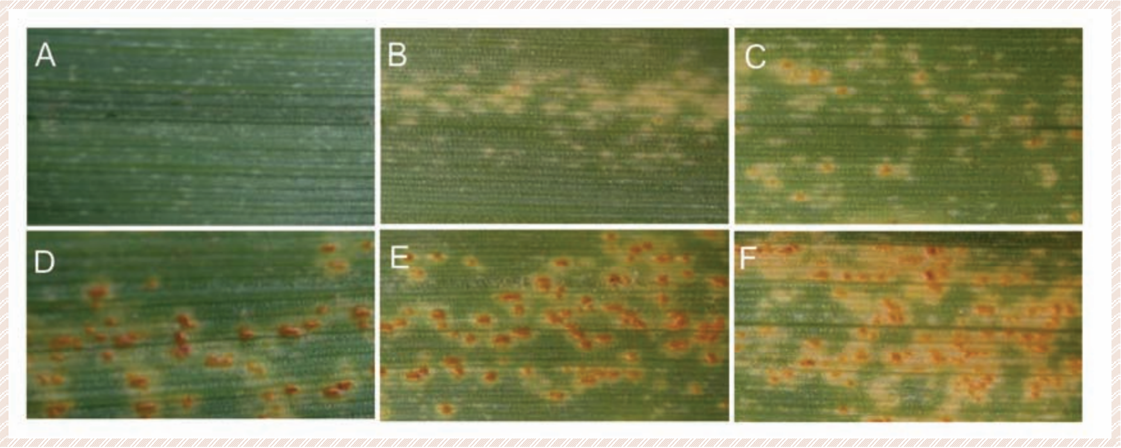
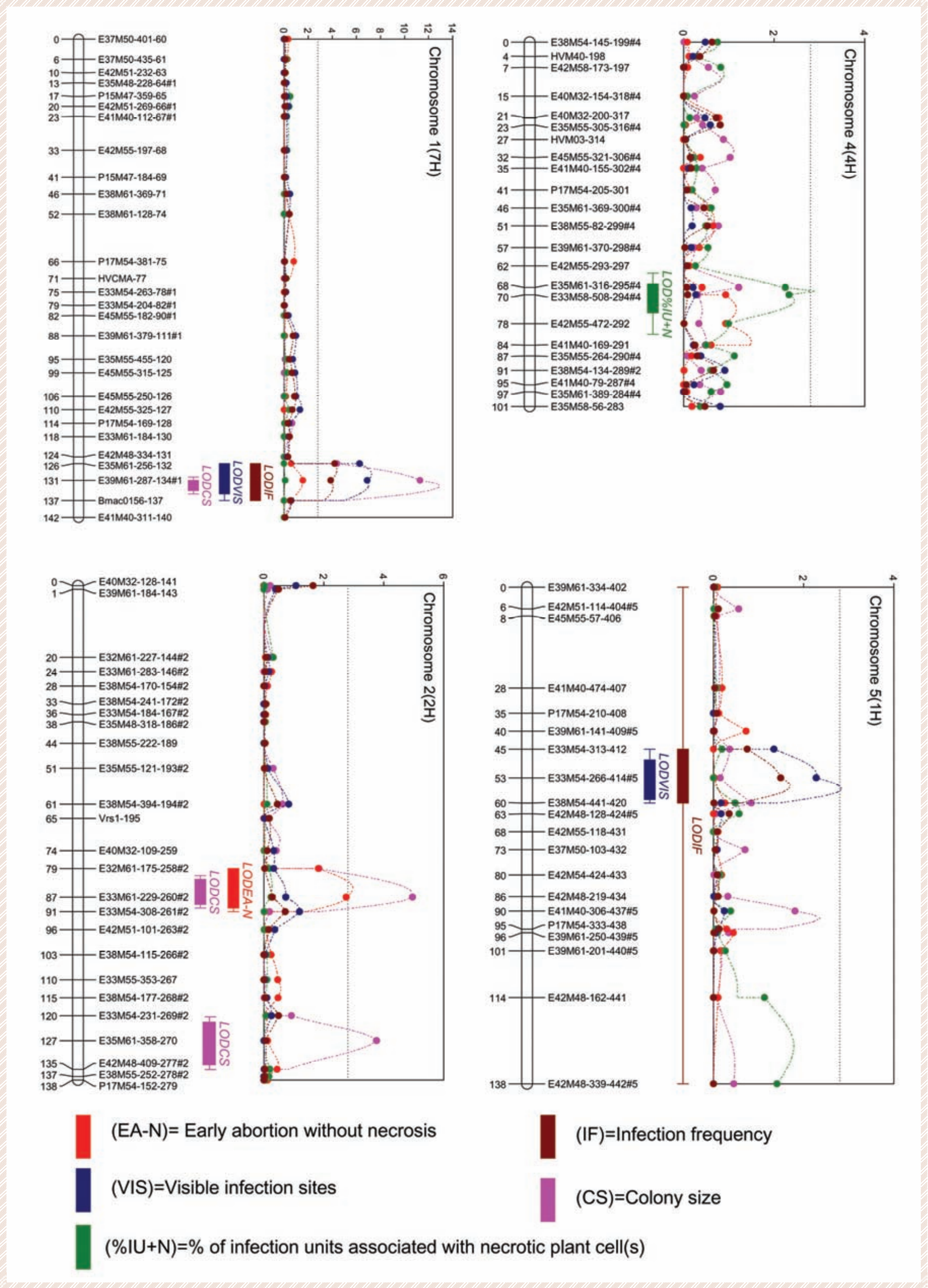
# Colour figures, chapter 5



Chapter 5, Page104, Figure 1

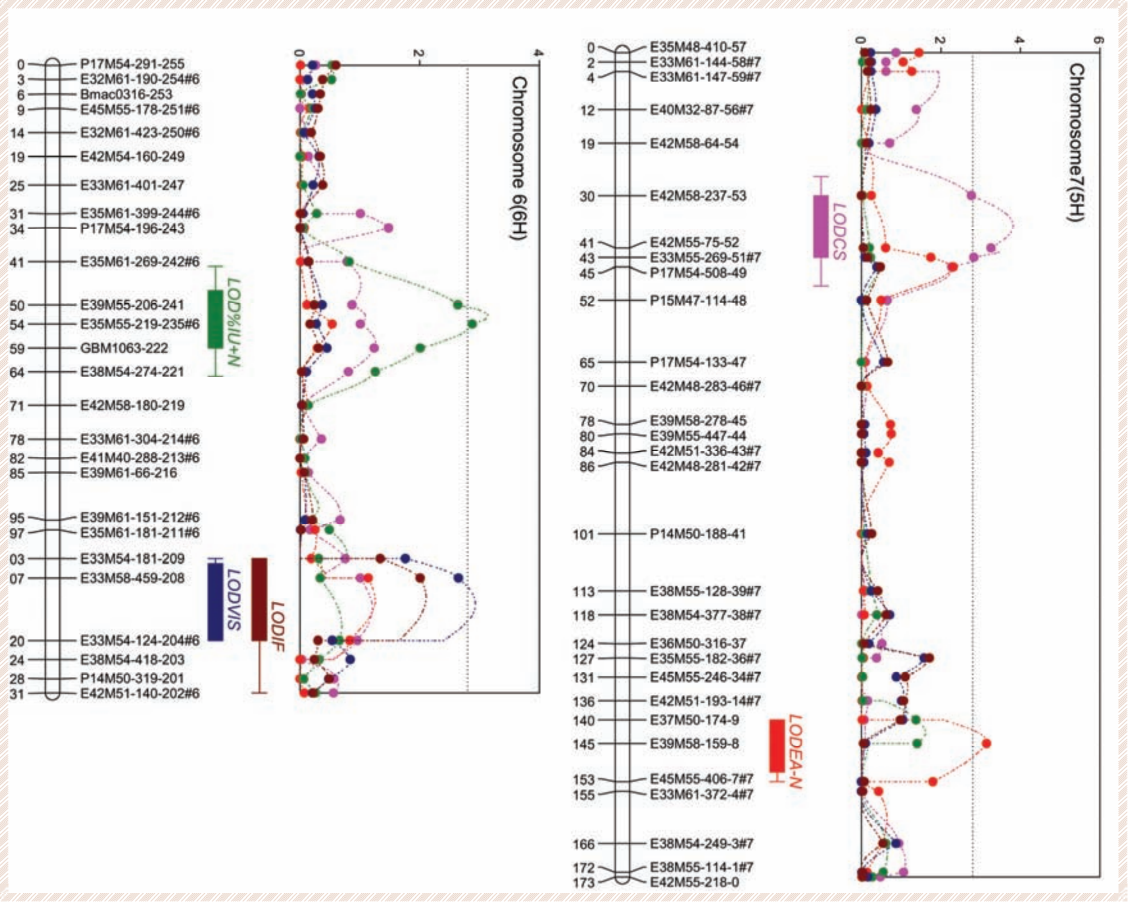


Chapter 5, Page 105, Figure 3

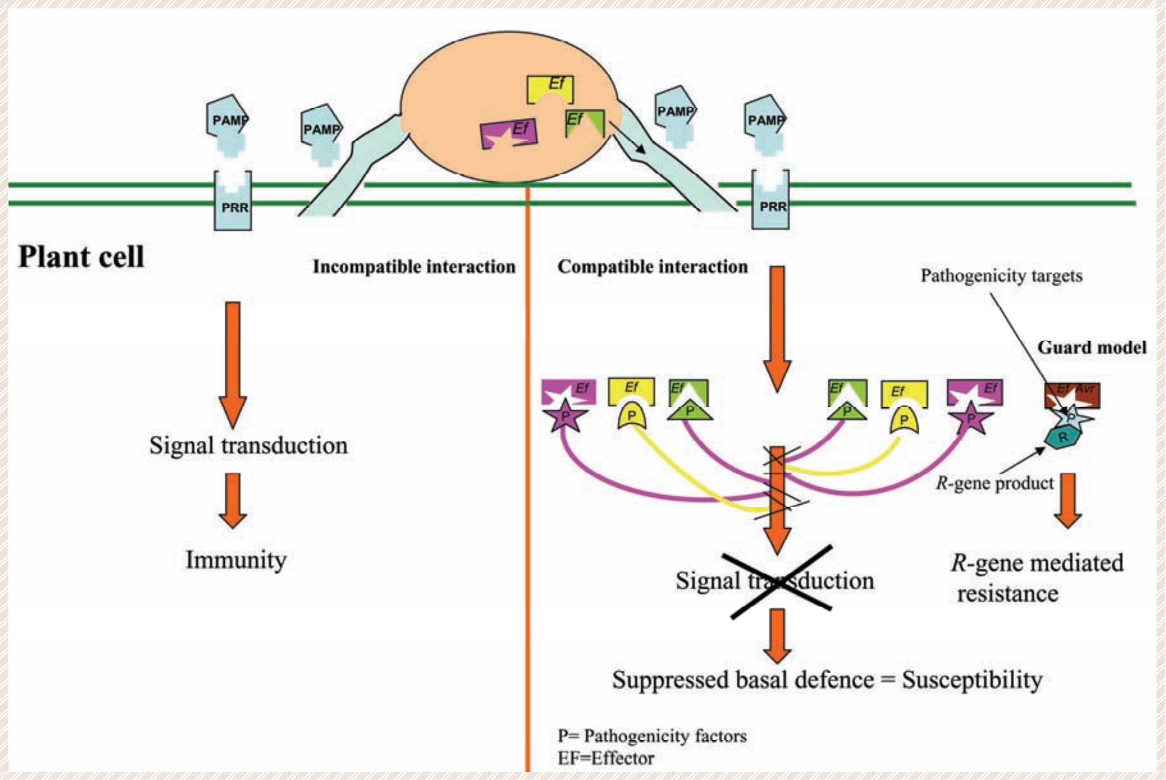


Chapter 5, Page108, Figure 4

# Colour figures, chapter 5 and 6



Chapter 5, Page 109, Figure 4 (continued)



Chapter 6, Page 120, Figure 1