Towards map-based cloning of partial resistance QTLs of barley to *Puccinia hordei*

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Chapter 1

General Introduction

General introduction

Basal defense of plants

Plants are exposed to a vast number of potential pathogens either in the phyllosphere (aerial plant part), rhizosphere (zone of influence by root system) or endosphere (internal transport system) (Berendsen et al. 2012; Bulgarelli et al. 2013; Lambers et al. 2009; Vorholt 2012). However, not all potential pathogens can invade successfully and inflict disease on plants. Plants usually possess layers of preformed and induced defense to resist the invasion of potential pathogens (Thordal-Christensen 2003).

The first line of defense in plants are the preformed physical and chemical barriers on the surface of plant cells. Surface structures such as, cuticle, trichome and cell wall provide the physical barrier which may prevent potential pathogens from penetrating into the cells (Łaźniewska et al. 2012). Various preformed chemicals, such as saponins, cyanogenic glucosides and glucosinolates, are toxic for potential pathogens (Iriti and Faoro 2009). The physical and phytochemical barriers are not full proof defenses. Some potential pathogens may have developed strategies to breach these defenses. Plants, however, have the ability to quickly detect these pathogens and trigger induced defense.

The induced defense mechanism requires the ability of plants to recognize invaders through pattern-recognition receptors (PRRs). Those well studied PRRs can be categorized into receptor-like kinases and receptor-like proteins (Liu et al. 2013; Monaghan and Zipfel 2012). The PRRs recognize invaders directly through the perception of microbe-associated molecular pattern (MAMPs) which is also known as pathogen-associated molecular pattern (PAMPs) (Ingle et al. 2006). The MAMPs/PAMPs, here on PAMPs, are conserved molecules that are vital for fitness or survival of entire groups of microbes and are not present in the host. Well described PAMPs includes chitin, peptidoglycans, lipopolysaccharides, elongation factor Tu and flagellin (Pel and Pieterse 2012). Indirect recognition of invaders is also possible through the perception of damage-associated molecular patterns (DAMPs) (Dodds and Rathjen 2010) also known as microbe-induced molecular patterns (MIMPs) (Mackey and McFall 2006). DAMPs are endogenous molecules such as plant peptides or cell wall fragments released during microbe invasion or wounding which are also known as host-derived danger signals (Boller and Felix 2009). Stimulation of PRRs will induce an immune response called PAMP-triggered immunity (PTI) (Jones and Dangl 2006). The preformed and induced defense generally is nonhost resistance which protects plants against non-adapted pathogens (Niks and Marcel 2009).

Pathogenicity

Pathogens have to negate the defenses of plants to infect plants successfully. To overcome the preformed defenses on the plant surface, pathogens have developed specialized infection structures or digestive enzymes to penetrate the physical barriers (Łaźniewska et al. 2012). Against the phytochemical defense, adapted pathogens acquired the ability to tolerate or detoxify the phytochemical compounds with antibiotic activity (Iriti and Faoro 2009). The ability of pathogens to neutralize the preformed defenses needs to be accompanied by the ability to escape or evade the recognition by the PRRs on the plant surface (Hoefle and Hückelhoven 2008; Łaźniewska et al. 2012). Escaping or evading the recognition by PRRs is necessary especially for biotrophic pathogens which need to exploit plants without inducing PTI or with effective suppression of PTI (Laluk and Mengiste 2010).

A pathogen can escape from the recognition by PRRs when the recognition domain of its PAMP is altered under selective pressure. Since PAMPs are vital for fitness or survival of pathogens, a mutation in PAMPs is likely to put pathogens in unfavorable condition for survival. However, variations have been observed in the recognition domain of PAMPs, viz. bacterial flagellin and lipopolysaccharide which impaired the recognition by PRRs without decreasing the fitness of the pathogen (Pel and Pieterse 2012). When a pathogen tries to evade the recognition by PRRs, the pathogen will produce proteins that prevent PAMP recognition by PRRs or that interferes with PTI. The proteins that interfere with PTI are called effectors which mostly interfere the downstream of defense signaling pathways after PRRs recognition of pathogens (Pel and Pieterse 2012). Effectors are "molecules secreted by plant-associated organisms that alter host-cell structure and function" (Win et al. 2012), i.e. effectors are secreted by adapted and non-adapted pathogens, and mutualistic microorganisms.

Pathogens will secrete effectors either into the apoplast or cytoplast. For successful colonization, pathogens need to secrete effectors to the right location and at proper timing to target plant defenses (Hogenhout et al. 2009; Win et al. 2012). The effectors will manipulate the operative targets in the plant to enhance pathogen fitness (van der Hoorn and Kamoun 2008). Effectors have specific operative targets in plants. Therefore, a pathogen may fail to infect a plant if the versions of the operative targets in the plant are incompatible with the effectors of the would-be pathogen. So, the targets may not be absent, but be present in a version the effector cannot address (Niks and Marcel 2009; O'Connell and Panstruga 2006; van der Hoorn and Kamoun 2008). This explains the strong host specificity of many biotrophic pathogens, i.e. pathogenic on certain plant

species, even if plant species taxonomically, physiologically and morphologically seem to be very similar (barley versus wheat).

Plants are able to recognize effectors of pathogens through their intracellular receptors which will signal the activation of effector-triggered immunity (ETI). Many resistance genes (*R* genes) encode such intracellular receptors (Dodds and Rathjen 2010; Jones and Dangl 2006). Under natural selection, pathogens will shed or will have new variant effectors to negate ETI. In turn, plants also will acquire new variance of R genes to reactivate the ETI. This evolutionary arm race continues (Jones and Dangl 2006).

The resistance confirmed by *R* genes is race specific and functions on a gene-for-gene basis (Flor 1971; Parlevliet 1983). This resistance is not durably effective (Dodds and Rathjen 2010; Niks and Marcel 2009). For example, *Puccinia striiformis* f. sp. *tritici* (*Pst*) can evolve rapidly in to new races and makes previously resistant wheat cultivars susceptible. In the United States, after 49 years from the first ever recorded *Pst* infection, now 118 *Pst* races have been documented (Chen 2005; Chen et al. 2010; Hovmøller et al. 2011). The rapid emergence of new virulent races is also true for *P. striiformis* f. sp. *hordei* on barley (Chen 2008; Wan and Chen 2011) – 82 races after 18 years from the first detection in the US. Low durability of *R* gene resistance is probably because: (1) many effectors are dispensable, (2) virulence is restored by only one arbitrary loss-of-function mutation in the cognate effector-*Avr* gene and (3) pathogens with restored virulence after mutation of the cognate *Avr* gene can reproduce much more efficiently than the avirulent genotypes, and have a tremendous selective advantage (Niks and Marcel 2009).

Genetic basis of host status in plants

A plant species is a host to a pathogen species if that pathogen can deal effectively with the basal defense mounted against them, i.e. basic compatibility is achieved. Otherwise, a plant species is a nonhost. The versions of operative targets of effectors in plants probably will determine the host status of a plant species.

Inheritance study is a strategy to identify the operative targets which determine the host status. Three different approaches are possible (Niks and Marcel 2009): (1) crossing a nonhost with a host species (Jeuken et al. 2008), (2) crossing individuals within a nonhost species with different degree of nonhost resistance (Ayliffe et al. 2011; Shafiei et al. 2007), and (3) within a marginal-host, crossing a rare susceptible individual with an accession with regular immunity (Jafary et al. 2006; 2008).

Inheritance studies by following the first approach are usually difficult because it involves interspecific crosses. The progenies obtained from interspecific crosses, if any, are associated with abnormalities which may hamper genetic studies. The second approach depends on the quantitative differences in the defense components. The quantitative differences can be relatively small which can obscure the observations and typically require laborious microscopic observations. The third approach is used in the barley-rust pathosystem (see details in the next section).

The model system: barley-rust pathosystem

Barley (*Hordeum vulgare*) is a host to barley leaf rust (*Puccinia hordei*), and a complete nonhost to most of the non-adapted rusts fungal species but a marginal-host to several others, such as *P. triticina*, *P. hordei-murini*, *P. hordei-secalini*, among others (Atienza et al. 2004). The marginal-host status of barley to some non-adapted rust fungi provides the opportunity to study the inheritance of nonhost resistance without resorting to interspecific crosses. The inheritance study assumes that the genetic basis of marginal-host status can help in the comprehension of full nonhost resistance by extrapolation.

Rare barley accessions with moderate susceptibility to *P. triticina* at seedling stage were identified. They served as genetic resources to develop an experimental line – SusPtrit. SusPtrit is, at seedling stage, not only exceptionally susceptible to *P. triticina* but also susceptible to at least nine other non-adapted rust fungi to which barley is a marginal-host (Atienza et al. 2004). Another experimental line is also available – SusPmur, which was developed using the same procedure, but under selection for increased susceptibility to *P. hordei-murini* (Atienza et al. 2004).

SusPtrit was used to develop mapping populations by crossing it with regular barley, viz. Vada (Jafary et al. 2006) and Cebada Capa (Jafary et al. 2008) which are immune to non-adapted rust fungi. The immunity of Cebada Capa and Vada inherits polygenically. Different sets of quantitative trait loci (QTLs) segregate in the two populations with few QTLs in common between the populations. Most of their QTLs confer resistance against one or two rust species which implies overlapping specificities of QTLs for resistance to non-adapted rust fungi (Jafary et al. 2006; 2008).

SusPtrit is also susceptible to *P. hordei* (Jafary et al. 2006). Hence, the Cebada Capa/SusPtrit and Vada/SusPtrit mapping populations were also used to study the inheritance of partial resistance to *P. hordei* in addition to other mapping populations of barley, viz. L94/Vada, Steptoe/Morex and Oregon Wolfe Barley (OWB). Partial resistance

to *P. hordei* is observed as low levels of infection despite a compatible infection type in the field due to a delay in epidemic build-up of *P. hordei* (Niks et al. 2011; Parlevliet 1979). Similar to nonhost resistance, partial resistance is inherited polygenically (Parlevliet 1976, 1978). To date, at least 20 QTLs against *P. hordei* were mapped in different mapping populations and seem to act according to a minor-gene-for-minor-gene model (González et al. 2012; Jafary et al. 2008; Marcel et al. 2007b; 2008; Niks et al. 2000; Parlevliet and Kuiper 1977; Qi et al. 1998; 1999).

On the high density consensus map of barley, the QTLs for nonhost resistance tend to co-localize significantly with the partial resistance QTLs (Jafary et al. 2006; 2008). The QTLs for nonhost and partial resistance also tend to coincide with barley peroxidase gene clusters (González et al. 2010). This observation suggests that partial and nonhost resistance may share some genes for resistance which supports an earlier suggestion of Zhang et al. (1994) and Hoogkamp et al. (1998).

The association between nonhost and partial resistance is also observed at microscopic level. Barley genotypes with partial resistance against *P. hordei* arrest a high proportion of infection unit at an early stage of development, coined early abortion (Niks, 1982), due to the failed attempts of haustorium formation associated to the formation of papillae. It is therefore a prehaustorial resistance, and it is not associated with a hypersensitive reaction of plant cells. The proportion of early aborted colonies is correlated to the level of partial resistance (Niks 1982, 1986). The mechanism of partial resistance is shared with, but less complete than, nonhost resistance of barley to non-adapted rust fungi (Niks 1983a, b). The evidences seem to suggest that partial resistance is a weak form of nonhost resistance (Niks and Marcel 2009; Niks et al. 2011).

The principle of partial and nonhost resistance

Many PAMPs, but not all, are highly conserved among microbes (Pel and Pieterse 2012; Thomma et al. 2011). Plausibly, *P. hordei* and other non-adapted rust fungi possess very similar or identical PAMPs. Therefore, *P. hordei* as well as non-adapted rust fungi can be recognized by the PRRs of barley and signal the activation of PTI.

P. hordei and non-adapted rust fungi will secrete effectors to suppress the PTI of barley. For haustoria-forming pathogens such as *P. hordei*, the large majority of effectors are assumed to be secreted through feeding structures called haustoria (Catanzariti et al. 2007; Voegele and Mendgen 2003, 2011). The mechanism of delivering effectors from haustoria to host cells remains a mystery but several hypothetical mechanisms have been proposed

(de Jonge et al. 2011; Kamoun 2007; Panstruga and Dodds 2009). The successfulness of effectors in manipulating the operative targets in barley will determine the intensity of PTI suppression, i.e. the level of susceptibility (Figure 1). For non-adapted rust fungi to which barley is a nonhost, the PTI of barley will not be suppressed by the effectors of non-adapted rust fungi. For some non-adapted rust fungi, barley is a marginal-host such as *P. triticina* and *P. hordei-murini* (Atienza et al. 2004). The effectors of these non-adapted rust fungi may have suppressed partly the PTI activated in some of the barley accessions, which result in some level of basic compatibility. Incomplete suppression of PTI is also expected when partially resistant barley genotypes are attacked by *P. hordei*, but for susceptible barley genotypes, the effectors of *P. hordei* fully suppress the PTI (Niks and Marcel 2009) and bring about high basic compatibility. The marginal host status and partial resistance of barley may represent the transitional stages during the evolution process of losing or acquiring host status to rust fungi.

The genes underlying the effect of resistance QTLs need to be cloned for further understanding of nonhost resistance, partial resistance and their possible association. The information can be applied for developing new crop varieties with efficient and durable resistance against different pathogens.

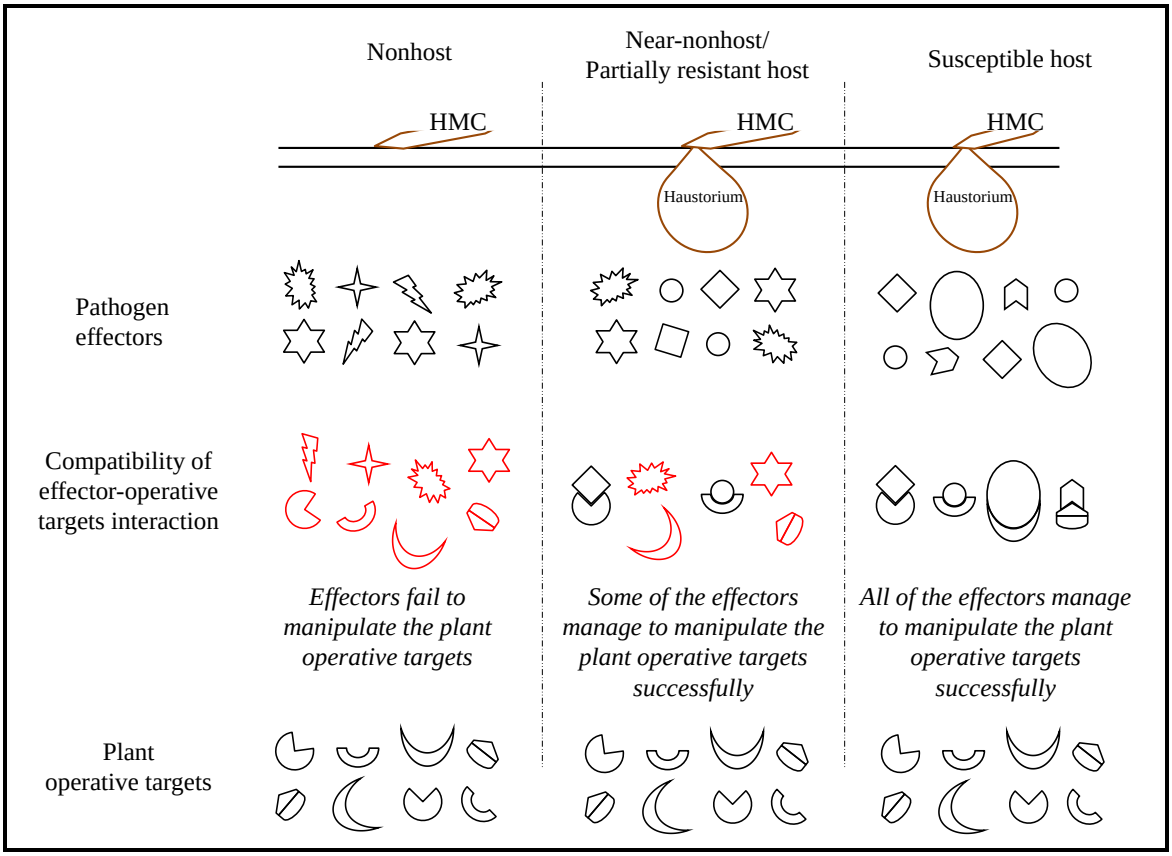

Figure 1: The ability of pathogen effectors in manipulating the plant operative targets in different barley-rust interactions.

Map-based cloning nonhost and partial resistance QTLs of barley to *P. hordei*

The identified nonhost and partial resistance QTLs of barley are presumed to be or to encode the operative targets of the pathogen's effectors (van der Hoorn and Kamoun 2008). They are expected to differ not only between plant species but also between members of a plant species as indicated by the different set of QTLs mapped for nonhost and partial resistance of barley in different mapping populations, with few shared QTLs when challenged with a particular rust pathogen (González et al. 2012; Jafary et al. 2008; Marcel et al. 2007b). Consequently, one plant genotype can be a more suitable host individual than another genotype of the same plant species, provided that the effectors-operative targets interaction is specific. The specificity of effectors-operative targets interaction is supported by the fact that the specificity of QTL effects is observed both for nonhost and for partial resistance of barley (Figure 3) (González et al. 2012; Jafary et al. 2008).

Partial and nonhost resistance are probably resting on similar principles. Hence, studying partial resistance of barley to *P. hordei* is relevant to understand nonhost resistance of barley to the related non-adapted rust fungi and *vice versa*. In barley over 100 QTLs for partial and nonhost resistance to rust fungi and powdery mildew have been mapped in different barley mapping populations. We need to identify and isolate the genes that are responsible for the effect of these QTLs because, only then we can know and understand the molecular basis of partial and nonhost resistance.

To begin with, the QTLs identified should be validated by using near isogenic lines (NILs). NILs allows the QTL effect to be tested in a uniform genetic background without the interference of other resistance QTLs. These isogenic lines are developed by recurrently backcrossing of the QTL donor to a recurrent recipient parent to "Mendelize" the QTL (Alonso-Blanco and Koornneef 2000). The process of developing NILs can be accelerated with the assistance of molecular markers (Collard et al. 2005; Moose and Mumm 2008). The prefered barley genotype to be used as the recipient parent is SusPtrit. In SusPtrit genetic background, each QTL effect can be tested against adapted and non-adapted rust fungi.

QTLs which are identified in a mapping study are usually positioned into a large interval, depending on the mapping population size and the resolution of the linkage map. Before the identification and cloning of a gene explaining a QTL is feasible, the QTL needs to be fine-mapped into a smaller interval. NILs are usually used for QTL fine-mapping by creating multiple sub-NILs which carry different recombination points at the QTL marker support interval (Han et al. 1999; Marcel et al. 2007a; Xue et al. 2010; Zhou et al. 2010).

The QTL interval after fine mapping needs to be sufficiently small to allow the construction of a Bacterial Artificial Chromosomes contig covering the region i.e., physical mapping. The co-segregating markers of the targeted QTLs are used to fish BAC clones from the BAC libraries and also act as anchor to construct the BAC contig. Up to now, BAC clones can be fished out from BAC libraries which are made available for four barley genotypes – Morex (Schulte et al. 2011; Yu et al. 2000), Cebada Capa (Isidore et al. 2005), Haruna Nijo (Saisho et al. 2007), and a doubled haploid barley line CS134 derived from Clipper/Sahara-3771 (Shi et al. 2010). The genomic sequence of Morex (The International Barley Genome Sequencing Consortium 2012) is also valuable for assisting in physical mapping of QTLs. The genomic libraries of the four barley genotypes mentioned above may not contain the genes for targeted QTLs if they are not the QTL donor. Therefore, a BAC library should be constructed for the barley accession that was donor of the QTL of interest (Saisho et al. 2007).

The sequence information of the BAC clones will allow the identification of candidate genes for QTLs. Validation of candidate genes may possibly be conducted via either transient overexpression and silencing through virus-mediated overexpression and virus-induced gene silencing (Lee et al. 2012). However, each transiently transformed plant is unique and makes replicated disease tests not possible to validate candidate genes for their effect on quantitative resistance as are relevant in nonhost and partial resistance of barley. Stable transformation is another option for candidate genes validation. For stably transformed plants, multiple identical plants with the transgene are available because the transgene is transmitted from one generation to another through grains. Hence, disease tests with replications are possible. Unfortunately, the efficiency of stable transformation in barley is genotype-dependent (Hensel et al. 2008). Functional study of candidate genes on the appropriate barley genotypes, such as SusPtrit, can possibly be restricted by the transformation efficiency of the barley genotype. One must have an alternative for validation of candidate genes if the barley genotype of interest is not transformable.

The scope of thesis

This thesis is working on map-based cloning of QTLs for nonhost and partial resistance. We got to know SusPtrit is a valuable barley genotype for nonhost and partial resistance study, but it is not amenable to *Agrobacterium*-mediated transformation. Validation of candidate genes is then not possible, although preferable, on SusPtrit. In Chapter 2, we aimed to develop a new barley experimental line which can replace SusPtrit as a valuable line for studies on nonhost and partial resistance, and can be used to validate candidate genes.

From the abundantly available QTLs for partial and nonhost resistance mapped in different mapping populations, five QTLs with major effect for partial resistance and one QTLcontributing to nonhost resistance are selected for our studies (Table 1). In Chapter 3, we aimed to develop NILs for the selected QTLs by using SusPtrit as recurrent parent. We expected to confirm the effect of each QTL against a selection of adapted and non-adapted rust fungi, in a uniform genetic background.

Table 1: The QTLs selected to study of partial and nonhost resistance

*QTL	Chromosome	Explained phenotypic variance	Donor	Mapping population	References
Rphq2	2H	36%	Vada	L94/Vada	Qi et al. (1998)
Rphq3	6H	17%	Vada	L94/Vada	Qi et al. (1998)
Rphq4	5H	25%	Vada	L94/Vada	Qi et al. (1998)
Rphq11	2H	34%	Steptoe	Steptoe/Morex	Marcel et al. (2007b)
Rphq16	5H	33%	Dom	OWB	Marcel et al. (2007b)
Rnhq	7H	30%	Vada	L94/Vada	Niks et al. (2000)

* *Rphq* – QTL for partial resistance, *Rnhq* – QTL for nonhost resistance

Among the QTLs in Table 1, only *Rphq2* and *Rphq4* are fine-mapped into a sufficiently small interval for physical mapping [(Marcel et al. 2007a); Y. Wang and X. Qi, Institute of Botany, Chinese Academy of Sciences, Beijing, China]. In Chapter 4, we aimed to fine-map another two QTLs, *Rphq11* and *Rphq16*, to get them ready for physical mapping. For *Rphq2*, we aimed to accomplish physical mapping and then to obtain the sequence information of the region from Vada and SusPtrit to identify candidate genes explaining the resistance effect of the QTL (Chapter 5).

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Chapter 2

Golden SusPtrit - a genetically well transformable barley line for studies on the resistance to rust fungi

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Golden SusPtrit – a genetically well transformable barley line for studies on the resistance to rust fungi

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Abstract

Nonhost and partial resistance to *Puccinia* rust fungi in barley are both polygenically inherited. These types of resistance are principally prehaustorial, show a high diversity between accessions of the plant species and are genetically associated. To study nonhost and partial resistance, as well as their association, the candidate gene(s) for resistance must be cloned and tested in susceptible material where SusPtrit would be the line of choice. Unfortunately, SusPtrit is not amenable to *Agrobacterium*-mediated transformation. In this study, a new bi-parental doubled haploid (DH) population (n=122) was created by crossing SusPtrit with Golden Promise to develop a 'Golden SusPtrit', i.e., a barley line combining SusPtrit's high susceptibility to non-adapted rust fungi with the high amenability of Golden Promise for transformation. At a logarithm of the odds (LOD) threshold of 10, a linkage map was constructed using 686 SNPs obtained from the ILLUMINA iSelect 9k barley infinium chip. The total map length is 1175 cM. Quantitative trait locus (QTL) mapping identified nine genomic regions occupied by resistance QTLs against four non-adapted rust fungi and *P. hordei* isolate 1.2.1 (*Ph*.1.2.1). Among the nine genome regions, five regions conferred resistance to different rust fungi, suggesting that the responsible genes have effects on multiple rust species. Four DH lines were selected for an *Agrobacterium*-mediated transformation efficiency test. They were among the 12 DH lines most susceptible to the tested non-adapted rust fungi. The most efficiently transformed DH line was SG062N (11 to 17 transformants per 100 immature embryos). The level of non-adapted rust fungi infection on SG062N is either similar to or higher than the level of infection on SusPtrit. Against *Ph*.1.2.1, the latency period conferred by SG062N is as short as that conferred by SusPtrit. SG062N, designated 'Golden SusPtrit', will be a valuable experimental line that could replace SusPtrit in future nonhost and partial resistance studies, especially for stable transformation using candidate genes that may be involved in rust resistance mechanisms.

Keywords: DH lines, *Agrobacterium*-mediated, QTL mapping, *Puccinia*

Introduction

Nonhost resistance implies immunity of all members of a plant species against a potential pathogen species (Niks et al. 2011). The resistant plant species is referred to as nonhost, and the would-be pathogen species is referred to as non-adapted pathogen. Classification of a plant species as nonhost or host against certain potential pathogen species is not easy (Niks 1987; Niks et al. 2011). Some plant species have few accessions with an intermediate level of susceptibility to a particular pathogen. Such plant species are referred to as having a near-nonhost status (Niks 1987; Niks et al. 2011). The rare susceptibility of those few accessions may occur only at the seedling stage or under a very severe infection pressure (Niks 1987). Barley appears to be a near-nonhost to several non-adapted rust fungal species, such as *Puccinia triticina* and *P. hordei-murini*. Through the accumulation of genes for susceptibility to *P. triticina* from rare barley accessions with moderate susceptibility at the seedling stage, an experimental barley line called SusPtrit was developed. This line is extraordinarily susceptible to several grass rusts that are non-adapted to barley (Atienza et al. 2004). SusPtrit facilitated the development of the barley-*Puccinia* rust fungus model system to study the inheritance of nonhost resistance in plants. Two mapping populations – Vada/SusPtrit (V/S) and Cebada Capa/SusPtrit (C/S) – were developed using SusPtrit as one of the parents (Jafary et al. 2006; 2008).

Partial resistance is a type of host resistance that slows down epidemic development despite a compatible infection type (Niks et al. 2011). On partially resistant barley, the pathogen realizes a lower infection frequency, has a lower sporulation rate and has a longer latency period (Parlevliet 1979). The partial resistance of barley against *P. hordei* (the adapted rust fungus) is one of the most extensively studied cases of this type of resistance [reviewed by St. Clair (2010)].

Nonhost and partial resistance to *Puccinia* rust fungi in barley are both polygenically inherited. Nonhost and partial resistance quantitative trait loci (QTLs) against different rust fungi have been mapped in different bi-parental mapping populations (Qi et al. 1998; 1999; Jafary et al. 2006; 2008; Marcel et al. 2007; 2008). Nearly all of the reported QTLs were effective against only one to three rust species, and, hence, were rust species-specific, and some QTLs were even rust isolate-specific (Atienza et al. 2004; González et al. 2012; Jafary et al. 2006; Marcel et al. 2008). The QTLs for nonhost resistance to rust fungi tended to map in the same genomic regions as the QTLs for partial resistance to *P. hordei* (González et al. 2010; Jafary et al. 2008). There is evidence that nonhost and partial resistance of barley to rust fungi share important features: both are principally prehaustorial (Hoogkamp et al. 1998; Niks 1983; Niks and Marcel 2009), show a high

diversity between accessions of the plant species (Qi et al. 2000; Jafary et al. 2006; 2008) and are genetically associated (Hoogkamp et al. 1998; Zhang et al. 1994). The association was also shown in a transcriptional study wherein barley (cv. Ingrid) was exposed to a pair of adapted and non-adapted rust fungi and a pair of adapted and non-adapted powdery mildews. The induced transcriptional changes overlapped not only for the responses of cv. Ingrid to the adapted and non-adapted fungal pathogen but also for the responses to the two different pathosystems (Zellerhoff et al. 2010).

SusPtrit is useful for studying the association between nonhost and partial resistance of barley. This valuable experimental line is not only susceptible to *P. triticina* and several other non-adapted rust fungi but also extremely susceptible to the adapted *P. hordei* (Atienza et al. 2004; Jafary et al. 2006). QTLs conferring nonhost and partial resistance in other barley accessions have been introgressed into SusPtrit to develop near isogenic lines (NILs) (Chapter 3, this thesis). These QTL-NILs allow the testing of individual QTLs against non-adapted rust fungi and *P. hordei* without the interference of other QTLs. Subsequently, sub-NILs are developed to fine-map the responsible gene(s) to a small genetic window, which may be spanned by bacterial artificial chromosome (BAC) clones. The candidate gene(s) explaining the resistance QTLs are identified from the sequenced BAC clones, isolated and verified through complementary functional studies.

Functional studies of candidate genes may be conducted via either transient overexpression or transient silencing of genes by particle bombardment, as applied in the studies of candidate resistance genes against powdery mildew (Douchkov et al. 2005; Miklis et al. 2007). For candidate genes of barley against rust fungi, this approach is not feasible because the carriers of the gene constructs cannot reach the mesophyll cells, which are the main cell types that rust fungi target. Transient overexpression and silencing in barley are also feasible through virus-mediated overexpression (VOX) and virus-induced gene silencing (VIGS) (Lee et al. 2012). These approaches, however, are difficult for quantitative resistance because phenotyping with replication is not possible as each individually treated plant is unique. Stable transformation is another alternative for the functional study of candidate genes, although also this approach has its caveats and limiations. Primary transformants transmit the transgene to their offspring through the grains. This transmission to offspring is important because it allows the transgenic line to have multiple identical plants with the transgene, which are necessary to test the line for its level of partial resistance. Stable transformation is possible in barley, but the efficiency of barley transformation is genotype-dependent (Hensel et al. 2008). To date, barley cv. Golden Promise (GP) is the first choice for the standard method of *Agrobacterium*-mediated transformation using immature embryos (IEs). The transformation efficiency of GP can be as high as 86.7 transformants per 100 IEs when the co-cultivation medium is

supplemented with L-cysteine and acetosyringone and the *Agrobacterium* strain AGL-1 is used (Hensel et al. 2008).

In a functional study, the resistance allele can be silenced in a resistant plant. In polygenic nonhost resistance, however, an immune plant may have several redundant genes for resistance. A barley accession, such as cv. Vada, may be immune to most, if not all, non-adapted rust fungi, and the resistance is encoded by several genes (QTLs) (Jafary et al. 2006). Silencing one of the resistance QTLs in Vada might be insufficient to compromise the nonhost resistance enough to alter the immunity to some level of susceptibility. Therefore, testing of the candidate genes for resistance in a susceptible line may be a better option when SusPtrit is the line of choice. SusPtrit is, unfortunately, not amenable to *Agrobacterium*-mediated transformation using the established procedure (Hensel et al. 2008) with minor modification (FKS Yeo and G Hensel, unpublished data). Although the non-inoculated IEs of SusPtrit appeared to be responsive to callus induction media, the callus growth of *Agrobacterium*-inoculated IEs ceased after approximately two weeks. A similar situation was observed by WA Harwood on cultivars Optic, Oxbridge and Tipple [unpublished data, reviewed by Harwood (2012)].

In this study, a new bi-parental population of doubled haploids was created by crossing SusPtrit with GP. The objective was to find a line, 'Golden SusPtrit,' that combines the susceptibility of SusPtrit to non-adapted rust fungi with the amenability of GP for *Agrobacterium*-mediated transformation. In addition, the population was used to map nonhost and partial resistance QTLs, which were compared with QTLs identified earlier in other mapping populations.

Materials and Methods

Development of recombinant doubled haploids and general outline

SusPtrit was crossed with cv. GP. F_1 grains were sent to PLANTA Angewandte Pflanzengenetik und Biotechnologie, Germany to develop a DH population. The haploid/DH plantlets were regenerated from embryogenic pollen cultures, with the DH plants obtained through spontaneous genome duplication. Fertility of spikes was used to indicate the restoration of the diploid condition. The population was inoculated with selected non-adapted and adapted leaf rust fungi, and QTLs were mapped (see below). The most susceptible DH lines were selected and tested for amenability to *Agrobacterium*-mediated transformation.

Whole genome genotyping and linkage map construction

Fresh young leaves of seedlings of the SusPtrit, GP and the DH populations were used to extract genomic DNA according to the CTAB-based protocol of Stewart and Via (1993). The DNA samples were sent for whole genome genotyping (TraitGenetics GmbH, Gatersleben, Germany). Genotyping was performed using an ILLUMINA iSelect 9k barley infinium chip which carries 7,864 SNPs. JoinMap 4.1 (van Ooijen 2006) was used for linkage analysis and map construction. Map distance calculations were made based on Kosambi's mapping function. The linkage groups were assigned to their respective chromosomes based on the linkage map for the Morex/Barke recombinant inbred line population, which was previously genotyped using the same chip (Comadran et al. 2012). The linkage map was used for QTL mapping. The biggest gap in the linkage map was 16 cM on chromosome 6H.

Mapping QTLs for nonhost and partial resistance at the seedling plant stage

Four non-adapted leaf rust fungi, *P. hordei-murini* isolate Rhenen (*Phm*.R), *P. hordei-secalini* isolate France (*Phs*.F), *P. persistens* isolate Wageningen (*Pp.*W), and *P. triticina* isolate Flamingo (*Pt*.F), as well as one adapated leaf rust fungus, *P. hordei* isolate 1.2.1 (*Ph*.1.2.1), were used for disease tests. Inoculums of these pathogens were produced on their respective host plants.

The disease tests were carried out in a greenhouse. The seedlings of the SusPtrit/GP (S/G) population were inoculated with the above-mentioned leaf rust fungi in consecutive experiments. For each leaf rust fungus, three consecutive disease tests (series) were performed. In each series, every DH line of the S/G population was represented by one seedling.

Grains of the DH lines, SusPtrit and GP were sown in boxes (37 x 39 cm). Twelve-day-old seedlings with unfolded primary leaves were fixed horizontally with the adaxial side facing up. For non-adapted leaf rust fungi, seven milligrams of spores per box per series were used, amounting to approximately 400 spores deposited per cm^2. For *Ph*.1.2.1, one milligram of spores (approximately 60 spores per cm^2) per box was applied. The spores were diluted with 10 times their volume of lycopodium spores before inoculating the box in a settling tower (Niks et al. 2011). The inoculated boxes were incubated overnight for eight hours in the dark in a dew chamber set at 18°C with 100% relative humidity. Following incubation, the boxes were moved to a greenhouse compartment set at 20 ± 3°C with 70% relative humidity.

For non-adapted leaf rust fungi, the infection frequency (IF; number of pustules per cm^2 leaf area) was scored at 12 days post-inoculation. For *P. hordei,* the latency period (LP50S) of the leaf rust was scored and calculated as described by Parlevliet (1975). Relative infection frequency (RIF) and relative latency period (RLP50S) were calculated relative to the average IF and LP50S, respectively, of all SusPtrit seedlings in each series.

The RIF and RLP50S data were used to map QTLs using MapQTL®6 (van Ooijen 2009). The logarithm of the odds (LOD) threshold to declare a QTL was determined by a permutation test. Only QTLs mapped in at least two of the three series and in the data averaged over the three series were reported. The confidence interval of a QTL is the estimated LOD-2 support interval. When the LOD-2 support interval of two QTLs overlapped, either between QTLs mapped in the different series of one rust fungus or of different rust fungi, they were considered one QTL. Declared nonhost resistance QTLs were designated as *Rphmq*/*Rphsq*/*Rppq*/*Rptq* followed by a number. Partial resistance QTLs to *P. hordei* were designated as *Rphq* (host QTL) followed by a number.

Selection of S/G DH lines for Agrobacterium-mediated transformation

The S/G DH lines were ranked based on their RIFs in the first series of disease tests against *Phs*.F and *Pt*.F. Preliminary data on the infection levels of *Phm*.R were available and were used to provisionally rank the DH lines for selection of the most susceptible DH lines. The ten most susceptible DH lines according to the ranking, with an infection severity similar to SusPtrit against the three rust fungi, were selected for *Agrobacterium*-mediated transformation efficiency tests.

Of the ten selected DH lines, four were used to test the amenability to genetic transformation. Growth of donor plants and the transformation protocol were essentially the same as described elsewhere (Hensel et al. 2009). Briefly, developing caryopses were harvested at 12-16 days post-pollination and surface sterilized. The IEs were excised and either pre-cultured on liquid barley co-culture medium for one day or directly inoculated with the *Agrobacterium tumefaciens* strain AGL-1 harboring the plasmid pGH215. The plasmid contains the selectable marker gene *HYGROMYCIN PHOSPHOTRANSFERASE* (*HPT*) driven by the doubly enhanced *CaMV 35S* promoter and the synthetic *green fluorescent protein* (*gfp*) gene under the control of the maize *UBIQUITIN 1* promoter with first intron. The agrobacteria were grown, inoculated and co-cultured with the IEs (Hensel et al. 2009). After co-culture, the IEs were transferred to barley callus induction medium supplemented with either 20 or 50 mg/L Hygromycin B (Roche, Mannheim, Germany) to induce calli under selective conditions. After 2 rounds of 2-week incubations in the dark at

24°C, the calli were transferred to barley regeneration medium supplemented with 25 mg/L Hygromycin B, then transferred to light. All regenerants (T_0) were transferred to soil, and genomic DNA was isolated and used for PCR with primers specific for the selectable marker and reporter genes, as described below (Table 1). The transformation efficiency was evaluated based on the number of independent transgenic regenerants per 100 IEs (transgenics/100 IEs).

Table 1: Primer sequences used for the PCR analysis of putative transgenic regenerants.

Primer	Sequence 5' – 3'	Primer binding site
35S-F2-Catrin	CATGGTGGAGCACGACACTCTC	Bp 331-352 of enhanced *CaMV 35S* promoter
Bie475	TTTAGCCCTGCCTTCATACG	Bp 1421-1440 of *ZmUBI1* promoter
GH-GFP-F1	GGTCACGAACTCCAGCAGGA	Bp 680-661 of *gfp* gene
GH-HYG-F1	GATCGGACGATTGCGTCGCA	Bp 896-877 of *HPT* gene

DNA gel blot analysis and analysis of reporter gene expression

Twenty one transgenic plants (T_0) from the most efficiently transformed line, SG062N, were randomly selected and subjected to DNA gel blot analysis to determine the transgene copy number. At least 25 µg of genomic DNA was digested with *Hin*dIII, separated by agarose gel electrophoresis and blotted onto a Hybond N membrane. A gene-specific probe (*GFP* or *HPT*) was labeled with DIG, as recommended by the supplier (Roche, Mannheim, Germany). The 21 T_0 plants of SG062N produced 21 T_1 populations by selfing. Between 21 and 59 T_1 grains harvested from the 21 transgenic T_0 plants of SG062N, as well as from the SG062N wild type control, were surface sterilized, germinated on solid B5 medium (Gamborg et al. 1968) and incubated under a 16/8 h light/dark regime at 24°C. After ten to fourteen days, root tips were screened for GFP fluorescence using a Leica MZFLIII fluorescence microscope equipped with the GFP Plant filter set (Leica Microsystems, Wetzlar, Germany). Genomic DNA of four plants from each T_1 population, preselected by the presence/absence of GFP fluorescence in the root tip, was extracted from ~100 mg of snap-frozen leaf tissue, as described in Pallotta et al. (2000). Multiplex-PCR was designed based on the amplification of 100 ng of template primed by the sequences listed in Table 1. Amplicons were separated by agarose gel electrophoresis and visualized by staining with Ethidium bromide. From each of the 21 T_1 populations, three plants that tested positive in the PCR assays, as well as one plant that had lost the transgene via segregation, were subjected to DNA gel blot analysis, as

described above, to estimate the transgene copy number and to characterize the integration site(s) regarding linked/unlinked copies.

Results

DH population and linkage map construction

Of the 308 *in vitro* cultured plantlets, 137 survived and were fertile, thus producing grains. Through whole genome genotyping, duplicate genotypes were identified and eliminated, resulting in a population of 122 unique DH lines.

From the 7,864 SNPs on the chip, 2,943 SNP markers were polymorphic between SusPtrit and GP. Before linkage analysis was performed, 2,257 markers with identical segregation patterns were removed from the data set. We used 686 markers to construct the linkage map. At LOD threshold 10, seven linkage groups corresponding to seven barley chromosomes were detected (Supplemental Figure 1). The total map length was 1,175 cM. The map length of individual linkage groups ranged from 130 cM (4H, 84SNP markers) to 202 cM (5H, 122 SNP markers). The marker order on the linkage map of S/G is in agreement with the linkage maps of V/S and C/S, which were also genotyped using the ILLUMINA iSelect 9k barley infinium chip (unpublished data). Of the 686 SNP markers, 351 (51%) exhibited segregation patterns that significantly deviated from the expected 1:1 ratio. Segregation was skewed towards the SusPtrit allele for 213 markers and towards the GP allele for 138 markers. The markers showing distorted segregation occurred in clusters (14 clusters). On linkage groups corresponding to chromosomes 2H and 3H, all of the distorted segregation was skewed towards the SusPtrit allele. In contrast, all of the distorted segregation for markers on chromosome 4H was skewed towards the GP allele.

Disease resistance of S/G recombinants against non-adapted and adapted leaf rust fungi.

SusPtrit is susceptible to all four non-adapted leaf rust fungi and to *Ph*.1.2.1. GP is immune to all four non-adapted leaf rust fungi and causes 5% higher RLP50S (approximately eight hours longer LP) of *Ph*.1.2.1 than SusPtrit. Segregation in the level of resistance among the S/G population was quantitative, suggesting a polygenic inheritance pattern (Figure 1). The infection levels observed in the S/G population with the four non-adapted leaf rust fungi ranged from immune to either as susceptible as or more

susceptible than SusPtrit. Correlations between the average RIF values for the four non-adapted rust species ranged from 0.5 to 0.7. These values indicate a moderate association in the genetic basis of resistance to these four rust species. This result is consistent with the conclusion made by Jafary et al. (2006; 2008) that genes underlying nonhost resistance have overlapping specificities. Due to the moderate association of susceptibilities to different rust fungi, we found 21 S/G DH lines that were, for more than one non-adapted rust fungi, among the 20 most susceptible lines (Table 2).

Generally, the pustules formed by the non-adapted rust fungi on the SG lines were of the compatible type, i.e., they were not associated with chlorosis or necrosis. This was true even for the lines on which few pustules appeared, i.e., lines with fair levels of resistance. For all non-adapted rust types, fewer than 15 lines with *Pt*.F, *Phs*.F, or *Phm*.R and fewer than 30 lines tested with *Pp*.W displayed some chlorosis or necrosis; however, in most cases, these reactions were inconsistent over experimental runs.

Nonhost and partial resistance QTL mapping in the S/G population

For each non-adapted rust fungus, the results obtained in the three disease test series correlated well (r = 0.6-0.9); however, the correlation between the three disease test series was low for *Ph*.1.2.1 (r = 0.2-0.4). Based on permutation tests, a LOD threshold between 2.9 and 3.2 was used for QTL declaration in each mapping attempt.

We found two nonhost resistance QTLs for *Phm*.R, four for *Pt*.F, five for *Phs*.F and six for *Pp*.W (Table 3). As previously described by Jafary et al. (2008), declaring QTLs from LOD profiles may become arbitrary when multiple peaks are observed in the same genomic region. Such was the case for *Pp-nhq5* and *Pp-nhq6* located on chromosome 7H (Table 3, Figure 2), which we chose to report as two QTLs. These QTLs co-localized with two QTLs against *Phs*.F, *Phs-nhq4* and *Phs-nhq5*, which were indicated by two clearly separated peaks in the LOD profile.

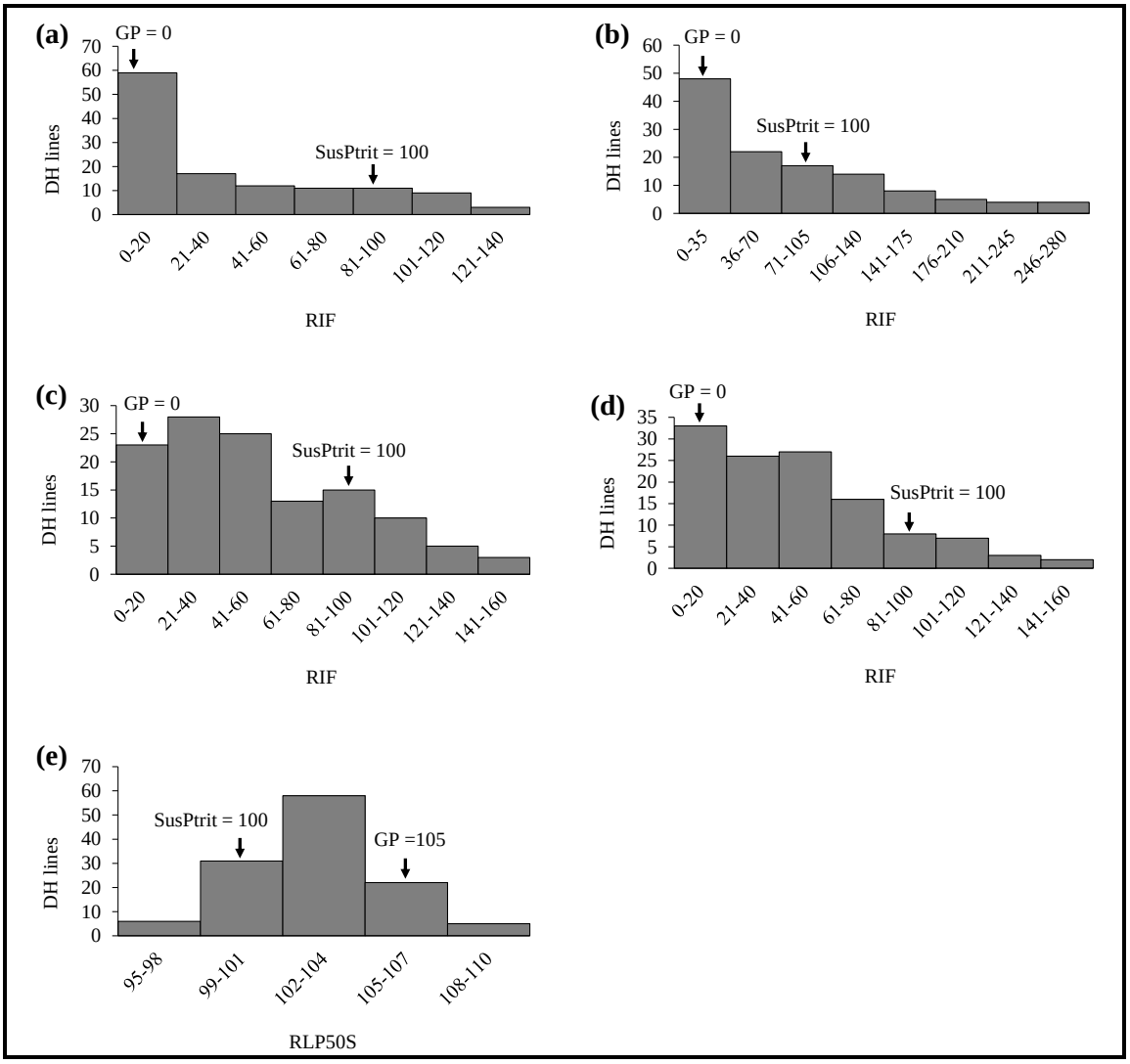

Figure 1: Frequency distribution of average RIF for (a) *Phm*.R, (b) *Phs*.F, (c) *Pp*.W, and (d) *Pt*.F, and (e) frequency distribution of average RLP50S for *Ph*.1.2.1. Values of the parental lines are indicated by arrows.

Table 2: Twenty-one S/G DH lines with levels of susceptibility similar to or higher than SusPtrit for more than one rust species. The ranking was based on the average RIF over three series of disease tests.

DH line	*Phm*.R	*Phs*.F	*Pt*.F	*Pp*.W	*Ph*.1.2.1	No. of species
SG019N	4	18	8	20	*	4
SG020N	7	1	11	1	*	
SG037N	14	2	4	7	*	
SG048N	19	16	12	9	*	
SG062N	10	10	9	19	*	
SG117N	2	15	17	3	*	
SG038N	14	7	*	16	8	
SG093N	8	5	*	6	15	
SG088N	20	19	6	*	*	3
SG130N	*	6	16	15	*	
SG109N	18	*	2	2	*	
SG047N	12	3	*	4	*	
SG010N	6	4	*	*	*	2
SG078N	17	8	*	*	*	
SG097N	*	14	20	*	*	
SG119N	*	9	18	*	*	
SG068N	*	*	5	8	*	
SG113N	*	*	1	10	*	
SG133N	*	*	7	11	*	
SG075N	11	*	*	17	*	
SG051N	*	12	*	12	*	
SusPtrit	13	38	13	21	17	

* The DH line ranked over 20 based on the average RIF over three series of disease tests

The QTLs mapped for the different non-adapted rust fungi occupied nine genomic regions, among which only one region on 7H affected resistance to all four non-adapted rust species. Four regions had a QTL that significantly contributed to resistance to only one particular non-adapted rust fungus. The four other regions had a QTL that was found to confer resistance to two or three rust fungi (Table 3, Figure 2). This tendency of QTL regions to affect susceptibility to more than one non-adapted rust fungus may explain why many DHs with high IF to one non-adapted rust fungus also exhibit relatively high IFs to the other non-adapted rust fungi.

Among the QTLs, *Phm-nhq2* at 7H had such a high LOD score (LOD = 21) and such a large effect (explaining 51% of the phenotypic variation) that it could be considered a major resistance gene. Of the remaining QTLs, seven explained 10-18% and nine explained less than 10% of the phenotypic variation. The immune parent, GP, is the sole resistance allele donor for all of the QTLs mapped for *Phm*.R, *Pp*.W and *Pt*.F. For the QTLs affecting resistance to *Phs*.F, GP donated a resistance allele for four of the QTLs and SusPtrit donated the resistance allele for one QTL. This observation is consistent with the intermediate resistance against *Phs*.F seen in SusPtrit (Figure 1b).

Only one QTL for partial resistance to *P. hordei* was detected; this QTL mapped to chromosome 6H and explained approximately 14% of the total phenotypic variation. The resistance allele is donated by GP, as expected. The QTL is designated as *Rphq3* (as explained below). This QTL co-localizes with *Pp-nhq4* and *Pt-nhq3* (Table 3).

Among the non-adapted rust fungi, Jafary et al. (2008) mapped nonhost resistance QTLs with the same isolates used in this study for *Phs* and *Pt* but with different isolates for *Phm* (*Phm* isolate Aragón) and *Pp* (*Pp* isolate RN-8) in the mapping populations V/S and C/S. Jafary et al. (2006) mapped QTLs for partial resistance to *Ph*.1.2.1 in the V/S mapping population. Recently, V/S and C/S were re-genotyped using the same ILLUMINA iSelect 9k barley infinium chip used for the present S/G map, and new SNPs linkage maps were generated for these two populations (unpublished data). The V/S, C/S and S/G maps were integrated (A. Martin-Sanz, R. Niks and P. Schweizer ERA-PG "TritNONHOST" project, ERAPG08.053; unpublished), and we compared the positions of nonhost and partial resistance QTLs mapped in S/G with those mapped in V/S and C/S. Seven out of the nine QTL regions detected in S/G coincided with QTL intervals detected in V/S and C/S. The coinciding QTLs are effective against either the same rust or to different rust species (Table 3, Figure 2). On chromosome 6H, the only QTL conferring partial resistance to *P. hordei* of S/G coincided with the nonhost resistance QTLs of Jafary et al. (2008). On the barley integrated map [Barley, Integrated, Marcel 2009 available at http://wheat.pw.usda.gov/GG2/index.shtml; (Aghnoum et al. 2010)], the nonhost resistance QTLs of Jafary et al. (2008) on chromosome 6H coincided with *Rphq3*, a QTL for partial resistance to *P. hordei* mapped in L94/Vada (Marcel et al. 2008; Niks et al. 2000; Qi et al. 1998). It is possible that the partial resistance QTL of S/G also coincided with *Rphq3*; hence, the same name was given.

Amenability of pre-selected DH lines for Agrobacterium-mediated transformation

The four DH lines chosen for the *Agrobacterium*-mediated transformation efficiency test were among the 12 most susceptible lines (average ranking) to *Phm.R*, *Phs.F* and *Pt*.F (Supplemental Table 1). Three of the four tested DH lines were amenable to transformation. The efficiency of transformation ranged from 1 to 17 T_0 plants/100 IEs (Table 4). The most efficient DH line was SG062N (11 to 17 T_0/100 IEs), and its T_0 plants were further analyzed to determine the number of T-DNA copies that were integrated (see below). The transformation efficiency for these lines was approximately 6% less than for the GP line. Compared to other barley genotypes tested for transformation efficiency using IEs, SG062N had transformation efficiency better than the 9 barley accessions tested by Hensel et al. (2008) and other barley accessions reviewed in Goedeke et al. (2007).

Twenty-one SG062N T_0 plants from three independent transformation attempts were randomly selected to determine the number of T-DNA copies integrated in their genomes (Figure 3). We detected one to five integrated T-DNA copies in the genomes of the 21 T_0 plants using *HPT* and *gfp* probes (Table 5). The *gfp* probe suggested that 8/17 of the T_0 plants had single-copy integrations, while the *HPT* probe suggested 10/21 T_0 plants had single-copy integrations. At T_1, GFP fluorescence (Figure 4) indicated that six out of the 17 T_1 populations, instead of the expected eight T_1 populations, were segregating for a single copy T-DNA. This result is because there were two T_1 populations (BG398E21 and BG398E22) that gave segregation ratios of 15:1 (with reporter gene expression : no reporter gene expression), indicating that two copies of T-DNA were segregating. It is possible that the two independently integrated T-DNAs in BG398E21 and BG398E22 cannot be distinguished based on *Hin*dIII DNA digestion. Nine other T_1 populations (excluding BG398E21 and BG398E22) segregated for two or more T-DNA copies, and among the nine, six showed segregation of linked T-DNA copies (Table 5 and Supplemental Figure 2).

Table 3: Summary of nonhost and partial resistance QTLs detected at the seedling stage in the S/G population in this study and the overlapping QTLs reported in Jafary et al. (2006; 2008).

Chr	Position (cM)	*Phm*.R	*Phs*.F	*Pp*.W	*Pt*.F	*Ph*.1.2.1	*Previously mapped QTLs for rust resistance*[#]
1H	43-68		*Rphsq1* (4,11%,G)[*]				*Phs-nhq* (6,12%,V)
2H	40-68			*Rppq1* (4,10%,G)	*Rptq1* (4,11%,G)		*Pp*^{RN}*-nhq* (4,6%,C)
	98-141		*Rphsq2* (4,10%,G)	*Rppq2* (3,8%,G)	*Rptq2* (5,12%,G)		*Pp*^{RN}*-nhq* (5,8%,V)
3H	112-176	*Rphmq1* (3,5%,G)					*Phm*^{A}*-nhq* (6,10%,C) *Phs-nhq* (7,12%,C) *Pp*^{RN}*-nhq* (7,14%,C) *Pt-nhq* (8,22%,C)
4H	52-75		*Rphsq3* (3,8%,S)				*Pt-nhq* (3,7%,S) *Phs-nhq* (5,11%,S) *Pp*^{RN}*-nhq* (6,12%,S) *Phm*^{A}*-nhq* (8,14%,S)
5H	73-110			*Rppq3* (4,10%,G)			
6H	56-88			*Rppq4* (4,9%,G)	*Rptq3* (4,9%,G)	*Rphq3* (4,14%,G)	*Pp*^{RN}*-nhq* (4,5%,C) *Pt-nhq* (11,19%,V) *Phm*^{A}*-nhq* (6,12%,C) *Rphq3* (16,21%,V)
7H	92-121		*Rphsq4* (4,9%,G)	*Rppq5*[!] (6,14%,G)			*Phs-nhq* (3,6%,V) *Pp*^{RN}*-nhq* (5,10%,V) *Pt-nhq* (11,21%,V) *Rphq8* (4,6%,V)
	141-168	*Rphmq2* (21,51%,G)	*Rphsq5* (5,12%,G)	*Rppq6*[!] (4,9%,G)	*Rptq4* (7,18%,G)		

[!] The LOD-2 interval of these QTLs overlapped, but they were still considered two QTLs.

[#]Results extracted from Jafary et al. (2006; 2008). *Rphq3* results extracted from Qi et al. (1998).

[*]In ( ), the QTL LOD score, percentage of explained phenotypic variation and the donor are given. The LOD score and the percentage of explained phenotypic variation for each QTL from this study were extracted from the mapping results for the averaged data. The QTL donor: C = Cebada Capa, G = GP, S = SusPtrit, V = Vada

Phm^{A} is a *P. hordei-murini* isolate from Aragón, Spain. *Pp*^{RN} is a *P. persistens* isolate from Netherlands collection number RN-8. These isolates were used in Jafary et al. (2008) and were different from those used in this study.

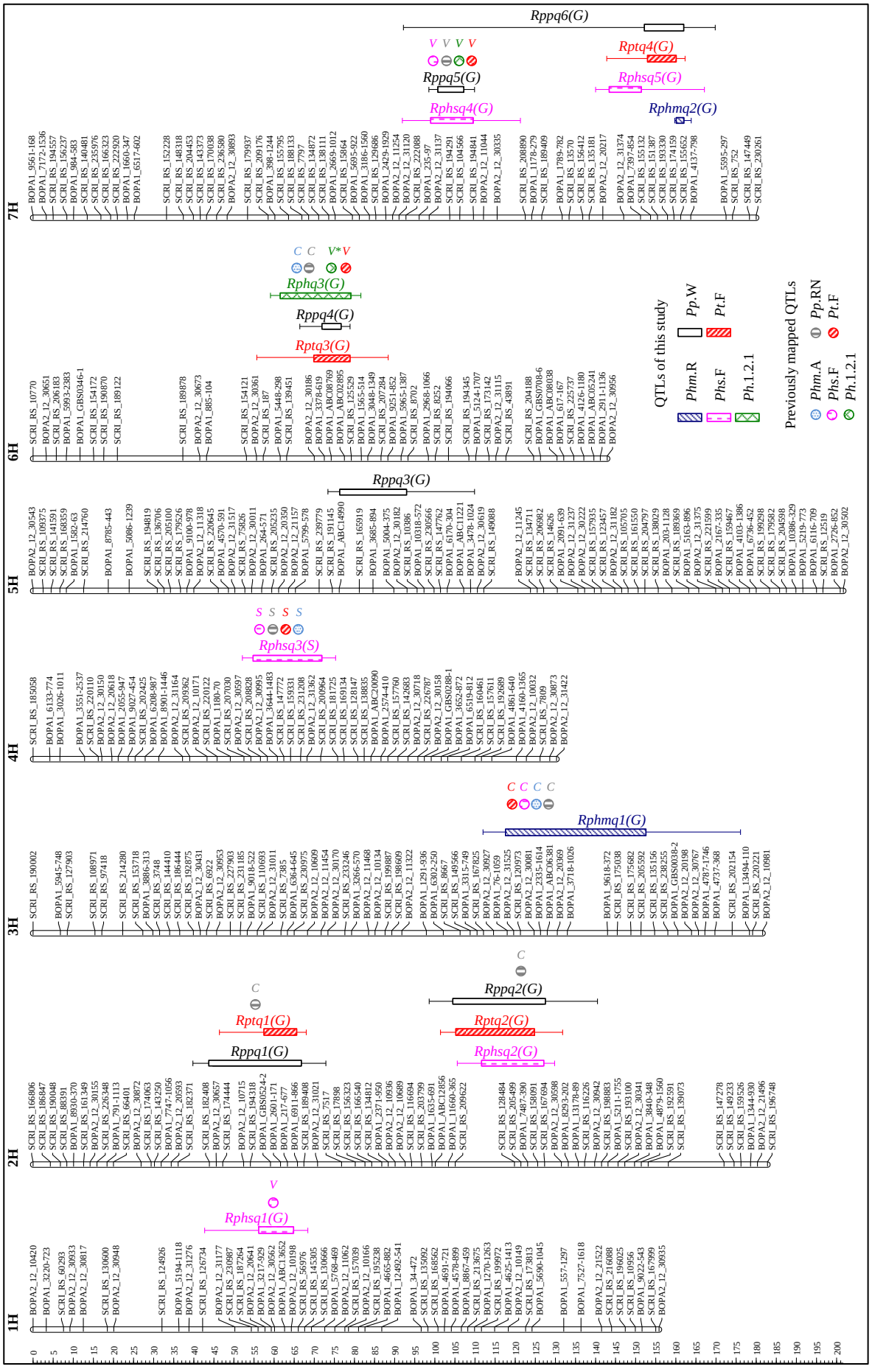
Rppq6(G)
Rptq4(G)
Rphsq5(G)
Rphmq2(G)
Rppq5(G)
Rphsq4(G)
Rphq3(G)
Rppq4(G)
Rptq3(G)
Rppq3(G)
Rphsq3(S)
Rphmq1(G)
Rppq2(G)
Rptq2(G)
Rphsq2(G)
Rptq1(G)
Rppq1(G)
Rphsq1(G)
QTLs of this study
Pp.W
Pt.F
Phm.R
Phs.F
Ph.1.2.1
Previously mapped QTLs
Pp.RN
Pt.F
Phm.A
Phs.F
Ph.1.2.1
7H
6H
5H
4H
3H
2H
1H

Figure 2 (previous page)**:** Skeleton linkage map with position of nonhost resistance QTLs and one partial resistance QTL mapped in this study and the co-localizing partial and nonhost resistance QTLs of Jafary et al. (2006; 2008). Only on chromosome 6H, ● V* is a QTL for partial resistance to *P. hordei* mapped in Qi et al. (1998). The QTL bars represent the QTLs of this study. The bars correspond to the rMQM LOD-1, and the extended lines correspond to the rMQM LOD-2 confidence interval. The letters inside ( ) indicate the QTL donor (*G* =GP; *S*=SusPtrit). The QTL dots represent the estimated position of the peak markers of previously mapped QTLs. The letters above the dots represent the QTL donor (*C*=Cebada Capa; *S*=SusPtrit; *V*=Vada). The ruler on the left side shows the distance in cM calculated according to Kosambi.

Table 4: Summary of transformation experiments for the four selected DH lines, expressed as the number of transformed plants per 100 plated IEs. Co-cultivation for 2-3 days and selection on 50 mg/L Hygromycin B.

DH line	Attempt	Total IEs	*Number of T_0 plants	T_0 plants/100 IEs
SG062N	BG398-1	210	35	17
	BG398-2	430	49	11
	BG398-3	122	15	12
			Average T_0 plants/100 IEs	**13**
SG047N	BG396-1	[a]420	36	9
	BG396-2	[b]300	7	2
			Average T_0 plants/100 IEs	**6**
SG093N	BG399-1	310	30	10
	BG399-2	180	16	9
	BG399-3	[b]110	1	1
			Average T_0 plants/100 IEs	**8**
SG133N	BG400-1	220	0	
	BG400-2	[b]420	0	
	BG400-3	[b]210	0	
GP	BG405-1	200	[c]36	18
	BG405-2	200	[c]40	20
			Average T_0 plants/100 IEs	**19**

* The number of regenerants positive for root GFP fluorescence and for PCR detection of *gfp* and *HPT* genes

[a] 210 IEs were pre-cultured one day before co-culture

[b] The IEs were pre-cultured one day before co-culture

[c] The number of regenerants positive for root GFP fluorescence detection. Ten randomly selected regenerants were positive for PCR detection of *gfp* and *HPT* genes

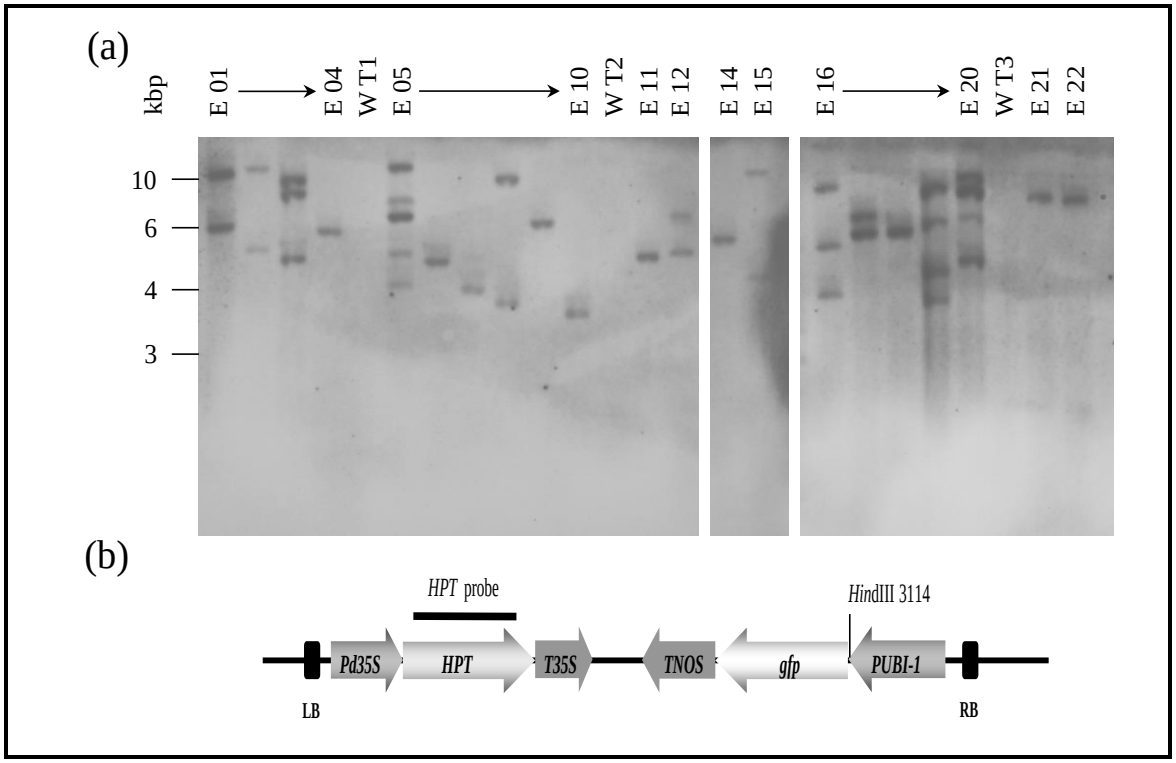

Figure 3: (a) Representative blot for analysis of T-DNA copy numbers in T_0 plants integrated with (b) *HPT* gene driven by *CaMV 35S* promoter and *gfp* gene driven by maize *UBIQUITIN 1* promoter with first intron (this study). The *Hin*dIII-digested genomic DNA from SG062N T_0 plants (E) and SG062N wild type (WT) was hybridized with DIG-labeled *HPT* probe.

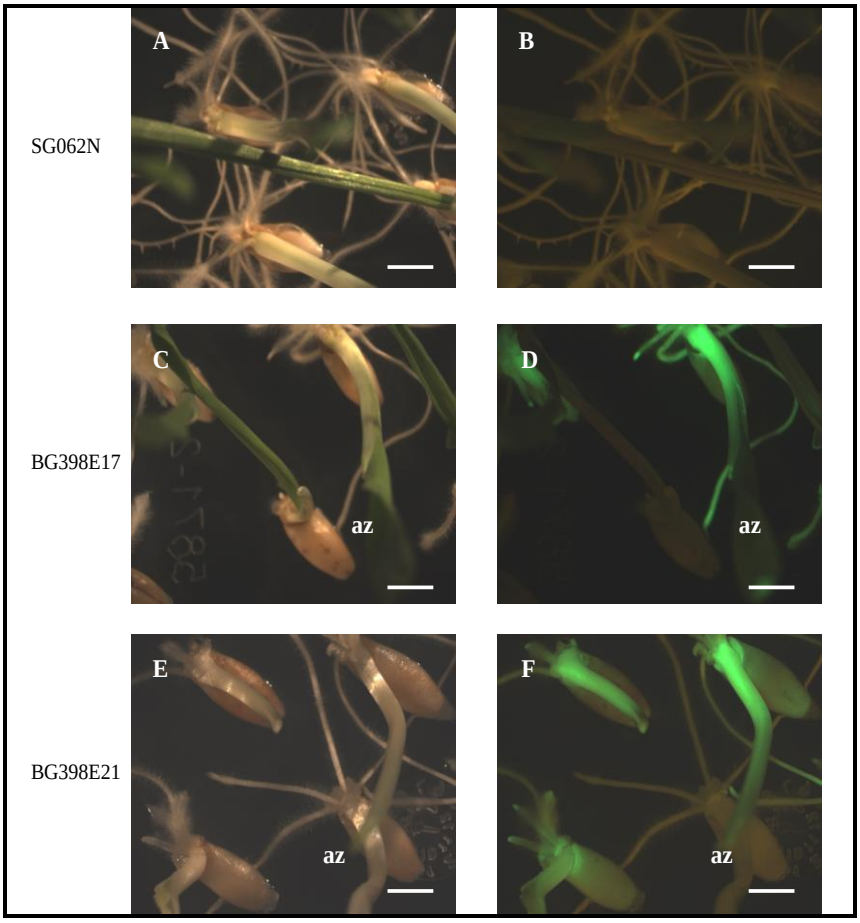

Figure 4: Representative microscopic analysis of GFP fluorescence in SG062N transgenic plants (BG398E17 & G398E21). Bright field (A, C, E) and epifluorescence (B, D, F) in germinating caryopses and roots of T_1 segregants. az – azygous plant that has lost the transgene. Bar = 0.5 cm

Table 5: Copy numbers of integrated T-DNA for the 21 SG062N T_0 plants and segregation of their T_1 populations.

T_0			T_1 populations			
Transformants (T_0)	**Copy number according to *gfp* probe**	**Copy number according to *HPT* probe**	**Reporter gene expression vs. no expression**	**Segregation observed (assumed)**	**X^2-Value**	**Likelihood (P) according to X^2- test**
BG398E06	1	1	39:18	2.2:1 (3:1)	1.92	> 0.10
BG398E07	1	1	42:17	2.5:1 (3:1)	0.64	> 0.40
BG398E09	1	1	27:12	2.2:1 (3:1)	0.70	> 0.40
BG398E10	1	1	37:12	3.1:1 (3:1)	0.03	> 0.90
BG398E11	1	1	36:15	2.4:1 (3:1)	0.54	> 0.40
BG398E14	1	1	25:14	1.8:1 (3:1)	2.43	> 0.10
BG398E21	1	1	35:3	12:1 (15:1)	1.33	> 0.20
BG398E22	1	1	48:1	48:1 (15:1)	1.53	> 0.20
BG398E01	2	2	21:0	21:1 (15:1)	3.40	> 0.05
BG398E12	2	2	44:4	11:1 (15:1)	0.36	> 0.60
BG398E03	2	3	26:12	2.2:1 (3:1)	2.17	> 0.10
BG398E18	3	1	44:0	44:0 (15:1)	3.02	> 0.05
BG398E17	3	2	46:9	5:1 (3:1)	2.17	> 0.10
BG398E16	3	3	53:0	53:0 (63:1)	2.59	> 0.10
BG398E20	3	4	52:6	9:1 (15:1)	4.09	> 0.05
BG398E19	4	4	45:0	45:0 (15:1)	3.00	> 0.05
BG398E05	5	5	36:12	3:1 (3:1)	0.00	> 0.99
BG398E04	n.d	1	n.d	n.d	n.d	n.d
BG398E02	n.d	2	n.d	n.d	n.d	n.d
BG398E08	n.d	2	n.d	n.d	n.d	n.d
BG398E15	n.d	2	n.d	n.d	n.d	n.d

Susceptibility of SG062N to non-adapted rust fungi and *Ph*.1.2.1

The selection of DH lines for *Agrobacterium*-mediated transformation tests was based on preliminary infection data. Additional series of experiments to quantify the susceptibility level were performed following the initiation of the transformation experiments.

This study identified nine genomic regions occupied by resistant QTLs against the four non-adapted rust fungi and *Ph*.1.2.1. At the nine genomic regions, SG062N carried six susceptibility alleles and three resistance alleles. The resistance alleles conferred resistance to *Pp*.W, *Pt*.F and *Phs*.F, and each explained approximately 10% of the phenotypic variation.

Based on the three series of disease tests for QTL mapping, the level of infection in SG062N for the four non-adapted rust fungi is either similar to or higher than the level of infection in SusPtrit (Table 6). The LP50S against *Ph*.1.2.1 conferred by SG062N is as short as that conferred by SusPtrit in additional disease tests (data not shown). This result is expected because SG062N has the susceptible allele for *Rphq3*, the only partial resistance QTL detected in this study.

Table 6: The susceptibility of SG062N, relative to SusPtrit, to the four non-adapted rust fungi tested over three series per rust species.

Non-adapted rust fungi	Average RIF	
	SusPtrit	SG062N
Phm.R	100	104
Phs.F*	100	189
Pp.W	100	100
Pt.F	100	105

* SusPtrit has one resistant QTL

Discussion

Genetics of nonhost and partial resistance

Nine chromosomal regions were found to segregate for nonhost resistance in S/G, and of these regions, one co-locates with the only partial resistance QTL, *Rphq3*, mapped in this study. Among the nonhost resistance QTLs mapped in this study, *Phm-nhq2* on chromosome 7H had the largest effect and may be considered a major gene for resistance. The resistance conferred by *Phm-nhq2* is not associated with a hypersensitivity response. The confidence interval of *Phm-nhq2* overlapped with the estimated position of the major gene for resistance to *P. hordei*, *Rph19.ah* (Marcel 2007). This result suggests that either *Phm-nhq2* is an allelic version of *Rph19.ah* or that they are simply at two closely linked loci. GP is not known to carry *Rph19.ah*.

Rphq3 was the only partial resistance QTL found in this study. GP gave an average of 5% (8 hours) longer LP50S than SusPtrit, a relatively low level of partial resistance to *P. hordei* compared to Vada, which has a high level of partial resistance. The LP50S of *P. hordei* on Vada is approximately 25% longer than on the susceptible accessions SusPtrit (Jafary et al. 2006) and L94 (Qi et al. 1998). Additionally, Vada has three QTLs effective at the seedling stage (Jafary et al. 2006; Qi et al. 1998). It is not surprising, then, to find only *Rphq3* in S/G; however, there may be other QTLs with effects too small to be detected.

GP is immune to non-adapted rust fungi and contains many genes (nine chromosomal regions) for resistance to the four non-adapted rust fungi species tested. This finding is very similar to the results obtained by Jafary et al. (2006; 2008) for Vada and Cebada Capa. It is, however, possible to find DH lines with susceptibility as high as, or higher than, that of SusPtrit.

Among the nine chromosomal regions with resistance QTLs identified in S/G, five regions conferred resistance to different rust fungi, suggesting that the responsible genes have effects on multiple rust species. Jafary et al. (2006) observed that QTLs affecting multiple rust species do not tend to be effective against taxonomically related rust species. Based on the phylogenetic tree of the rust species constructed by Jafary et al. (2006), we observed three genomic regions where the co-localization only involved QTLs affecting resistance to closely related rust species (*Phs*.F, *Pp*.W and *Pt*.F) (Table 3). In the other three regions, the QTLs were effective against less closely related rust species (QTLs for *Phm*.R and *Ph*.1.2.1 overlapped with QTLs for *Phs*.F, *Pp*.W and *Pt*.F). Co-localization of the QTLs for nonhost and partial resistance suggests an overlap of gene sets for these types of resistance in barley. Jafary et al. (2008) also observed similar QTL co-localization. Furthermore, several other studies (Hoogkamp et al. 1998; Zellerhoff et al. 2010; Zhang et al. 1994) have suggested that nonhost and partial host resistance may partly involve the same genes.

The tendency for co-localization of QTLs for different rust fungi may be due to either several closely linked genes, each involved in resistance to only one or two rust species, or to a single gene that contributes to resistance to multiple rust species. Fine-mapping is required to distinguish between these two possibilities.

SG062N, a new experimental line for nonhost and partial resistance studies

As in most monocotyledonous plant species, barley transformation efficiency is limited by genotype, explant, and media components, among other factors [reviewed in (Cheng et al. 2004; Goedeke et al. 2007; Harwood 2012)]. To improve transformation efficiency, adjusting treatment and tissue culture variables can be tried [e.g., the use of different *Agrobacterium* strains or the application of acetosyringone and L-cysteine (Hensel et al. 2008)]. Improving transformation efficiency for one genotype (e.g., GP) is helpful, but the transformable line may not be ideal for studying specific traits – in this case, GP is not suitable for the functional study of nonhost resistance. The line of choice to study nonhost resistance, SusPtrit, was unsuccessfully tested for amenability to *Agrobacterium*-mediated transformation (data not shown). Therefore, we applied a breeding approach to combine the amenability of GP for *Agrobacterium*-mediated transformation with the susceptibility of SusPtrit to non-adapted rust fungi.

Theoretically, the S/G mapping population can be used to locate genetic factors affecting transformation efficiency, as described in Cogan et al. (2002; 2004). It is not practical, however, to apply the *Agrobacterium*-mediated transformation procedure used in our study to a mapping population because of the labor and greenhouse space required. The high non-genetic variation in the transformation efficiency of a single line between experimental runs is another factor that complicates the mapping of such genes in barley. This variation can be attributed to variables such as the actual environmental conditions for transformation and tissue culture, the quality of explant donor plants and the individual handling of the experiment (Hensel et al. 2008). The high transformation efficiency of GP is likely a result of several genes, as in *Brassica oleracea* (Cogan et al. 2002; 2004). Hence, quantitative variation was observed in the transformability of the four pre-selected DH lines. We compared the genotypes of SG062N (highest transformation efficiency) and SG133N (not transformable) and found six chromosomal regions potentially involved in the transformation efficiency of barley (Supplemental Figure 3).

To date, GP is the line of choice for standard barley transformation. Notably, GP is a gamma-ray-induced mutant derived from cultivar Maythorpe (Forster 2001). The efficiency for transformation of GP is most likely not a result of the mutation, as Maythorpe can be transformed approximately as efficiently as GP. The transformation efficiency of Maythorpe ranges from 6 to 19% (G Hensel and J Kumlehn, unpublished data) and has been reported to reach 25% in one experiment [WA Harwood, unpublished data (John Innes Centre, Norwich, UK)]. In the Germplasm Resources Information Network (GRIN, http://www.ars-grin.gov/npgs/holdings.html), the ancestors of GP/ Maythorpe are traced back to Chevalier, Hana and Gull (Supplemental Figure 4). Tracing

the ancestor that has donated the genetic factors for efficient transformation can provide valuable information.

By crossing SusPtrit with GP, the susceptibility of SusPtrit to non-adapted and adapted (*P. hordei*) rust fungi and the amenability of GP to *Agrobacterium*-mediated transformation were easily combined. Simple screening of the progeny for individual lines that had inherited traits of both SusPtrit and GP was sufficient to verify that we had achieved our objective of obtaining the valuable new experimental line – SG062N (Golden SusPtrit). The optimized transformation procedure for GP can be applied directly to Golden SusPtrit to obtain approximately 47% of transformants with single-copy T-DNA integration (based on the *gfp* probe), which is fairly comparable to the proportion (50%) reported by Hensel et al. (2008).

Golden SusPtrit is as susceptible as SusPtrit to *P. hordei* and to the four tested non-adapted rust fungi. As such, Golden SusPtrit will replace SusPtrit as a valuable experimental line for future nonhost and partial resistance studies, especially for stable transformation with candidate genes that might be responsible for resistance.

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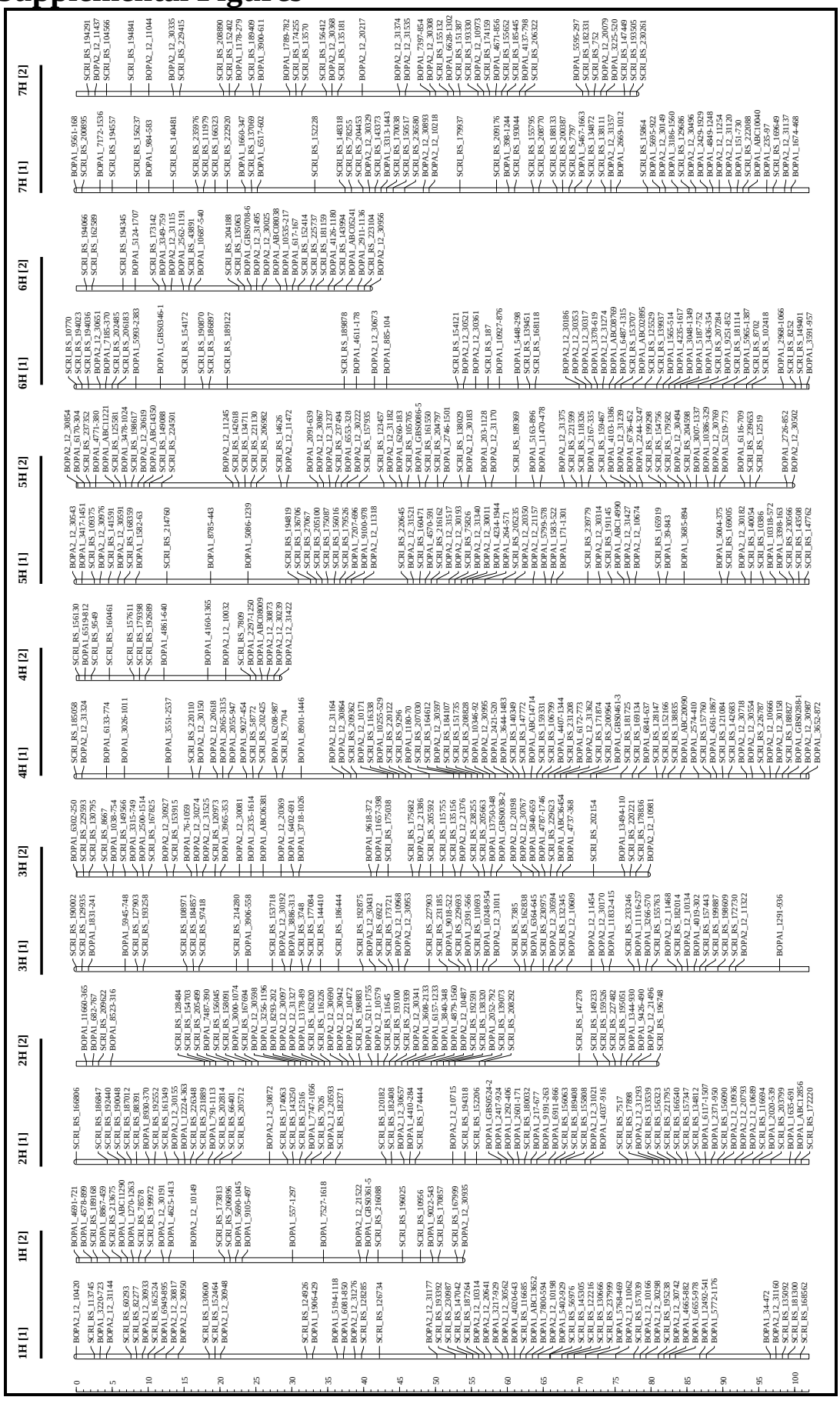
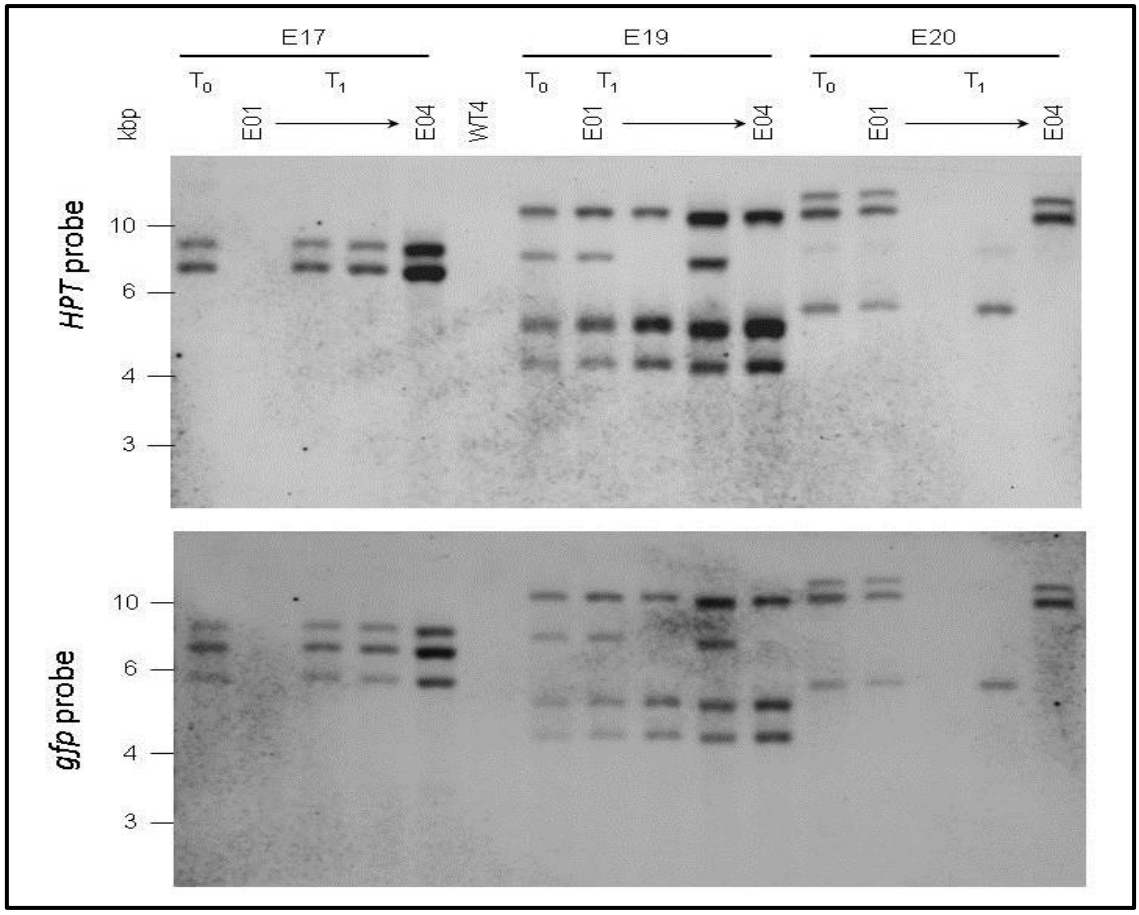
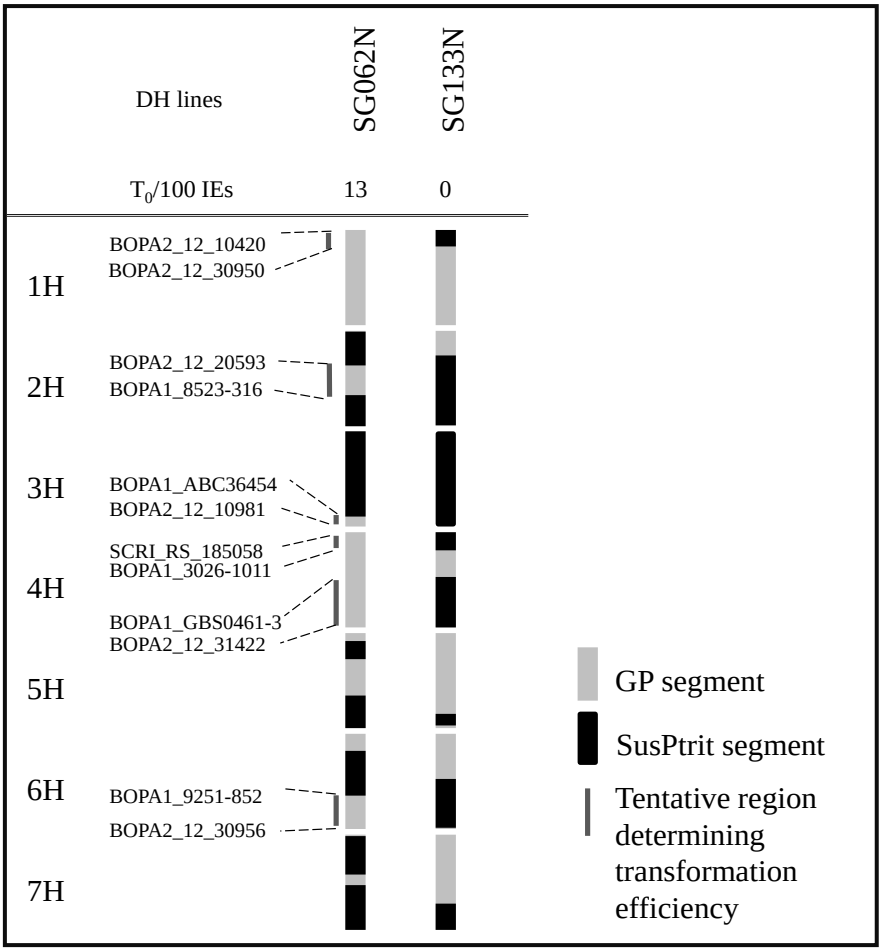
Supplemental Figures



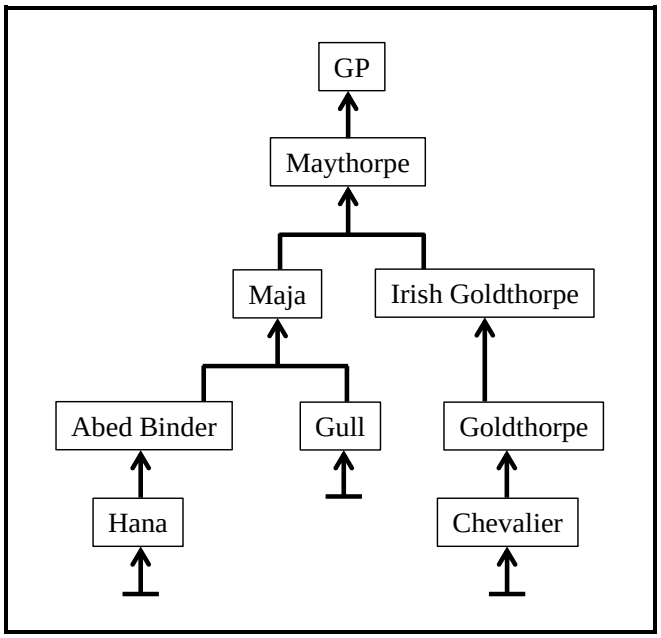
Supplemental Figure 1: Linkage map generated for S/G population containing 686 SNP marker loci identified in ILLUMINA iSelect 9k barley infinium chip. The reference ruler on the left side shows the distance in centiMorgans based on Kosambi.



Supplemental Figure 2: Transgenic loci linkage analysis of three segregating T_1 progenies. Genomic DNA of four T_1 plants from each primary transgenic line (T_0) were digested by *Hin*dIII, separated, blotted and hybridized with DIG-labeled *HPT* or *gfp* probes.



Supplemental Figure 3: The six tentative chromosomal regions determining the transformation efficiency of GP.



Supplemental Figure 4: The pedigree of GP. Information extracted from GRIN. Symbol ↥ indicates that the pedigree was not traced further.

Supplemental Table

Supplemental Table 1: The average ranking of susceptibility against *Pt*.F, *Phm*.R and *Phs*.F for the four DH lines selected for transformation efficiency tests.

Line	Pt (IF)	Phm (Severity score)*	Phs (IF)	Average ranking!	No. of susceptible QTL alleles
SG093N	20	4	21	8	6
SG062N	***13***	***4***	***49***	***8***	***6***
SG133N	18	4	17	12	6
SG047N	11	5	17	16	6
Line on rank 1	33	5	49	-	-
SusPtrit	20	5	18	8	9

* Severity score

0: Immune

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

Ranking involved 137 DH lines and SusPtrit.

!SusPtrit ranked number 1 in the average ranking.

Chapter 3

Specificity of barley near-nonhost and partial resistance QTLs towards adapted and non-adapted rust fungi

To be submitted

Specificity of barley near-nonhost and partial resistance QTLs towards adapted and non-adapted rust fungi

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Abstract

Partial resistance of barley to *Puccinia hordei* and nonhost resistance to non-adapted rust fungi inherits polygenically. The two types of resistance seem to share some genes and have a similar prehaustorial mechanism of resistance, but partial resistance is less complete than nonhost resistance of barley. Partial resistance to adapted rusts fungi seems, therefore, like a weak form of nonhost resistance to non-adapted rust fungi. If partial resistance and nonhost resistance are indeed based on the same principles, one can understand nonhost resistance by studying partial resistance and *vice versa*. Four partial resistance QTLs and one for nonhost resistance were selected to study their effect in near isogenic lines (NILs). SusPtrit and L94 are appropriate recurrent parents for NIL development because they are extremely susceptible to *P. hordei* and unusually susceptible to some non-adapted rust fungi at seedling stage. This allows the QTL effect in L94 and SusPtrit genetic backgrounds to be tested against different isolates of *P. hordei* and different species and isolates of non-adapted rust fungi. NILs of *Rphq2, Rphq3, Rphq4* and *Rnhq* in L94 background were already available. In this study, we developed NILs in SusPtrit background for *Rphq2, Rphq3, Rphq11, Rphq16* and two alleles of *Rnhq*. Whole genome genotyping of the NILs with the ILLUMINA iSelect 9k barley infinium chip showed some NILs to be free from unwanted donor genomes but some are not. The L94- and SusPtrit-NILs were inoculated with selected adapted and non-adapted rust fungi. Some QTLs were rust isolate and rust species specific but others have a broader resistance spectrum, such as *Rphq3* and *Rphq11*. However, the NILs may overestimate the spectrum of effectiveness for the gene underlying the QTL effect. If the spectrum of effectiveness for a QTL is confirmed, this study suggests some genes may be involved in partial as well as nonhost resistance. The NILs are suitable materials to start fine-mapping of the responsible genes for the QTLs they carry.

Keywords: NILs, Quantitative Trait Locus (QTL), Specificity, *Puccinia*

Introduction

Nonhost resistance is defined as resistance shown by all genotypes of a plant species to all genotypes of a potential pathogen species (Niks et al. 2011). It is impossible to test all genotypes of a plant species to all genotypes of potential pathogens. Hence, all 'nonhost' qualifications are virtually based on limited evidence.

Under certain conditions, such as under very high inoculum dose applied to seedling leaves, a low percentage of accessions of a putative nonhost species may turn out to be somewhat susceptible to a potential pathogen (Niks 1987). This marginal host status may qualify the plant species as near-nonhost to a certain tested potential pathogens. For example, after screening a set of 109 spring barley (*Hordeum vulgare*) accessions with different rust fungi, barley was classified as nonhost to most non-adapted rust fungi but as a marginal host, or near-nonhost, to some other non-adapted rust fungi like *Puccinia triticina* and *P. hordei-murini* (Atienza et al. 2004). In the context of our work, non-adapted rust fungi are rust fungi that are poorly or not adapted to barley, but primarily to one or few other plant species. The near-nonhost status of barley to some non-adapted rust fungi has opened up the opportunity to study barley nonhost resistance without resorting to interspecific crosses. The assumption is that the genetic basis of near-nonhost status may help to understand, by extrapolation, the genetic basis of full nonhost resistance. Rare barley accessions which were at the seedling stage moderately susceptible to *P. triticina* were intercrossed to develop an experimental line – SusPtrit, which is at the seedling stage exceptionally susceptible to *P. triticina*. SusPtrit is at seedling stage also susceptible to at least nine other non-adapted rust fungi to which barley is a near-nonhost (Atienza et al. 2004). Mapping populations developed by crossing SusPtrit with regular barley – Cebada Capa/SusPtrit (C/S) and Vada/SusPtrit (V/S) – showed that the immunity of Cebada Capa and Vada to the non-adapted rust fungi inherited polygenically. The two mapping populations segregated for different sets of quantitative trait loci (QTLs) with only few QTLs in common between the populations. Most mapped QTLs in the C/S and V/S were effective to only one or two rust species indicating high but overlapping specificities of QTLs for resistance to non-adapted rust fungi (Jafary et al. 2006; 2008).

Partial resistance is defined as resistance that delays the epidemic development despite a compatible infection type (Niks et al. 2011; Parlevliet 1979). Partial resistance of barley accessions to the barley leaf rust fungus (*P. hordei*) is due to a lower infection frequency, lower sporulation rate and longer latency period of the pathogen (Parlevliet 1979). As with the resistance of barley to non-adapted rust fungi, partial resistance of barley to *P. hordei* inherits polygenically and to date, more than 20 QTLs for partial resistance to *P. hordei*

have been mapped in different bi-parental populations at seedling and adult plant stages. Each mapping population segregates for a different set of QTLs with few QTLs shared (Marcel et al. 2007b; Qi et al. 2000). Some of the mapped QTLs were shown to have an isolate specific effect (González et al. 2012; Marcel et al. 2008; Niks et al. 2000; Qi et al. 1999).

Nonhost resistance of barley to non-adapted rust fungi and partial resistance to *P. hordei* are both based on reduced success in haustorium formation by the pathogen (Niks 1983a, b). Partial resistance to *P. hordei* seems, therefore, like a weak form of nonhost resistance to non-adapted rust fungi (Niks and Marcel 2009; Niks et al. 2011). Partial and nonhost resistance may share some genes because QTLs for partial resistance mapped in C/S and V/S tend to co-localise significantly with QTLs for resistance to non-adapted rust fungi (Jafary et al. 2008). For both resistances QTLs tend to coincide with peroxidase gene clusters (González et al. 2010). Transcriptomics on powdery mildew-inoculated barley suggested that partial resistance and nonhost resistance to powdery mildew are functionally associated (Zellerhoff et al. 2010). We hypothesize that nonhost and partial resistance are both based on the pathogen-associated molecular pattern-triggered (PAMP-triggered) defense system. The near-nonhost resistance and partial resistance of barley would result from incomplete suppression of PAMP-triggered defense by effectors of the pathogen (Niks and Marcel 2009). In the view of co-evolution, partial resistance may represent a transitional stage of losing or acquiring host status to a certain rust fungus.

If partial and nonhost resistance are indeed based on the same principles, one can understand nonhost resistance by studying partial resistance and *vice versa*. Five major QTLs for partial resistance to *P. hordei* were selected to study the molecular basis this resistance. Map-based cloning of the responsible genes at these QTLs has been initiated (Marcel et al. 2007b; Chapter 6 (*Rphq2*), this thesis; Y. Wang and X. Qi, Institute of Botany, Chinese Academy of Sciences, Beijing, unpublished). The effect of each QTL can be tested using QTL-near isogenic lines (QTL-NILs). A QTL is Mendelized when it is introgressed into a NIL (Alonso-Blanco and Koornneef 2000). The QTL effect is then evaluated in a uniform genetic background without the interference of other genes influencing the same trait. Such QTL-NILs are an important stage in map-based cloning of the responsible gene(s) determining the effect of the QTL.

SusPtrit and L94 served as recurrent parents. Both SusPtrit and L94 are extremely susceptible to *P. hordei* (Jafary et al. 2006). They are also susceptible to some non-adapted rust fungi at seedling stage, although L94 is not as susceptible as SusPtrit (Atienza et al. 2004). The QTL effect in L94 and SusPtrit genetic backgrounds can be tested not only against different isolates of *P. hordei* but also against different species and isolates of non-

adapted rust fungi to test whether genes for partial resistance may also play a role in resistance to non-adapted rust fungi.

The current study aims to develop NILs in SusPtrit genetic background (SusPtrit-NILs) for four partial resistance QTLs (*Rphq*) mapped in various mapping populations and derived from various donors. Two different alleles of nonhost resistance QTL (*Rnhq*) are also included to develop SusPtrit-NILs. The newly developed SusPtrit-NILs in this study together with the L94-NILs developed by van Berloo et al. (2001) and Marcel et al. (2007a) were inoculated with different adapted and non-adapted rust fungi to test the specificity of the introgressed QTLs against the different rust fungi.

Materials and Methods

SusPtrit-NILs development and whole genome genotyping

The plant materials used to develop SusPtrit-NILs for each QTL are listed in Table 1. SusPtrit was crossed with each donor accession to obtain an F_1 generation. F_1 individuals were backcrossed to SusPtrit for five or six rounds to obtain near-isogenic lines in BC_5 to BC_6. SusPtrit was used as the female plant throughout the NILs development program.

Table 1: Plant materials for SusPtrit-NIL for resistance QTLs of interest

QTL	Immediate donor	Original donor	Recurrent parent	References
Rphq2	L94-*Rphq2*	Vada	SusPtrit	van Berloo et al. (2001) Marcel et al. (2007a)
Rphq3	L94-*Rphq3*	Vada	SusPtrit	van Berloo et al. (2001) Marcel et al. (2007a)
Rphq11	Steptoe	Steptoe	SusPtrit	Marcel et al. (2007b)
Rphq16	DOM	DOM	SusPtrit	Marcel et al. (2007b)
Rnhq.V	L94-*Rnhq.V*	Vada	SusPtrit	Niks et al. (2000)
Rnhq.L	L94	L94	SusPtrit	

At each round of backrossing, the donor allele of the QTL was selected by molecular markers, i.e. marker assisted selection (MAS). No whole genome selection against donor background was applied. For *Rphq2*, *Rphq3*, *Rnhq.L*. and *Rnhq.V*, their donor (L94 or L94-NILs) was not expected to contribute any other allele(s) for quantitative resistance to

rust fungi. Hence, only selection for the respective target QTLs was required. The MAS for *Rphq11* and *Rphq16* required positive selection for those QTLs and negative selection against a second QTL detected in the same donor. Steptoe, the donor of *Rphq11* and DOM, the donor of *Rphq16*, each carry one additional partial resistance QTL – *Rphq15* and *Rphq17*, respectively (Marcel et al. 2007b). The markers used for the selection in favour or against each QTL are listed in Table 2.

At generation $F_2BC_5S_1$ or BC_6S_1, two SusPtrit-NILs (Sister NILs) were selected for each QTL (only one selected for *Rphq2* and *Rphq16*). These SusPtrit-NILs together with the L94-NILs (Marcel et al. 2007a; van Berloo et al. 2001), Vada, SusPtrit, Steptoe, Dom and L94 were subjected to whole genome genotyping using the ILLUMINA iSelect 9k barley infinium chip which carries 7864 SNPs. Total DNA was extracted from young leaves using a CTAB-based method (Stewart and Via 1993).

The barley SNP integrated map (A. Martin-Sanz, R. Niks and P. Schweizer ERA-PG "TritNONHOST" project, ERAPG08.053; unpublished), was used as a guide to estimate the position and the size of the donor genome introgression in the NIL.

Table 2: The list of markers used for QTL selection in the NIL development program

QTLs	Chrom	Position (cM)[b]	No. of backcrossing cycles	Markers [!]	Position (cM)[b]	Types	References of marker
Rphq2	2H	148	6	k00345 [c]	147	CAPs	Hori et al. (2005); Marcel et al. (2007a)
				scP15M51-204	150	SCAR	Marcel et al. (2007a)
				besV76P5D5AR	-	SCAR	Chapter 5, this thesis
				GBMS216 [c]	150	SSR	Li et al. (2003); Varshney et al. (2007)
Rphq3	6H	61	6	GBM1212 [c]	54	SSR	Varshney et al. (2006); Varshney et al. (2007)
				WBE201	58	CAPs	Marcel et al. (2007a)
				HVM14	61	SSR	Liu et al. (1996); Varshney et al. (2007)
				HVM22a	61	SSR	Liu et al. (1996); Varshney et al. (2007)
				ABG388 [c]	72	CAPs	Marcel et al. (2007a)
Rphq11	2H	90	5	GBM1062	91	SSR	Thiel et al. (2003); Varshney et al. (2007)
				GBS0512 [c]	92[*]	CAPs	Stein et al. (2007); Chapter 4, this thesis
				GBMS244 [c]	97	SSR	Li et al. (2003); Varshney et al. (2007)
Rphq15 [β]	6H	22	-	scssr09398 [c]	5	SSR	Ramsay et al. (2004); Varshney et al. (2007)
				MWG966	18	CAPs	Graner et al. (1991); Chapter 4, this thesis
				GBMS033 [c]	26	SSR	Li et al. (2003); Varshney et al. (2007)
Rphq16	5H	170	6	scsnp03275 [c]	-	CAPs	Rostoks et al. (2005); Chapter 4, this thesis
				Dst-33	163	SCAR	BarleyWorld.org; Chapter 4, this thesis
				GMS002 [c]	179	SSR	Struss and Plieske (1998); Varshney et al. (2007)
Rphq17 [β]	3H	64	-	Bmag0136	64	SSR	Ramsay et al. (2000); Varshney et al. (2007)
				Bmac0067	67	SSR	Ramsay et al. (2000); Varshney et al. (2007)
Rnhq.V	7H	89	5	WBE101 [c]	83[+]	CAPs	Marcel et al. (2007a)
				SKT1	-	CAPs	Kikuchi et al. (2003)
Rnhq.L			6	MWG2031	87	CAPs	Kikuchi et al. (2003)
				GBM1303 [c]	88[+]	SSR	Varshney et al. (2007); Marcel et al. (2007a)

[b] The position is based on the integrated map, Marcel 2009 (Aghnoum et al. 2010)

[c] Flanking marker

* In Chapter 4, this thesis and [+] in van Dijk (2007) the marker is flanking the QTL.

[β] QTL selected against

[!] Primer sequences and annealing temperature of the different markers are available in Supplemental Table 1

Inoculum and disease tests

The levels of resistance of the NILs were determined for eight adapted and non-adapted *Puccinia* species and *formae speciales*. Four of these, *P. hordei*, *P. hordei-secalini*, *P. hordei-bulbosi* and *P. triticina*, were represented by more than one isolate (Table 3).

Table 3: Rust isolates used in this study.

Adapted			
Puccinia sp	Isolate	Origin	Abbreviation
P. hordei	1.2.1	Netherlands	*Ph*.1.2.1
P. hordei	Cordoba 4	Spain	*Ph*.Co4 [α]
P. hordei	Uppsala	Sweden	*Ph*.Upp [β]
P. graminis f. sp. *tritici*		Hungary	*Pgt*
Non-adapted			
P. graminis f. sp. *lolii*	Rhenen	Netherlands	*Pgl*
P. hordei-murini	Rhenen	Netherlands	*Phm*.R
P. hordei-secalini	French	France	*Phs*.F
P. hordei-secalini	Groningen	Netherlands	*Phs*.G
P. hordei-secalini	Wageningen	Netherlands	*Phs*.W
P. hordei-bulbosi	Iran	Iran	*Phb*.Ir [γ]
P. hordei-bulbosi	Israel	Israel	*Phb*.Is [δ]
P. triticina	BWR96258	Switzerland	*Pt*.B [ε]
P. triticina	Flamingo	Netherlands	*Pt*.F
P. triticina	INRA	France	*Pt*.I [η]
P. persistens	Wageningen	Netherlands	*Pp*.W

[α] Kindly provided by Dr D. Rubiales. Collected at Còrdoba, Spain.
[β] Kindly provided by Dr F. Martínez. Collected at Uppsala, Sweden.
[γ] Kindly provided by Dr H. Jafary. Collected from Kalaybar, Iran.
[δ] Kindly provided by Prof Dr Y. Anikster. Collected at Ariel, Israel.
[ε] Kindly provided by Dr G. Schachermayer, Zürich-Reckenholz, Switzerland.
[η] Kindly provided by Dr H. Goyeau, INRA, France
For [ε] and [η], the places of collection are unknown.

The disease tests were carried out at seedling stage in a greenhouse compartment with two to three series. The L94-NILs and SusPtrit-NILs together with the reference lines L94 and SusPtrit were sown in boxes (37 x 39 cm). Each NIL and the relevant reference line were represented by five seedlings in each disease test. Twelve days after sowing, the unfolded primary leaf of each seedling was fixed horizontally with adaxial side up and inoculated in a settling tower (Niks et al, 2011).

For *P. hordei*, one milligram of inoculum per box (about 60 spores/cm^2) was used. For *P. graminis* f. sp. *tritici* (*Pgt*) and the non-adapted rust fungi, two milligram of inoculum per box was applied. *Lycopodium* spores were used to dilute the inoculum about 10 times. The inoculated boxes were subjected to overnight incubation in a dew chamber, set at 18^oC with 100% relative humidity, for 8 hours overnight, in the dark. After the incubation, the boxes were transferred to a greenhouse compartment. The temperature in the compartment was set at 20 ± 3^oC with 70% relative humidity.

Latency period (LP50S) of the *P. hordei* isolates was scored as described in Niks et al. (2011). From the day the first pustules became visible, a mid-section of each seedling leaf was delimited by marker pen, and mature pustules in this section were counted daily using a pocket lens (×10), until the number did not increase anymore (5 or 6 days). The LP of the pathogen on each seedling was evaluated by estimating the number of hours from inoculation to the moment at which 50% of the ultimate number of uredinia was visible. For *Pgt*, the percentage of area covered with lesions (ACL) was scored for SusPtrit and SusPtrit-NILs (as in Jafary et al. 2006). This is because the neighboring pustules and surrounding halos often merged. Such a merge of colonies was not observed in L94 and L94-NILs. Therefore, for *Pgt* infection frequency (IF – total number of pustules/cm^2) was scored on L94 and L94-NILs, at 12 days post inoculation. For non-adapted rust fungi, IF was scored at 12 days post inoculation. The relative latency period (RLP50S) and relative infection frequency (RIF) were calculated relative to the LP50S and IF on SusPtrit for SusPtrit-NILs and on L94 for L94-NILs. The data were tested for significant differences by the linear mixed model using GenStat® 14th edition (VSN International Ltd. 2011). The least significant difference, $P<0.05$ ($LSD_{0.05}$) was used to declare if the L94-NILs and SusPtrit-NILs were significantly different from L94 and SusPtrit, respectively. The reported RLP50S and RIF data are the predicted means according to the linear mixed model.

Preliminary histological observations on non-adapted rust fungi in QTL-NILs

For six QTL-NIL/rust isolate combinations in which the macroscopic data suggested a relatively strong effect of the QTL on resistance level, histology of the infection was characterized. The selected combinations were sown and inoculated as for the macroscopic tests. For each QTL-rust interaction, two leaf segments were sampled on the fifth day post inoculation (5dpi). The collected leaf segments were bleached in acetic/ethanol (1:3) for a week, and stained with Uvitex 2B (Ciba-Geigy) (Rubiales and Niks 1996). A fluorescence microscope (Zeiss Axiophot, exciter filter BP 395-440, chromatic beam splitter FT 460 nm and barrier filter LP 420) was used to observe the leaf

segments. Approximately, 50 infection units were screened for each leaf segment and were classified into different stages of development (Niks 1982). Infection units with six or less haustorial mother cells were considered as early aborted. Infection units with more than six haustorial mother cells were classified as established, and their longest diameter was measured using an eyepiece micrometer. The number of infection units associated with autofluorescent cells, indicating plant cell necrosis, was recorded. For testing the diameter of established infection units for differences between barley accessions, a linear mixed model was performed using GenStat® 14th edition (VSN International Ltd. 2011) unless indicated otherwise.

Results

Genome purity of L94- and SusPtrit- NILs

The whole genome genotyping for L94-*Rphq2*, L94-*Rnhq.V*, Sus-*Rphq11.6* and Sus-*Rphq16* did not indicate any donor marker allele away from the target introgression, and hence these NILs may be clear from unwanted segments from the donor. The other NILs contained segment(s) of unwanted donor genome with size ranges from approximately 2 cM to 30 cM (Figure 1). The SusPtrit sister NILs did not tend to have the same inadvertent undesired donor fragments.

Marcel et al. (2007a) reported on the basis of 226 amplified fragment length polymorphism (AFLP) markers that L94-*Rphq2* and L94-*Rphq4* are free of unwanted donor segments, and L94-*Rphq3* has a segment at the end of chromosome 3HL. The present genome-wide screen with SNP markers from the ILLUMINA iSelect 9k barley infinium chip did not reveal other donor fragments in L94-*Rphq2*, and confirmed the donor fragment at the end of chromosome 3HL for L94-*Rphq3*, but indicated three unwanted donor fragments in L94-*Rphq4*.

We compared the estimated size of QTL introgressions based on the integrated SNP map with the size estimated based on the position of their flanking markers on the barley integrated map [Marcel 2009, (Aghnoum et al., 2010)]. No discrepancy was observed except for the *Rphq3*- and *Rnhq.V*-introgressions in L94; *Rphq2*-, *Rphq11*- and *Rnhq.V*-introgressions in SusPtrit, which may have donor DNA beyond the flanking markers of the QTL.

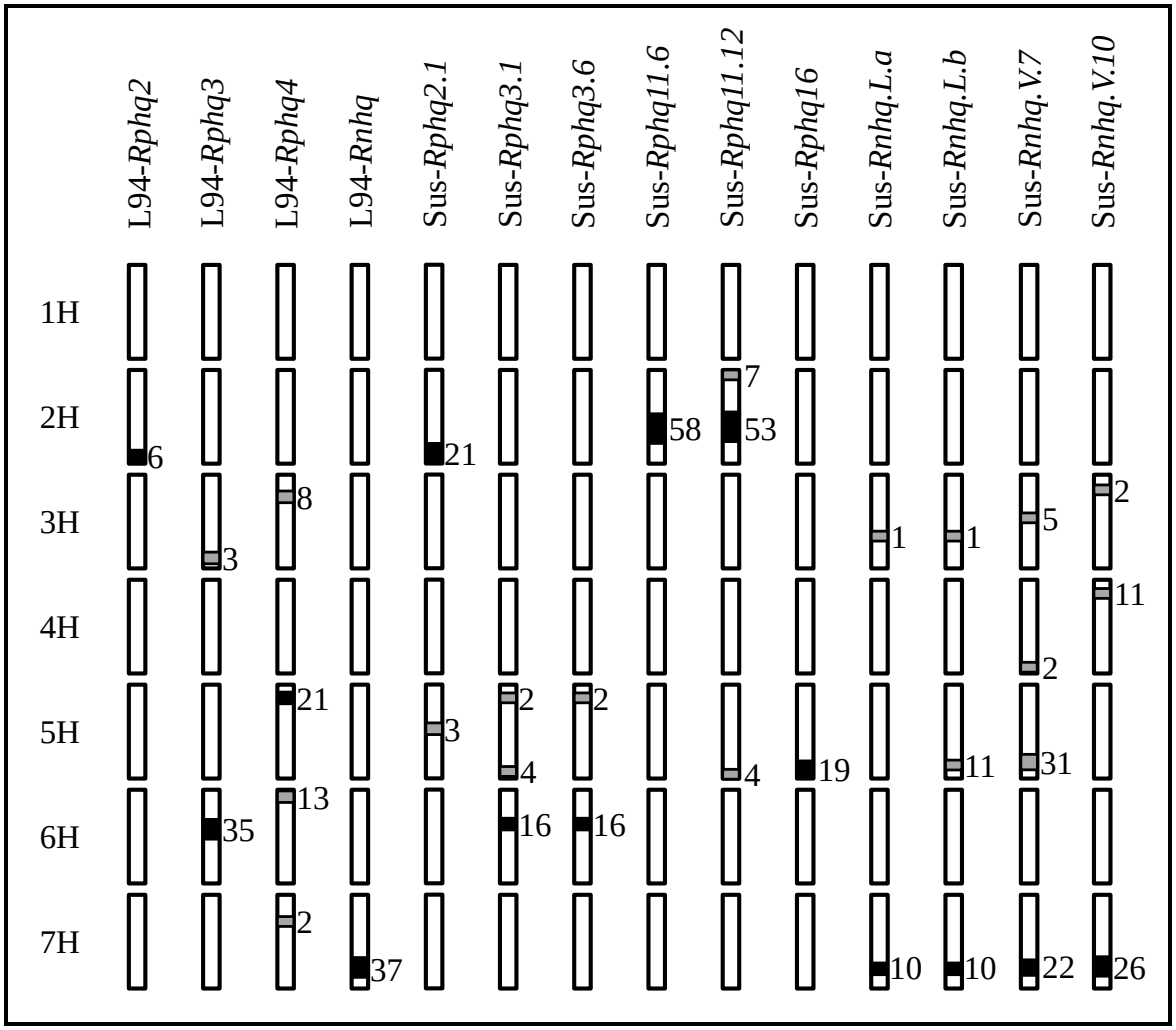

Figure 1: The graphical genotypes of L94- and SusPtrit-NILs carrying partial and nonhost resistance QTLs of interest. The introgression size in cM is indicated on the right hand side of the chromosome bars. The grey boxes in the chromosome bars indicate unwanted donor genome introgressions. The black boxes in the chromosome bars indicate the targeted introgressions carrying the QTLs.

Partial and nonhost resistance QTLs against adapted and non-adapted rust fungi

Table 4 summarizes the infection tests on the L94-NILs and SusPtrit-NILs with adapted and non-adapted rust fungi. Compared to the susceptible recurrent parent, some NILs were significantly more resistant only to *P. hordei*, others also to one or more non-adapted rust fungi (for example, compare SusPtrit-*Rphq11* versus SusPtrit-*Rphq16*). L94-*Rnhq* gave a similar latency period of *P. hordei* as L94, but showed a significantly higher level of resistance than L94 when inoculated with four of the non-adapted rust species tested. These observations indicate that the responsible genes varied in spectrum of effectiveness to rust species and rust isolates. The data also indicate that QTLs may be effective to some rust isolates and not to others. Such isolate specificity occurred for resistance to the adapted *P. hordei* as well as to some non-adapted rust fungi. It seems surprising that for

some rust fungi relatively small differences in RIF values are significant, whereas in others large differences are not. The lack of significance in some cases where the difference is large may be due to a large experimental error in the experiment (see *Pt*.B and *Pt*.I in Table 4).

L94-Rphq4 was not included in the disease test against *Ph*.1.2.1 and *Ph*.Co4 because *Rphq4* is only effective against *Ph*.1.2.1 at adult stage (Qi et al. 1998) and it was not detected in the L/V mapping population against *Ph*.Co4 at seedling stage (González et al. 2012). We could not confirm a significant effect of *Rphq4* at the seedling stage to *Ph*.Upp, as reported by Marcel et al. (2008). L94-*Rphq4* also showed a similar level of infection as L94 to *Phm*.R, the three isolate of *Pt* and the three isolates of *Phs*, and hence at the seedling stage *Rphq4* did not appear to be effective to any of the non-adapted rust fungi tested here (data not shown).

Rphq2, *Rphq3* and *Rnhq.V*-introgressions were available in two genetic backgrounds, viz. in SusPtrit and in L94. Some background effect was suggested. For example, the *Rphq2*-introgression was effective against *Ph*.Co4 in L94 background, but not in SusPtrit background; *Rnhq.V* was effective against *Ph*.Upp in SusPtrit but not in L94 background. In other cases isolate specificity was consistently found in both backgrounds (e.g. *Rphq3* against *Ph*.Co4 and the three isolates of *Phs*). Among the adapted rust fungi, *Ph*.Upp was the isolate to which the highest number QTL-introgressions were effective (five QTL-introgressions), and among the non-adapted rust fungi, it was isolate *Pt*.F (four QTL-introgressions).

The Sus-*Rphq11* sister NILs had a higher level of resistance than SusPtrit to almost all the rust fungi tested and they were nearly completely resistant to *Phb*.Ir (Figure 2). No hypersensitivity response was observed macroscopically on Sus-*Rphq11* sister NILs against all the rust fungi tested.

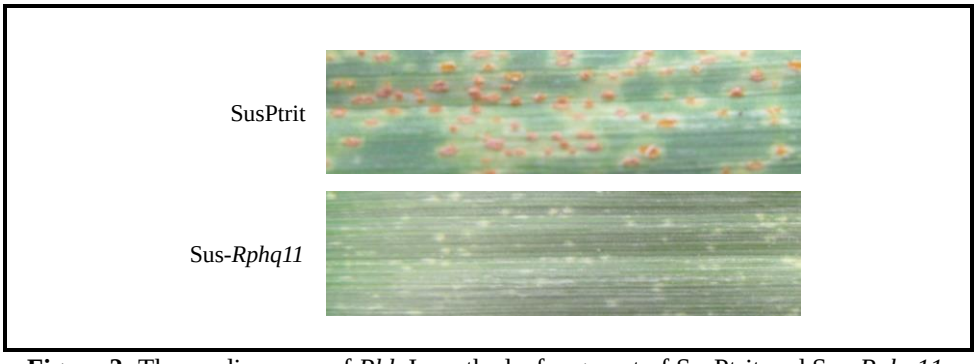

Figure 2: The urediospores of *Phb*.Ir on the leaf segment of SusPtrit and Sus-*Rphq11*.

Table 4: The resistance of L94-NIls and SusPtrit-NILs against adapted and non-adapted rust fungi.

Nils	*Adapted rust fungi*				*Non-adapted rust fungi*										
	Ph.1.2.1 RLP50S	*Ph*.Upp RLP50S	*Ph*.Co4 RLP50S	*Pgt* * RIF/ ACL (%)	*Pgl* RIF	*Phs*.F RIF	*Phs*.W RIF	*Phs*.G RIF	*Phm*.R RIF	*Pt*.B ! RIF	*Pt*.F RIF	*Pt*.I ! RIF	*Pp*.W RIF	*Phb*.Is RIF	*Phb*.Ir RIF
L94	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**n.d**	**n.d**	**100**
L94-*Rphq2*	104	106	106	99	89	24	96	53	67	60	88	109	n.d	n.d	84
L94-*Rphq3*	103	105	106	36	9	19	52	30	77	36	35	31	n.d	n.d	86
L94-*Rnhq.V*	n.d	101	100	97	4	n.d	15	10	26	48	75	61	n.d	n.d	93
SusPtrit	**100**	**100**	**100**	**60**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**
Sus-*Rphq2.1*	103	102	99	68	83	159	79	153	109	112	116	126	155	134	136
Sus-*Rphq3.1*	105	105	101	53	22	131	105	120	74	60	54	76	104	74	85
Sus-*Rphq3.6*	105	104	102	52	35	113	113	170	84	63	60	73	135	94	90
Sus-*Rphq11.6*	104	106	102	38	27	69	44	83	46	36	98	56	65	52	3
Sus-*Rphq11.12*	104	106	102	43	38	42	43	42	42	43	75	58	49	37	2
Sus-*Rphq16*	107	107	106	63	102	163	109	119	112	105	91	91	200	100	91
Sus-*Rnhq.La*	101	99	100	56	59	91	71	77	89	198	85	139	77	61	86
Sus-*Rnhq.Lb*	100	100	99	53	54	63	68	68	95	114	85	119	76	66	87
Sus-*Rnhq.V7*	100	104	102	54	44	70	60	68	79	105	82	94	55	53	70
Sus-*Rnhq.V10*	101	104	101	58	64	77	71	66	95	119	91	101	80	69	73

Shaded cells contain values that differ significantly from the value of the respective recurrent parent for that rust fungus ($\alpha = 0.05$).

* IF was scored for L94 and L94-NILs, ACL for Sus and SusPtrit-NILs

! Due to the great experimental error in some experiments, some NILs with fairly low RIF value were not significantly different from the reference line L94 or SusPtrit.

n.d , not determined

Preliminary histological observations on partial and nonhost resistance QTL NILs against non-adapted rust fungi

The NILs with *Rphq3*, *Rphq11* and *Rnhq.V*-introgression had a higher level of resistance than NILs containing one of the other introgressions when inoculated with a certain non-adapted rust fungi (Table 4). For those introgressions and a selection of rust fungi we determined the fate of about 50 infection units on two leaf samples per NIL/rust combination by UV microscopy (Table 5).

Rphq11-*introgression in SusPtrit*

The resistance observed on the NIL Sus-*Rphq11* to non-adapted rust fungi was for *Phb*.Ir and *Phs*.W due to high early abortion without hypersensitivity but for *Phm*.R and *Pp*.W no enhanced level of early abortion was observed. The *Rphq11*-introgression was not associated with high levels of autofluorescence near early aborted or established colonies. The established colonies on the sister NILs also did not necessary have smaller diameter than those on SusPtrit.

Rphq3-*introgression in SusPtrit and L94*

The *Rphq3*-introgression in SusPtrit and L94 affected resistance against *Pt*.F (Table 4) by increasing the proportion of early aborted colonies, with hardly or no induction of hypersensitivity. The introgression seemed to slow down the growth of established *Pt*.F colonies more obviously in L94 than in SusPtrit.

The background resistance present in L94 against *Phs*.G already caused a substantial early abortion of that rust fungus. The *Rphq3*-introgression did not increase early abortion further. The introgression seemed, however, to slow down the growth of established *Phs*.G colonies. We did not test the effect of the introgression in SusPtrit background, since in that background it did not seem to cause resistance (Table 4).

Rnhq.V-*introgression in L94*

The *Rnhq.V*-introgression affected resistance against non-adapted rust fungi such as *Phs*.W, *Phs*.G and *Phm*.R in L94 genetic background but not in SusPtrit background (Table 4). The resistance against *Phs*.W, *Phs*.G and *Phm*.R conferred by the *Rnhq.V*-introgression was not due to an enhanced proportion of early aborted colonies and was not associated with high levels of autofluorescence near

early aborted or established colonies. The introgression seemed to slow down the growth of established colonies of those non-adapted rust fungi.

In general, the resistance observed either increases the proportion of early abortion or restricts the growth of colonies or both. We also observed resistance confer by a QTL which seems to affect neither the proportion of early abortion nor the diameter of established colony, such as *Rphq11*-introgression against *Phm*.R and *Pp*.W. There is no systematic difference in the mechanism between the QTL-introgressions.

Table 5: The histology of the resistance to a selection of non-adapted rust fungi, conferred by introgressions carrying *Rphq11* or *Rphq3* or *Rnhq.V* in either SusPtrit or L94 background

QTL	Rust fungus	Line	EA % (EA+N) [α]	Est % (Est + N) [β]	Diameter of Est infection units (μm)
Rphq11	*Phb*.Ir [!]	SusPtrit	4 (0)	96 (0)	346
		Sus-*Rphq11.6*	**24 (0)**	76 (4)	232*
	Phs.W	SusPtrit	4 (0)	96 (0)	245
		Sus-*Rphq11.6*	**29 (0)**	71 (4)	265
		Sus-*Rphq11.12*	**31 (4)**	69 (3)	237
	Phm.R	SusPtrit	17 (17)	83 (36)	247
		Sus-*Rphq11.6*	19 (10)	81 (29)	228
		Sus-*Rphq11.12*	27 (35)	73 (50)	259
	Pp.W	SusPtrit	46 (0)	54 (0)	251
		Sus-*Rphq11.6*	34 (0)	66 (8)	266
		Sus-*Rphq11.12*	60 (0)	40 (7)	234
Rphq3	*Phs*.G	L94	50 (16)	50 (22)	199
		L94-*Rphq3*	40 (13)	60 (43)	139*
	Pt.F	L94	17 (0)	83 (7)	338
		L94-*Rphq3*	**41 (6)**	59 (16)	172*
		SusPtrit	23(0)	77 (1)	360
		Sus-*Rphq3.1*	**49(0)**	51(0)	336
		Sus-*Rphq3.6*	**60(0)**	40(0)	324
Rnhq.V	*Phs*.W	L94	17 (0)	83 (42)	336
		L94-*Rnhq.V*	25 (19)	75 (41)	203*
	Phs.G	L94	50 (16)	50 (22)	199
		L94-*Rnhq.V*	56 (9)	44 (38)	129*
	Phm.R	L94	37 (6)	63 (5)	344
		L94-*Rnhq.V*	39 (3)	61 (11)	241*

n.d. not determined
EA, early aborted colonies
Est, established colonies
+N, associated with autofluorescence
[α] in ( ) is the percentage of EA+N colonies from the total EA colonies
[β] in ( ) is the percentage of Est+N colonies from the total Est colonies
* The means of L94-NILs significantly smaller than the mean of L94 or SusPtrit-NILs from SusPtrit ($\alpha = 0.05$)
[!] t-test was used to analyse the data
Bold: The proportion of EA on the NIL is more than in the recurrent parent (not statistically tested)

Discussion

The susceptibility of L94 and SusPtrit to *P. hordei* made these lines suitable for development of NILs to study partial resistance. These NILs could also be studied to determine a possible pleiotropic effect of partial resistance genes on nonhost resistance to non-adapted rust fungi. SusPtrit is an experimental line which is at the seedling stage susceptible to at least nine non-adapted rust fungi (Atienza et al. 2004), and L94 has a remarkable level of susceptibility to several non-adapted rust fungi (Niks 1983a), but less extreme than SusPtrit in direct comparison (Atienza et al 2004). The substantial level of nonhost resistance in L94 makes it less ideal than SusPtrit to study a possible pleiotropic effect of partial resistance genes on nonhost resistance to non-adapted rust fungi.

The NILs can serve as materials to fine-map the QTLs in the introgressions, and later to map-based clone and functional characterization of the responsible genes. In this study, the NILs developed were used to confirm the resistance QTLs found in earlier mapping studies, to quantify their effect without the interference of other QTLs, and to study their spectrum of effectiveness to several adapted and non-adapted rust fungi.

The QTL-NILs suggested that the effects of the partial resistance genes depended on rust species and rust isolate. Some introgressions conferred a broader resistance spectrum than others, the broadest being *Rphq11*-introgression. This introgression was selected to be introduced into SusPtrit on the basis of its relatively large contribution (30% explained variation) to partial resistance to *P. hordei* in the population Steptoe x Morex (Marcel et al, 2007b). In the NILs the introgression appeared to confer broad spectrum resistance to nearly all non-adapted rust fungi included in the test. The NILs may overestimate the spectrum of effectiveness of the partial resistance genes for two reasons. First, some NILs contain inadvertent donor genome in the background (away from QTL region, Figure 1) which by chance may have additional resistance genes to the same or other rust isolate/species. Second, the introgressed QTL region may contain several resistance genes, each with a narrow spectrum of effectiveness, but together conferring a broad resistance spectrum against different rust isolates/species. Therefore, individual genes may be more specific than suggested by the data in Table 4. If a certain QTL-NIL has resistance to one rust isolate but not to another, the specificity should be real, since the same QTL-NIL was tested against different rust isolates, such as the *Rphq2*, *Rphq16* and *Rnhq.V*-introgressions in SusPtrit against *Ph*.1.2.1. Isolate and species specificity of nonhost and partial resistance QTLs were observed in previous studies as well (González et al. 2012; Jafary et al. 2006; 2008; Marcel et al. 2008; Parlevliet 1977; 1978).

We compared the effect of *Rphq2*, *Rphq3* and *Rnhq.V* against adapted and non-adapted rust fungi in SusPtrit and in L94 background. If an introgressed QTL is effective in L94 but not in SusPtrit and vice versa to a certain rust fungus (species/isolate), this may be due to the size of the introgressions in the two recurrent parents that may not cover an identical stretch of the chromosome, and hence absence or presence of additional resistance gene(s) on either of the introgressions. Possible inadvertent undesired donor segments in the background can also cause the different QTL effect observed in L94 and SusPtrit (as explained above). For example, *Rphq3* confers resistance to the three isolates of *Phs* in L94 but not in SusPtrit background. The resistance may be due to the extra length of *Rphq3*-introgression in L94 (35 cM) compared to SusPtrit (16 cM) or the inadvertent undesired donor segments on 3H and 5H respectively (Figure 1).

The SusPtrit and L94 NILs are also different in recurrent genome and cytoplasmic materials since SusPtrit and L94 were the female recurrent parents during the NIL development program. The difference in the spectrum of effectiveness for a gene in two backgrounds may be due to interactions between the introgressed gene with other gene elsewhere on the genome (Holland 2007; Lagudah 2011) or with a cytoplasmic factor (Akula et al. 2012; Levings and Siedow 1992; Mazouz et al. 2002) present in one of the two backgrounds.

The resistance of the NILs against different non-adapted rust fungi does not seem to be associated with a hypersensitive response but ranges from occasionally to heavily associated with hypersensitive response at macroscopic level (Figure 3). This hypersensitive response can also be observed on the reference lines viz. L94 and SusPtrit against some non-adapted rust fungi.

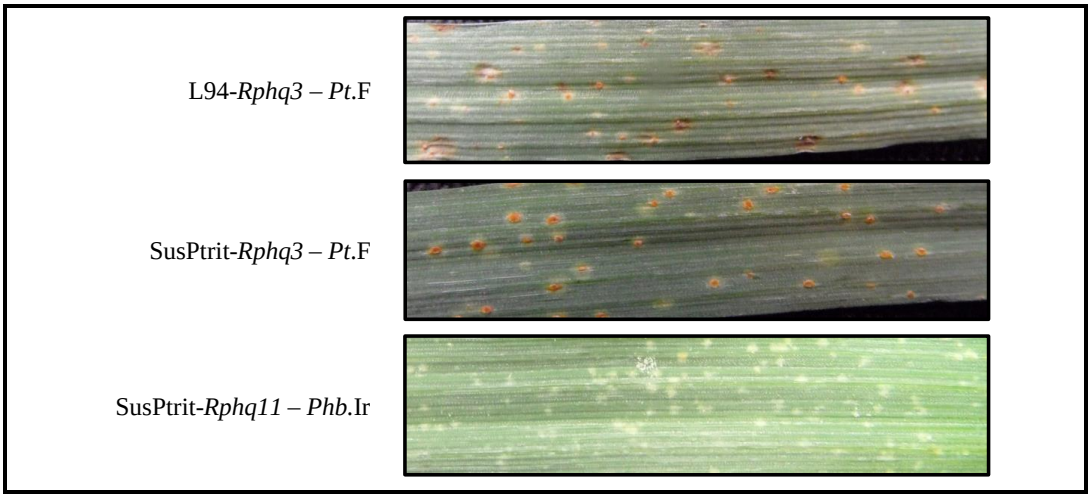

Figure 3: The range of hypersensitive response on NILs inoculated with non-adapted rust fungi.

At microscopic level, the resistance observed on the NILs carrying *Rphq11* and *Rphq3* to non-adapted rust fungi involved for some rust fungi a prehaustorially acting mechanism and may involve some level of hypersensitivity in the L94-*Rphq3*-*Pt*.F interaction. For other rust fungi (*Phm*.R, *Pp*.W and *Phs*.G) the resistance is not due to a high proportion of early aborted colonies and this is also true for L94-*Rnhq.V* inoculated with *Phs*.W, *Phs*.G and *Phm*.R. The resistance conferred by the introgressions against the respective rust fungi can probably be due to slower growth of infection units, leading to smaller colony size such as the interaction between L94-*Rphq3* with *Phs*.G and L94-*Rnhq.V* against *Phs*.W, *Phs*.G and *Phm*.R, but not on the sister NILs of *Rphq11* inoculated with *Pp*.W and *Phm*.R. The resistance conferred by *Rphq11*-introgression against *Pp*.W and *Phm*.R probably is due to late abortion, i.e. abortion of colonies after establishment, but before sporulation (Niks 1982). The growth and development of colonies may have been arrested at a later stage than the moment we collected the leaf segments (5dpi). Further histological investigation is needed to establish the fungal development stage in which the resistance interferes with the infection process.

Our disease tests show that QTLs such as *Rphq2* and *Rphq16* in SusPtrit background affect resistance specifically to *P. hordei*. Other QTLs such as *Rphq3* in L94 background and *Rphq11* in SusPtrit background seem to have broader resistance spectrum. However, the NILs that we developed may overestimate the spectrum of effectiveness for a QTL. If the spectrum of effectiveness of the introgressions reflects indeed the pleiotropic effect of a single (quantitative) gene is true, this study would add evidence that partial resistance and nonhost resistance are (partly) based on the same genes, and hence are evolutionary and mechanistically part of the same principal (González et al. 2010; Jafary et al. 2008; Niks and Marcel 2009; Zellerhoff et al. 2010).

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Supplemental Table

Supplemental Table 1: The markers used for marker assisted selection in the development of NILs for targeted QTLs

QTLs	Name	Type	Chrom	Restriction enzyme	Tm (°C)	Primer sequences
Rphq2	k00345	CAPs	2H	SduI	56	*F*: TTCCTTCCATGGCTTTTGAC *R*: AAGGCACACATCCACCTTTC
	besV76P5D5AR	SCAR	2H		56	*F*: GAGGAGCCGTGTCGTCTTGT *R*: CCGTTTCCGTTCACTGGTTAT
	scP15M51-204	SCAR	2H		56	*F*: CGGAGGAAACATGGACAACGAA *R*: AGCGAGCTCACTGCCAATCTACC
	GBMS216	SSR	2H			Confidential
Rphq3	ABG388	CAPs	6H	NlaIII		*F*: GCACTGGCATAGTCTCACAA *R*: CGATGCTGGTTCGGTCATAC
	WBE201	CAPs	6H	MnlI	58	*F*: GGTCAGCAATTCCCCAAAGTT *R*: AATGCCGAAATCTCCCAAATGA
	GBM1212	SSR	6H			*F*: TGTTGCAAGAAGCAAGGATG *R*: GCGCTTACTCTCTCGTCGTC
	HVM14	SSR	6H			*F*: CGATCAAGGACATTTGGGTAAT *R*: AACTCTTCGGGTTCAACCAATA
	HVM22	SSR	6H			*F*: TTTTGGGGGATGCCTACATA *R*: TTTCAAATGGTTGGATTGGA
Rphq11	GBS0512	CAPs	2H	AciI	58	*F*: CCACATGCTGCGGAGGT *R*: CGTTGAGGATGATGCTGAGG
	GBM1062	SSR	2H			Confidential
	GBMS244	SSR	2H			Confidential
Rphq15	MWG966	CAPs	6H	NlaIV	57	*F*: ATGCGTGCCCTTTGGAACA *R*: TGGCCTGCGATATGGAGACC
	GBMS033	SSR	6H			Confidential
	scssr09398	SSR	6H			*F*: AGAGCGCAAGTTACCAAGC *R*: GTGCACCTCAGCGAAAGG
Rphq16	scsnp03275	CAPs	5H	BglII	65	*F*: AACGGCCAGGCTATAACCATCACA *R*: CGGCGGCTTCATCAATTTCACTAA
	Dst-33	SCAR	5H		45	*F*: GCACACATATTATCATGAAAAAGAGC *R*: ACCCCAAATGAGTTTCGATG
	GMS002	SSR	5H			*F*: CCGACAACATGCTATGAAGC *R*: CTGCAGCAAATACCCATGTG
Rphq17	Bmag0136	SSR	3H			*F*: GTACGCTTTCAAACCTGG *R*: GTAGGAGGAAGAATAAGGAGG
	Bmac0067	SSR	3H			*F*: AACGTACGAGCTCTTTTTCTA *R*: ATGCCAACTGCTTGTTTAG
Rnhq.V *Rnhq.L*	MWG2031	CAPs	7H	MwoI	55	*F*: TGTGACCTGTCAGACTGTTCAAGTT *R*: TACGTCGGCATAATTGGCA
	SKT1	CAPs	7H	AluI	60	*F*: TGGACCTCATAGCAGCCTTT *R*: GGTGCCACTGAGATTCACCT
	WBE101	CAPs	7H	HpyCH4IV	52	*F*: CGAGCGCCTGACGGACGAT *R*: CTCACGGCCCAGACATAGC
	GBM1303	SSR	7H			*F*: TCTTTTTGGAGGGGTTTCCT *R*: ATCATCTTCACGCTTCCTCC

Chapter 4

High resolution mapping of genes involved in plant stage specific, partial resistance of barley to leaf rust

To be submitted

High resolution mapping of genes involved in plant stage specific, partial resistance of barley to leaf rust

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Abstract

Partial resistance QTLs, *Rphq11* and *Rphq16*, were first mapped against *Puccinia hordei* isolate 1.2.1 in seedlings of the mapping populations Steptoe/Morex (S/M) and Oregon Wolfe Barleys (OWB), respectively. In this study, QTL mapping was performed at adult plant stage for the two mapping populations challenged with the same rust isolate. In none of the series of disease tests for S/M and OWB we detected our target QTLs *Rphq11* and *Rphq16*. Therefore, it is likely that these two QTLs are effective only at the seedling stage, and not at the adult plant stage. Cloning of several genes responsible for partial resistance of barley to *P. hordei* is undertaken in order to elucidate the molecular basis of this type of plant defence. A map-based cloning approach implies first to fine-map the QTL in a narrow genetic window. The commonly followed fine-mapping strategy makes use of near isogenic lines (NIL). NIL development is very laborious and time consuming. For barley, the development of a NIL with approximately 95% genome of the recurrent parent at generation BC_4 will take approximately two years and four months. Another year will be necessary to obtain enough seeds of homozygous recombinant plants (*i.e.* sub-NILs) to allow fine-mapping. In total, about three years and four months are needed to fine-map a QTL to a certain genetic window. In this study, fine-mapping of partial resistance QTLs *Rphq11* and *Rphq16* was carried out using an approach aiming at speeding-up the development of plant material and simplifying its evaluation. The plant materials for fine-mapping were identified from early breeding materials (F_2 for *Rphq11* and BC_1/BC_2 for *Rphq16*) developed to produce QTL-NILs. The material was first selected to carry the targeted QTL in heterozygous condition and susceptibility alleles at other resistance QTLs in homozygous condition. This strategy took four to five generations to obtain fixed QTL-recombinants (i.e. homozygous recombinants at the *Rphq11*/*Rphq16* QTL alleles, homozygous susceptible at the non-targeted QTL alleles). Their genomic background was still segregating, but expected not to be of influence on the resistance level. In less than 2 years, *Rphq11* was fine-mapped into a 0.2 cM genetic interval and a 1.4 cM genetic interval for *Rphq16*. The strongest candidate gene for *Rphq11* is phospholipid hydroperoxide glutathione peroxidase (PHGPx). This gene corresponds to the new *Rphq11* peak marker – WBE129, located within the refined 0.2 cM genetic interval and was one of the candidate genes for *Rphq11* identified through eQTL mapping on S/M challenged with the same rust isolate. There was no clear candidate gene identified for *Rphq16*.

Keywords: High Resoltuion Mapping, Quantitative Trait Locus (QTL), Specificity, *Puccinia*, Barley

Introduction

Partial resistance of barley against barley leaf rust (*Puccinia hordei*) results in a reduced epidemic, despite a compatible infection type (Parlevliet 1979). The epidemic reduction is due to a lower infection frequency, lower sporulation rate and longer latency period of the pathogen on barley accessions with high levels of partial resistance (Parlevliet 1979). Partial resistance is a prehaustorial resistance, where failed attempts to form haustoria are associated with cell wall reinforcements, called papillae (Niks 1986; O'Connell and Panstruga 2006). The failure of a proportion of the haustorium formation reduces the capacity for nutrient extraction from the plant and for delivery of pathogenicity promoting effectors into the plant cells (Catanzariti et al. 2007; de Jonge et al. 2011). This mechanism of resistance is similar to, but less complete than, nonhost resistance of barley to non-adapted rust fungi like *P. recondita* and *P. triticina* (Niks 1983, 1989).

Partial resistance of barley to *P. hordei* is polygenically inherited and is supposed to act on a minor-gene-for-minor-gene model (González et al. 2012; Marcel et al. 2008; Niks et al. 2000; Parlevliet and Zadoks 1977; Qi et al. 1999). There is an abundance of quantitative trait loci (QTL) for partial resistance against barley leaf rust. To date, at least 20 partial resistance QTLs against barley leaf rust have been mapped in different bi-parental mapping populations. In each barley mapping population, a different set of QTLs was identified, with few QTLs shared among the populations. The explained phenotypic variation per QTL ranges from around three to 50 per cent (Jafary et al. 2008; Marcel et al. 2007b; 2008; Niks et al. 2000; Qi et al. 1998; 1999; Yeo et al. 2014).

QTL mapping studies indicated that the resistance QTLs can be effective across different stages of plant development or only at specific stages (Qi et al. 1998), which was confirmed on QTLs that were introgressed into near isogenic lines (NILs) (Wang et al. 2010). Plant growth stage dependent effects of resistance QTLs have also been observed in other plant-pathosystems (Aghnoum et al. 2010; Dedryver et al. 2009; Shankar et al. 2008; Steffenson et al. 1996). Consequently, different sets of QTLs will protect barley plants against leaf rust at different growth stages. It is important to know the effect of QTLs at different growth stages before to design breeding strategies or to engage in a positional cloning procedure.

Cloning of several genes responsible for partial resistance of barley to *P. hordei* is undertaken in order to elucidate the molecular basis of this type of plant defence (Marcel et al. 2007a). Fine-mapping and positional cloning requires the evaluation of very large numbers of plants in a controlled environment and a similar physiological condition,

which is often only feasible at the seedling stage. To date, no QTLs for resistance to rust fungi or powdery mildew have been cloned in barley. However, three large-effect resistance QTLs have been cloned in rice; two against *Magnaporthe oryzae* (Fukuoka et al. 2009; Hayashi et al. 2010) and one wide-spectrum QTL against *Rhizoctonia solani* and *M. oryzae* (Manosalva et al. 2009). There are two cloned QTLs in wheat; one against *P. striiformis* (Fu et al. 2009) and one wide-spectrum QTL against *P. triticina, P. striiformis* and *Blumeria graminis* (Krattinger et al. 2009). All the genes cloned so far belong to different gene families and are involved in different functions, suggesting a wide diversity of mechanisms underlying partial resistance.

A map-based cloning approach implies first to fine-map the QTL in a genetic window sufficiently narrow to make physical mapping feasible. This approach requires the effect of the QTL to be sufficiently clear to infer the QTL genotype from its phenotype; the phenotypic variation explained by the QTL should be more than 10% according to Kou and Wang (2012). The commonly followed fine-mapping strategy makes use of near isogenic lines (NIL). In non-isogenic plant materials, other QTLs may be segregating in the genetic background blurring the determination of the phenotypic effect of the QTL of interest. The NIL carrying the targeted QTL is crossed with its recurrent parent to "Mendelize" the QTL in the resulting progeny. Then, a selection of plants recombining at the QTL containing chromosome region (*i.e.* sub-NILs) and the evaluation of their phenotype allow to pin-point the targeted QTL into a refined genetic position (Han et al. 1999; Marcel et al. 2007a; Xue et al. 2010; Zhou et al. 2010). Fine-mapping using this strategy is efficient but very laborious and time consuming in generating the plant materials, marker development and genotyping.

Rphq11 and *Rphq16* are partial resistance QTLs that were first mapped against *P. hordei* isolate 1.2.1 in seedlings of the mapping populations Steptoe/Morex (S/M) and Oregon Wolfe Barleys (OWB), respectively (Marcel et al. 2007b). They are effective at seedling stage in their respective mapping population, each explaining approximately 30% of the phenotypic variance. *Rphq11* was mapped at seedling stage near the middle of chromosome 2HL and the resistance allele was contributed by Steptoe. It was also detected by Chen et al. (2010) as an expression QTL (eQTL) co-locating with the phenotypic QTL (pQTL) in the same mapping population. Six candidate genes were suggested by those authors that may explain *Rphq11*. *Rphq16* was mapped at seedling stage near the telomeric region of chromosome 5HL and the resistance allele was contributed by Dom.

The first objective of this study was to test whether *Rphq11* and *Rphq16* are also effective at adult plant stage. The second objective was to fine-map *Rphq11* and *Rphq16* using an

approach aimed at speeding-up the development of plant material and simplifying its evaluation with the final aim of cloning them. *Rphq11* and *Rphq16* qualify for map-based cloning to study partial resistance because of sufficient effect size at seedling stage.

Materials and methods

Inoculum

All the disease tests in this study were done with *P. hordei* isolate 1.2.1 (*Ph*.1.2.1), the same isolate used in (Marcel et al. 2007b). This isolate is a monospore purification of the isolate 1.2 collected in the Netherlands in 1971 (Parlevliet 1976).

QTL mapping on adult plants

The Doubled Haploids (DH) mapping populations S/M (Kleinhofs et al. 1993) and OWB (Costa et al. 2001) were used to map QTLs for non-hypersensitive quantitative resistance at the adult plant developmental stage (heading stage Z51-Z55 on Zadoks' growth scale). Marker segregation data of S/M (150 DH lines) and OWB (94 DH lines) were extracted from the barley integrated map [Barley, Integrated, Marcel 2009 available at http://wheat.pw.usda.gov/GG2/index.shtml; (Aghnoum et al. 2010)]. Data comprise 3561 segregating markers in S/M and 882 in OWB. Skeletal maps were generated for S/M and OWB by selecting markers homogeneously distributed over the integrated map, spaced at approximately 1-5 cM intervals.

The parental lines Steptoe and Morex or Dom and Rec and the reference barley lines L94 and Vada were included in each experiment. Parental and reference lines were sown continuously every 3 days from one week before to one week after the sowing of the DH lines of the two mapping populations. For each line, three seeds were sown in a pot. To ensure the uniformity of the developmental stage of the plants at the time of inoculation, plants of a mapping population were divided into 3-4 subgroups based on their heading date. For each subgroup, plants of parental and reference lines with a similar developmental stage were added. Three series – at different times of the year, were performed with three individuals per DH line for the first and second series, and one individual per DH line for the third series. The first and second series were conducted at a different greenhouse facility than the third series.

Plants were inoculated after the flag leaf was unfolded (around heading stage Z52). Per pot, one milligram of spores diluted 10 times with lycopodium spores were used as inoculum. Before the inoculation, the pots were lined-up two by two. Then, the inoculum was dusted over the plants as uniformly as possible. The inoculated plants were then placed in a humidity chamber overnight (8 hours) at 100% relative humidity in the dark at 18^{o}C to allow the spores to germinate. After incubation, the plants were transferred to a greenhouse compartment where the temperature was set at 20 ± 3^{o}C with 30-70% relative humidity.

The flag leaf (F) of the three plants in each pot was scored for latency period (LP50A). It was scored daily by counting the mature pustules on a marked area of the F leaf until all the pustules matured. Latency period estimates the period of time in hours at which 50% of the total number of pustules is mature. It is among the most informative components of barley partial resistance to leaf rust and is relatively easy to measure (Niks et al. 2011; Parlevliet 1979). Relative latency period (RLP50A) was calculated relative to LP50A of Steptoe for S/M and relative to LP50A of Dom for OWB.

ANOVA was performed using GenStat® 14th edition (VSN International Ltd. 2011). QTLs were mapped using MapQTL®6 (van Ooijen 2009). A permutation test was performed to set the LOD threshold to declare a QTL. The confidence interval of a QTL is the estimated LOD-2 support interval.

The data for heading date (HD) and plant height (PH) of S/M were downloaded from GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml). The QTLs for HD and PH were mapped on the S/M skeletal map generated from the barley integrated map [(Barley, Integrated, Marcel 2009; (Aghnoum et al. 2010)].

Selecting plant material segregating for a single target QTL

Previous QTL mapping studies at seedling stage revealed in the S/M population two QTLs contributed by Steptoe, *Rphq11* and *Rphq15*, and in the OWB population two QTLs contributed by Dom, *Rphq16* and *Rphq17* (Marcel et al. 2007b). Steptoe and Dom were first crossed, and then recurrently backcrossed with the susceptible experimental line SusPtrit (Atienza et al. 2004). Molecular markers flanking the QTLs were used to select for the susceptible QTL alleles of *Rphq15* and *Rphq17*, and to select for the resistance QTL alleles of *Rphq11* and *Rphq16* at each generation. *Rphq11* and *Rphq16* were finally introgressed into SusPtrit by backcrossing over five generations for *Rphq11* and six

generations for *Rphq16* to obtain NILs (Figure 1). Details and results obtained with the NILs will be published elsewhere.

For *Rphq11*, the backcross of F_1 plants to SusPtrit yielded a low number of BC_1 seeds. Therefore, some F_1 individuals were selfed to obtain the F_2 generation. At the F_2 generation, the resistance allele of *Rphq11* was selected with three simple sequence repeat (SSR) markers (Bmag0125, GBM1062 and GBMS244) while the susceptibility allele (from SusPtrit) of *Rphq15* was selected with two SSR markers (scssr09398 and GBM033) and a cleaved amplified polymorphic sequence (CAPS) marker (MWG966) (Table 1). The selected plants were heterozygous for the target QTL but lacked the second gene for resistance of the donor line at the other QTL. They were selfed, and 97 F_3 seedlings were inoculated with *Ph.*1.2.1 (Figure 1). Their latency period was scored (LP50S) and the relative latency period (RLP50S) was calculated relative to SusPtrit. The F_3 seedlings were also genotyped with the markers flanking *Rphq11*. The plants were grouped according to their QTL allele, homozygous Steptoe (AA), heterozygous (AB) and homozygous SusPtrit (BB), to estimate the effect and the dominance/recessiveness of *Rphq11* at the seedling stage. Plants that had a recombination between the QTL flanking markers were excluded from the analysis. The data were analysed with Unbalanced One-way ANOVA using GenStat® 14th edition (VSN International Ltd. 2011).

For *Rphq16*, the backcross of F_1 plants to SusPtrit was successful and a sufficient number of BC_1 seeds were obtained. The resistance allele of *Rphq16* was selected with two CAPS markers (ABG390 and ABG391) and two SSR markers (GMS002 and scssr09041) while the susceptibility allele (from SusPtrit) of *Rphq17* was selected with two SSR markers (Bmac0067 and Bmag0136) (Table 1). Similar to *Rphq11*, the selected plants were heterozygous for *Rphq16* but lacked the other resistance QTL *Rphq17* allele. Those plants were selfed and 52 BC_1S_1 seedlings were inoculated with *Ph.*1.2.1 (Figure 1). The QTL effect and the dominance/ recessiveness of *Rphq16* were estimated as described for *Rphq11*.

Fine-mapping Rphq11 and Rphq16

Among the 97 F_3 plants for *Rphq11* and among the 52 BC_1S_1 plants for *Rphq16*, there were 12 and 18 recombinant plants, respectively. These plants were grown to set seeds which were then used to identify plants with homozygous recombination. These plants were then homozygous recombinants at the *Rphq11*/*Rphq16* QTL alleles, homozygous susceptible at the *Rphq15*/*Rphq17* QTL alleles, but their genomic background was still segregating. For simplicity, these plants will be called "fixed QTL-recombinants" from

this point onwards. These fixed QTL-recombinants were already used to refine the positions of *Rphq11* and *Rphq16* (data not shown). Based on the refined positions, new flanking markers were selected for *Rphq11* (GBS0512 and GBMS244) and for *Rphq16* (scsnp03275 and GMS002) (Table 1).

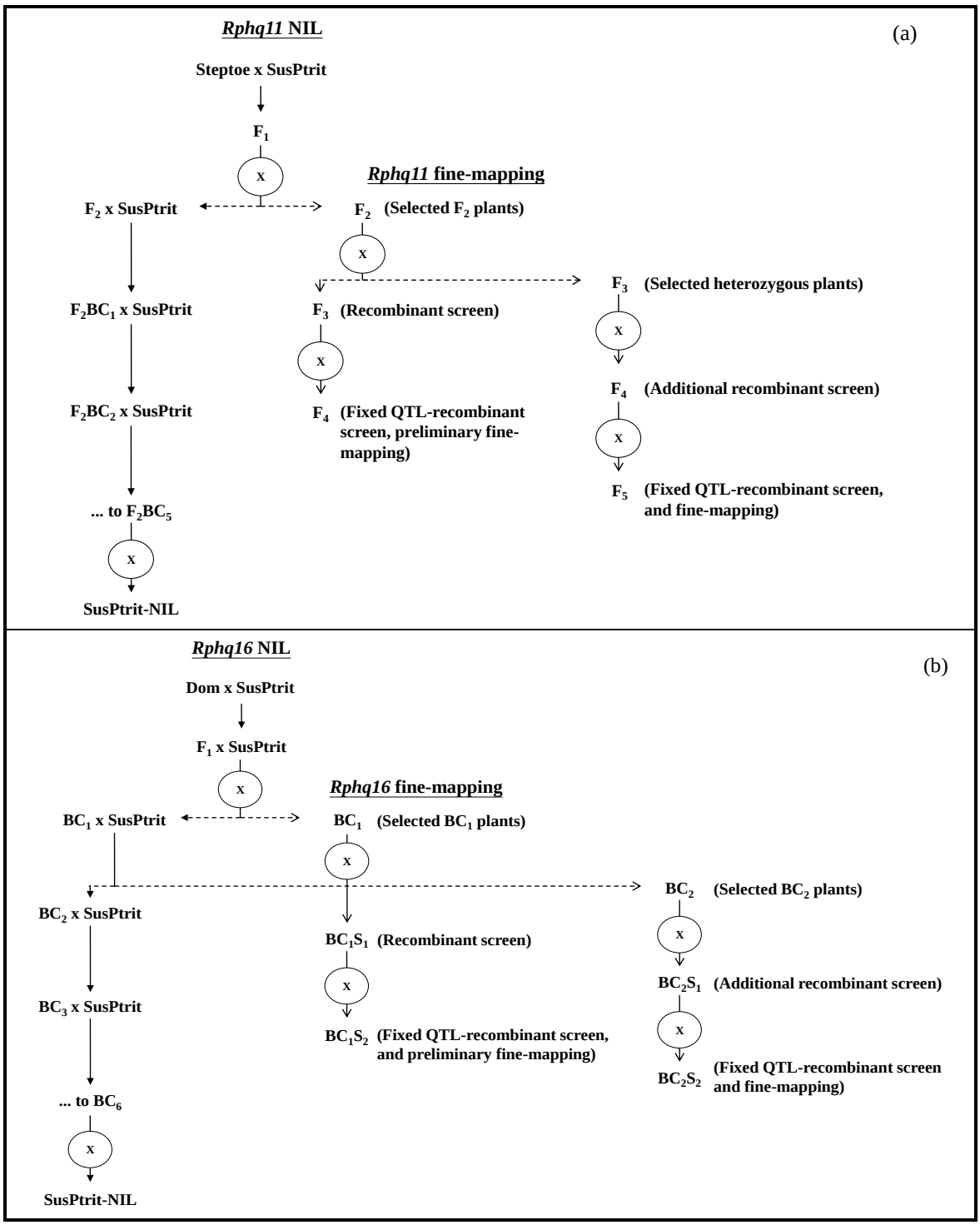

Figure 1: Procedure for fine-mapping a) *Rphq11* and b) *Rphq16* in parallel with their NIL development programs.

Table 1: Molecular markers used to select alleles at QTLs for resistance to *P. hordei* from barley cultivars Steptoe and Dom.

QTLs	Chrom.	Position[a]	Markers	Position[a]	Types	Ref.[b]
Rphq11[$]	2H	90	Bmag0125	84	SSR	Varshney et al. (2007)
			GBM1062	91	SSR	Varshney et al. (2007)
			GBS0512	92	CAPS	Stein et al. (2007)
			GBMS244	97	SSR	Varshney et al. (2007)
Rphq15[*]	6H	22	scssr09398	5	SSR	Varshney et al. (2007)
			MWG966	18	CAPS	Graner et al. (1991)
			GBMS033	26	SSR	Varshney et al. (2007)
Rphq16[$]	5H	170	ABG391	154	CAPS	Rostoks et al. (2005)
			ABG390	158	CAPS	Rostoks et al. (2005)
			scsnp03275	-	CAPS	Rostoks et al. (2005)
			GMS002	179	SSR	Varshney et al. (2007)
			scssr09041	179	SSR	Varshney et al. (2007)
Rphq17[*]	3H	64	Bmag0136	64	SSR	Varshney et al. (2007)
			Bmac0067	67	SSR	Varshney et al. (2007)

[a] The position is based on the integrated map (GrainGenes: Marcel 2009)

[b] References for the CAPS markers give the origin of the sequences obtained for marker development. Primer sequences and annealing temperature are available in supplemental data

[$] Resistance allele selected for

[*] Resistance allele selected against

In order to further fine-map *Rphq11* and *Rphq16*, the F_3 plants heterozygous for *Rphq11* and BC_2 plants heterozygous for *Rphq16* were selfed to produce a large number of seeds. New recombinants for *Rphq11* and *Rphq16* were identified by screening the F_4 and BC_2S_1 plants with the new flanking markers for *Rphq11* (GBS0512 and GBMS244) and *Rphq16* (scsnp03275 and GMS002). The same markers were used to identify fixed-QTL recombinants for both QTLs in the subsequent generation. The fixed QTL-recombinants were then genotyped with all molecular markers located in the QTLs vicinities to generate high resolution genetic maps around *Rphq11* and *Rphq16*.

The fixed QTL-recombinants were subjected to four rounds of disease tests for *Rphq11* and three for *Rphq16* (Figure 2). At each round, a different subset of the fixed QTL-recombinants was strategically selected based on previous results of disease tests in order to progressively refine the map position of the QTL. This strategy allowed more individuals to be tested per fixed QTL-recombinant at each round, increasing the confidence in the phenotype. SusPtrit was included in all disease tests as susceptible reference.

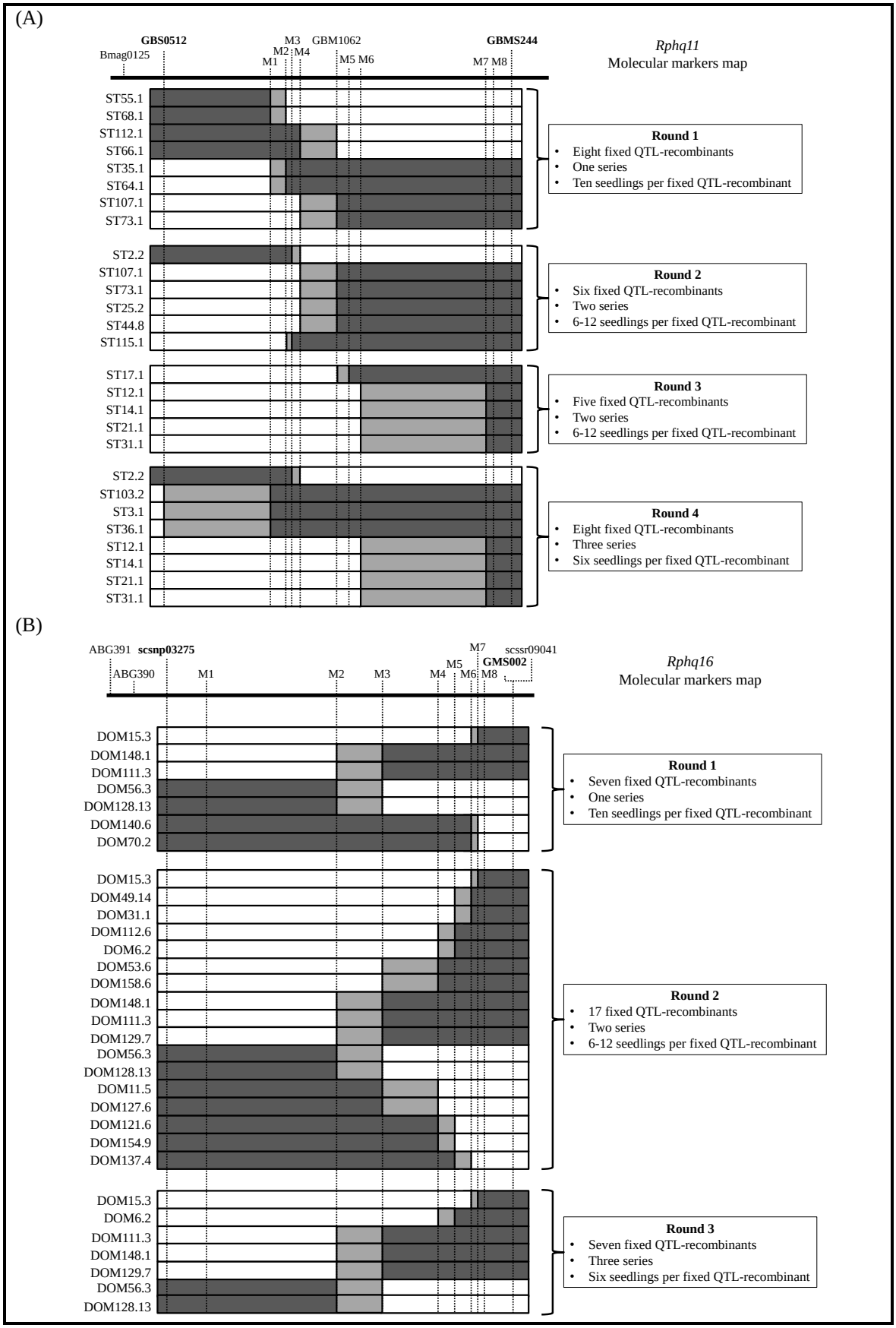

Figure 2: Rounds of disease test with fixed QTL-recombinants for (a) *Rphq11* and (b) *Rphq16*. The graphical genotypes represent the fixed QTL-recombinants tested in each round. The white bars represent homozygous SusPtrit; black bars represent homozygous Steptoe (a) or Dom (b); grey bars represent intervals where recombination occured. Markers in bold are the flanking markers used after preliminary fine-mapping. M – New markers obtained (Figure 7).

Disease tests were performed at seedling stage following the method of Qi et al. (1998). The latency period (LP) was measured. The relative latency period on seedlings (RLP50S) was calculated by setting SusPtrit at 100. Data from different rounds of disease test were analyzed together under a linear mixed model with GenStat® 14th edition (VSN International Ltd. 2011). The significant difference in mean RLP50S between fixed QTL-recombinants and SusPtrit was determined based on the least significant difference (LSD, $P < 0.05$).

Genomic DNA of the plant materials for recombinant screening was extracted following the method of Wang et al. (1993), adjusted for a 96-well format. This method allows quick genotyping of large numbers of plants, to recover rare recombinant plants. sbeadex® maxi plant kit (LGC Genomics) was used to isolate DNA of recombinant plants for selection of fixed QTL-recombinants.

Marker saturation of Rphq11 and Rphq16 intervals

Two approaches were followed to develop 20 molecular markers in the approximately 13 cM interval of *Rphq11* and to develop 27 molecular markers in the approximately 25 cM interval of *Rphq16*. All the markers developed are polymorphic in SusPtrit/Steptoe as well as in SusPtrit/Dom.

Approach I: Molecular markers that mapped within the intervals of *Rphq11* and *Rphq16* on the integrated map (GrainGenes: Barley, Integrated, Marcel 2009) were targeted for generating new PCR based markers segregating in our material. Sequence information of targeted Restriction Fragment Length Polymorphism (RFLP) markers and Transcript Derived Markers (TDM) were used to design specific primer pairs. For RFLPs, sequences were downloaded from the GrainGenes database (http://wheat.pw.usda.gov/GG2/index .shtml). For TDMs, unigene sequences were downloaded from the Barley SNP Database (http://germinate.scri.ac.uk/barley_snpdb/dbStats_contig.html) (Potokina et al. 2008). For Sequence Tagged Sites (STS) markers, the primer sequences were obtained directly from the GrainGenes database. The primer sequences of Simple Sequence Repeat (SSR) were obtained from literature (Varshney et al. 2007). Sequence for the Diversity Array Technology (DArT) marker ctg15632 and primers for the Cleaved Amplified Polymorphic Sequence (CAPS) marker Uni19962 have been reported elsewhere (Boyd et al. 2007).

Approach II: Conserved microsynteny between barley, rice and *Brachypodium distachyon* was also used to generate new markers closely linked to *Rphq11* and *Rphq16*. The sequences of EST-based markers mapped in the vicinity of *Rphq11* and *Rphq16* were

used for blast searches of rice and *Brachypodium distachyon* (*B. distachyon*) homologous genes, respectively, in the Rice Genome Annotation Project blast search (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml) and in the *B. distachyon* blast portal (http://blast.brachypodium.org/). Rice and *B. distachyon* gene sequences within the identified synteny blocks were in turn blasted against the barley EST tentative consensus (TC) sequences from the barley TIGR Gene Indices database (http://www.tigr.org/tdb/tgi/index.shtml). Only barley TC sequences with a blast hit having an E-value $\leq 10^{-15}$ were further considered for primer design and marker development. To maximise the chance of developing markers that map in the target regions of the barley genome, only barley TC sequences having an homologous gene in the syntenic regions of both rice and *B. distachyon* were further considered for primer design and marker development.

Primers were designed using the Lasergene software (DNASTAR® 8 Inc., Madison, WI, USA). For each primer pair a gradient PCR was performed to determine the optimal annealing temperature. Sequence Characterized Amplified Region (SCAR) markers were obtained by finding length polymorphism or allele-specific amplification directly after PCR on parental lines (SusPtrit, Steptoe – *Rphq11* donor, and Dom – *Rphq16* donor). For primers that amplified bands of the same size in parental lines, CAPS markers were developed. The PCR products were sent for sequencing (BaseClear, Leiden, the Netherlands). SNPs were identified from the sequence obtained, using the Lasergene software. The dCAPS finder program [http://helix.wustl.edu/ dcaps/dcaps.html; (Neff et al. 2002)] was then used to find discriminating restriction enzymes.

Markers developed based on TDMs, synteny and eQTL candidate genes were named as WBE for Wageningen Barley ESTs.

Results

Mapping QTL for partial resistance at adult stage in S/M and OWB populations

A significant series x genotype effect was observed for the adult plant disease tests of both S/M and OWB mapping populations. Consequently, QTL mapping was performed for each series independently. In both populations and in each series, RLP50A showed a continuous distribution of phenotypes with transgressive segregation (Supplemental Figure 1). On Steptoe and Rec RLP50A was always higher than on Morex and Dom, respectively, except in the first series for the OWBs where Rec and Dom had nearly the same RLP50A. A permutation test suggested a LOD threshold of 3 for each series. A QTL

was declared only when its LOD profile surpassed this threshold in at least two series of the same population.

Two partial resistance QTLs were mapped in S/M, *viz.* on chromosomes 1H and 3H (Table 2). These two QTLs were mapped in regions where no partial resistance QTL was reported before (Supplemental Figure 2). They are designated as *Rphq22* and *Rphq23*, respectively. *Rphq22* was mapped in all three series of the disease test. *Rphq23* was mapped in two series of the disease test while its LOD score in the third series was just below the threshold. *Rphq22* explained approximately 26% of the phenotypic variation and *Rphq23* explained around 22%. For both QTLs, the resistance allele was donated by Steptoe. Also for another possible QTL mapped on chromosome 6H the resistance allele was donated by Steptoe. But that third QTL was only detected in one series, and is therefore not reported in Table 2. No QTL resistance allele was found to be contributed by Morex despite the observed transgressive segregation in the mapping population. This most probably indicates the presence of QTLs with effects too small to be detected in this experiment.

The QTLs for heading date (HD) and plant height (PH) segregating in S/M were also positioned on the integrated map. *Rphq22* and *Rphq23* collocate neither with the HD nor the PH QTLs.

Table 2: Summary of partial resistance QTLs against barley leaf rust isolate 1.2.1 detected at adult plant stage in S/M DH mapping population. The QTL features are based on the series with the highest LOD score using MAPQTL®6 (van Ooijen 2009)

QTL	Chrom.	Peak marker	cM[1]	LOD	Exp%[2]	Donor
Rphq22	1H	Contig8593	134.4	8.6	26.1	Steptoe
Rphq23	3H	Contig10370	101.9	6.1	21.7	Steptoe

[1] Peak marker position on the integrated map "Barley, Integrated, Marcel 2009"

[2] Percentage of explained phenotypic variance (MapQTL®6)

For OWB, the correlation between series was very weak (data not presented). There was no QTL identified in at least two series of the disease test. In each series, a different unique QTL was identified on chromosomes 2H, 5H and 7H, respectively.

In none of the three series of disease tests for S/M and OWB we detected our target QTLs *Rphq11* and *Rphq16*. Therefore, it is likely that these two QTLs are effective only at the seedling stage (Marcel et al. 2007b), and not at the adult plant stage.

Markers developed for *Rphq11* and *Rphq16*

Twenty markers were developed that supposedly mapped on chromosome 2HL in the region of *Rphq11* flanked by the markers Bmag0125 and GBMS244 (Supplemental Table 1). Among those, 16 markers mapped between the flanking markers while the other four markers (one CAPS and three SSR) mapped near but outside the flanked QTL interval. The 16 markers consist of two SSR, two SCAR and 12 CAPS markers. Seven of the linked markers are synteny-based markers, developed using rice and *B. distachyon* annotated genes. The rice syntenic region on chromosome 4 was identified by blast with Uni19962 and GBM1062 sequences. Uni19962 is homologous to Loc_Os04g47040 in rice and GBM1062 is homologous to Loc_Os04g46820 in rice. However, there is no *B. distachyon* homolog for Uni19962 and GBM1062. Therefore the *B. distachyon* syntenic region on chromosome Bd5 was based on the rice homolog of Uni19962 and GBM1062, as well as two EST based markers, WBE144 and WBE129, which were flanked by Uni19962 and GBM1062. WBE144 is homologous to Bradi5g17980 in *B. distachyon* and WBE129 is homologous to Bradi5g18000 in *B. distachyon*

For *Rphq16*, twenty-seven markers were developed that supposedly mapped on chromosome 5HL in the QTL confidence interval flanked by the markers ABG391 and GMS002 (Supplemental Table 2). Among those, 18 markers mapped between the flanking markers of the QTL while the other nine markers (one SCAR, five CAPS and three SSR) mapped near but outside the flanked QTL interval. Three of the markers closely linked to *Rphq16* are synteny-based markers. The rice syntenic region on chromosome 3 and *B. distachyon* syntenic region on chromosome Bd1 was identified by blast with WBE320 and GBS0408 sequences. WBE320 is homologous to Loc_Os03g63450 in rice and Bradi1g01500 in *B. distachyon*. GBS0408 is homologous to Loc_Os03g63940 in rice and Bradi1g00990 in *B. distachyon*.

High-resolution genetic map for *Rphq11* and *Rphq16*

There were 89 fixed QTL-recombinants identified for *Rphq11* and 135 for *Rphq16* (described in the next section). These fixed QTL-recombinants were genotyped with the newly developed markers and high-resolution genetic maps were generated (Figure 3 and 4).

On the new high-resolution genetic map of the *Rphq11* region, the distance between markers GBS0512 and GBMS244, flanking *Rphq11*, is approximately 6 cM. Their interval on the barley integrated map [Barley, Integrated, Marcel 2009; (Aghnoum et al. 2010)] is

comparable (5 cM). Sixteen markers were mapped in this interval, providing an average marker density of one marker per 0.4 cM. Marker positions of GBS0512, WBE301 and GBM1062 around *Rphq11* in the new high resolution genetic map were slightly different compared to the integrated map, Marcel 2009. GBS0512 (Stein et al. 2007) and WBE301 (Potokina et al. 2008) were originally mapped in S/M, and GBM1062 in OWB (Varshney et al. 2007), which can explain the inaccuracy of their order on the integrated map. The 6 cM genetic window comprising *Rphq11* is in synteny with rice chromosome 4 and *B. distachyon* chromosome Bd5. The orientation of the syntenic block delimited by Uni19962 and WBE307 in barley is inverted compared to rice and *B. distachyon*, and microsyntenic rearrangements in marker order are also observed within the block. The orientation of the syntenic block and the order of markers are perfectly conserved between rice and *B. distachyon* (Figure 3).

On the new high-resolution genetic map of the *Rphq16* region, the distance between markers scsnp03275 and GMS002, flanking *Rphq16*, is approximately 11 cM. Their interval is approximately 20 cM on the barley integrated map [Barley, Integrated, Marcel 2009; (Aghnoum et al. 2010)]. The estimated 20 cM interval was based on MWG2193, WBE310 and WBE314 which shared the same position as scsnp03275 on the high-resolution genetic map because scsnp03275 was not mapped in the integrated map.

There were 18 markers mapped in this interval, providing an average marker density of one marker per 0.6 cM. Marker order at *Rphq16* was in agreement with marker order on the integrated map, Marcel 2009. The 11 cM genetic window comprising *Rphq16* is in synteny with rice chromosome 3 and *B. distachyon* chromosome Bd1. The orientation of this syntenic region and marker order are perfectly conserved between barley and rice but inverted in *B. distachyon* (Figure 4).

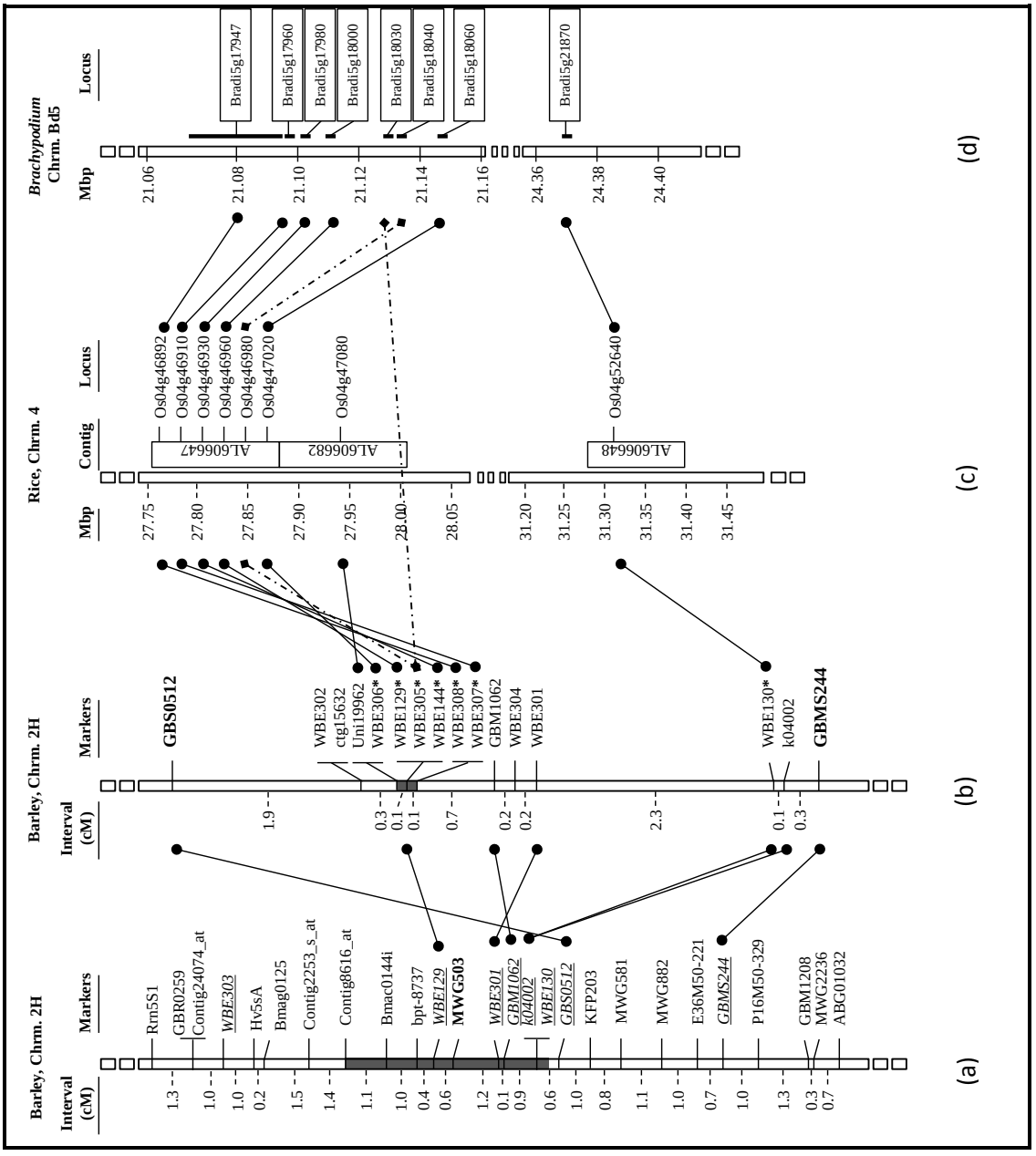

Figure 3: Alignment of (a) the integrated map, Marcel 2009 and (b) the high resolution map generated in this study, at the *Rphq11* region on barley chromosome 2HL with (c) the physical map of rice chromosome 4, and (d) the physical map of *B. distachyon* chromosome Bd5. The filled grey areas inside chromosome bars indicate the position of *Rphq11*. The bold marker on (a) is the peak marker of *Rphq11*. The bold markers on (b) are the flanking markers used for recombinant screening and the markers with an asterisk on (b) are synteny-based markers. The dashed lines show homologous sequences found between only two of the three species barley, rice and *B. distachyon*.

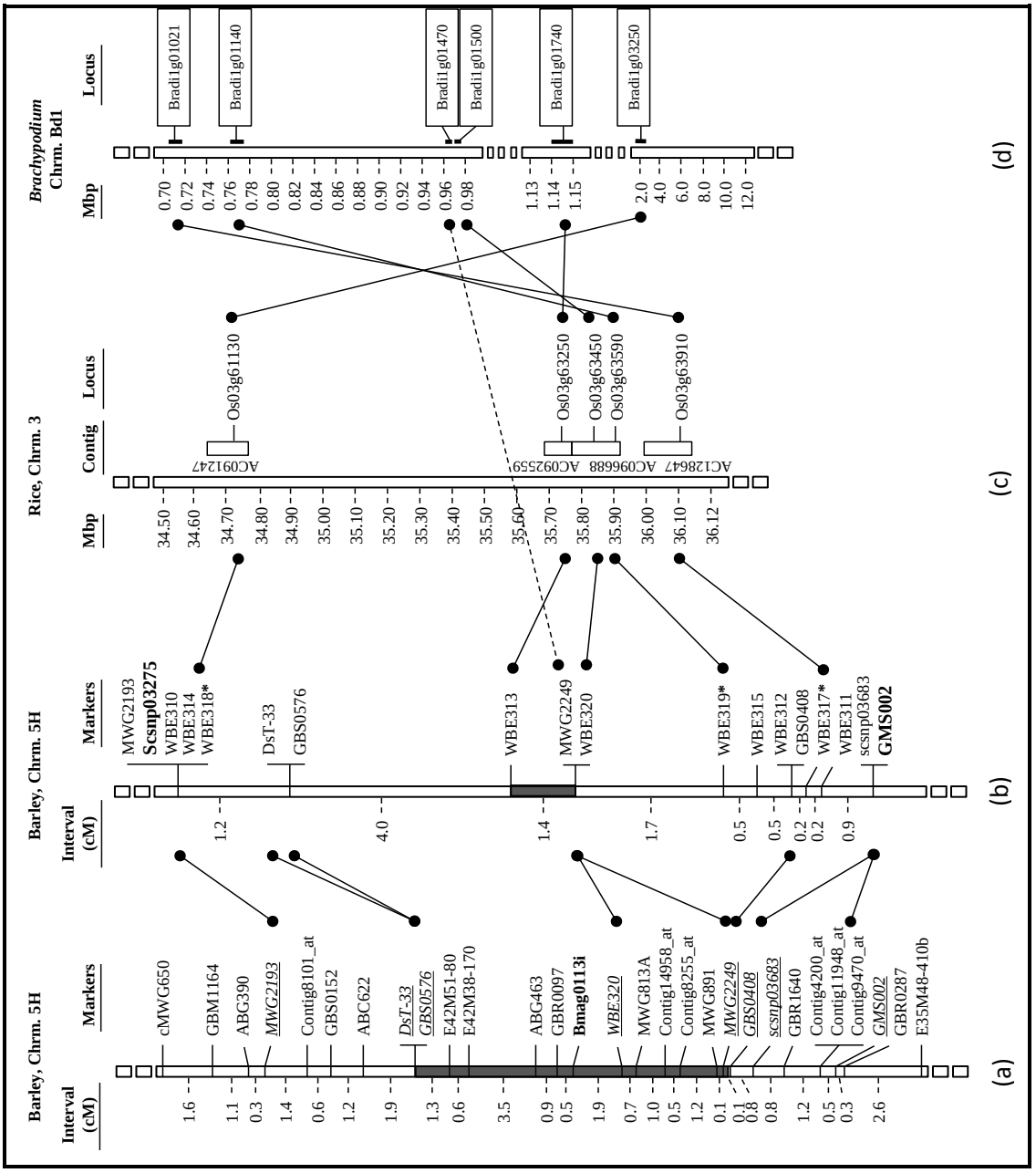

Figure 4: Alignment of (a) the integrated map, Marcel 2009 and (b) the high resolution map generated in this study, at the *Rphq16* region on barley choromosome 5HL with (c) the physical map of rice chromosome 3, and (d) the physical map of *B. distachyon* Bd1. The filled grey areas inside chromosome bars indicate the position of *Rphq16*. The bold marker on (a) is the peak marker of *Rphq16*. The bold markers on (b) are the flanking markers used for recombinant screening and the markers with an asterisk on are synteny-based markers. The dashed line shows a homologous sequence between barley and *B. distachyon*, which was only found in rice on another chromosome.

Fine-mapping of *Rphq11* and *Rphq16*

At the early backcross generations, the disease test on 97 F_3 plants segregating for *Rphq11* showed that *Rphq11* is an incompletely dominant gene, while the disease test on 52 BC_1S_1 plants segregating for *Rphq16* shows that *Rphq16* behaves predominantly as a recessive gene (Figure 5). The RLP50S comparison between the group of plants with homozygous donor allele (AA) and the group of plants without the donor allele (BB) shows that *Rphq11* gives an approximately 21 hours (11%) prolongation of latency period on the seedling leaves and *Rphq16* an approximately 14 hours (7%) prolongation.

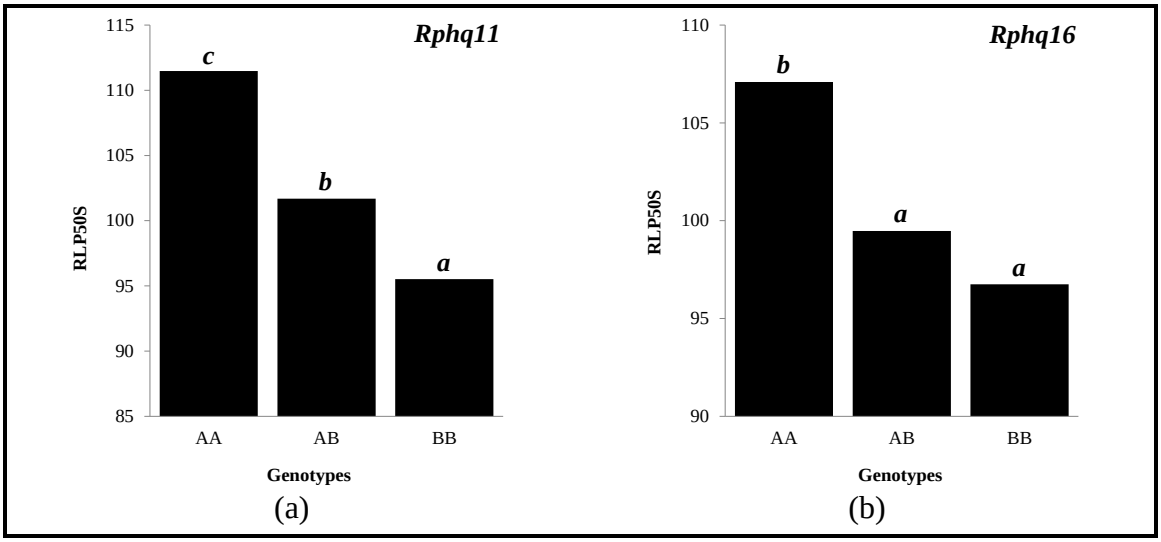

Figure 5: Histograms of the averaged RLP50S of (a) F_3 seedlings segregrating for *Rphq11* and (b) BC_1S_1 seedlings segregating for *Rphq16*. 'A' is the allele from Steptoe/ Dom and 'B' is the allele from SusPtrit. Similar letters above the bars indicate that the variance do not differ significantly according to the unbalanced one-way ANOVA analysis.

Recombinant plant screening of 730 plants at F_4/F_5 resulted in 89 fixed QTL-recombinants at *Rphq11* representing ten recombination points between all the markers mapped between Uni19962 and GBM1062. For *Rphq16*, recombinant plant screening of 655 plants at BC_2S_1/BC_2S_2 resulted in 135 fixed QTL-recombinants representing nine recombination points between all the markers mapped between WBE320 and GBS0408.

After several rounds of disease tests on a subset of the fixed QTL-recombinants, *Rphq11* was fine-mapped into a genetic window of 0.2 cM flanked by markers Uni19962/WBE306 proximal and WBE307/WBE308 distal. Indeed, the peak of the LOD profile generated by performing QTL mapping on the fixed QTL-recombinants supports this position of *Rphq11* (Figure 6a). This is consistent with an RLP50S between 107 and 110 for fixed

QTL-recombinants having the *Rphq11* allele, which is always significantly longer than the RLP50S on SusPtrit. And this is consistent with an RLP50S between 101 and 107 for fixed QTL-recombinants having the *rphq11* allele, which is however not always significantly shorter than those having the *Rphq11* allele.

Similarly, *Rphq16* was fine-mapped into a genetic window of 1.4 cM flanked by markers WBE313 proximal and MWG2249/WBE320 distal. The peak of the LOD profile generated by performing QTL mapping on the fixed QTL-recombinants supports this position of *Rphq16* (Figure 6b). This is consistent with RLP50S on fixed QTL-recombinants having the *Rphq16* allele ranging from 106 to 111, which is always significantly longer than the RLP50S on SusPtrit. And this is also consistent with RLP50S on fixed QTL-recombinants having the *rphq16* allele ranging from 100 to 105, which is not always significantly shorter than those having the *Rphq16* allele.

The refined position of *Rphq11* in a 0.2 cM interval corresponds to the syntenic region inverted between barley and rice (Figure 4). The 0.2 cM in barley corresponds to physical distances of 161 kb with 18 annotated genes in rice and 79 kb with nine annotated genes in *B. distachyon*. Concerning *Rphq16*, its refined position of 1.4 cM in barley corresponds to physical distances of 118 kb with 20 annotated genes in rice and 188 kb with nine annotated genes in *B. distachyon*.

Discussion

Plant stage specific QTLs in S/M and OWB

The seedlings and adult plants of S/M and OWB mapping population were challenged with *Ph*.1.2.1. None of the partial resistance QTLs that were detected at seedling stage (Marcel et al. 2007b) were also detected in any of the three series of disease test at the adult plant developmental stage (this study). This indicates that *Rphq11* and *Rphq16* are plant growth stage dependent and that their characterization can only be performed at the seedling stage. *Rphq22* and *Rphq23*, which were detected in this study at the adult stage in S/M, are also plant growth stage dependent, since they were not detected in the earlier seedling tests. This plant stage dependence of QTLs for partial resistance against *P. hordei* was also reported for other partial resistance QTLs (Qi et al. 1998; Wang et al. 2010).

Plant growth characteristics, such as heading date and plant height may influence the resistance of plants (Klahr et al. 2007; Williams 2003). In rice, the germin-like protein 1

was demonstrated to be involved in regulating plant height and disease resistance (Banerjee and Maiti 2010). *Rphq22* and *Rphq23* did not collocate with any heading date and plant height QTLs, which suggest that the resistance conferred by these two QTLs is not a pleiotropic effect of genes affecting heading date or plant height.

Adult plant QTLs from S/M and OWB are affected by the environment

In both populations, a clear series x genotype effect was observed, which was especially strong in the OWBs. From the three QTLs mapped in S/M, one was identified in only one of the three series. And all three QTLs mapped in OWB were identified in only one of the three series.

Environmental effect may be one of the contributing factors for the inconsistency of QTL identification. The disease tests were carried out at different time periods of the year and the third series was carried out at a different greenhouse facility. The performance of DH lines can be sensitive to the difference in fluctuations of temperature and light intensity or quality in the greenhouse, leading to the detection of different QTLs depending on the conditions. For example, detection of partial resistance QTL *Rphq4* is generally detected only at adult plant stage, but in plants that germinated at low temperature, the gene is also clearly effective at young plant stages (Xiaoquan Qi, Institute of Botany, Chinese Academy of Sciences, Beijing, pers. comm.). In S/M, the one extra QTL mapped on chromosome 6H in only one of the series can also be due to an interaction between genotype and environment (GxE).

The high morphological variation between the OWB lines may also have contributed to the inconsistency of QTLs mapped in this population. Indeed, the parental lines of the OWB population have been developed by systematically crossing recessive alleles for morphological and physiological trait into one parent and dominant alleles into the other parent, maximizing the genetic, morphological and agronomic diversity segregating in the population (Costa et al. 2001). The variation in plant height may affect the uniformity of inoculum deposition. Heading date variation can also influence the result of a disease test as the sequential inoculation of different groups of lines according to their heading date may also compromise uniformity and randomisation of DH lines over batches. Notably, the disease resistance of DH lines may vary based on the age of the flag leaf used. It is difficult to homogenise the disease tests for this population due to the high morphological variation between lines.

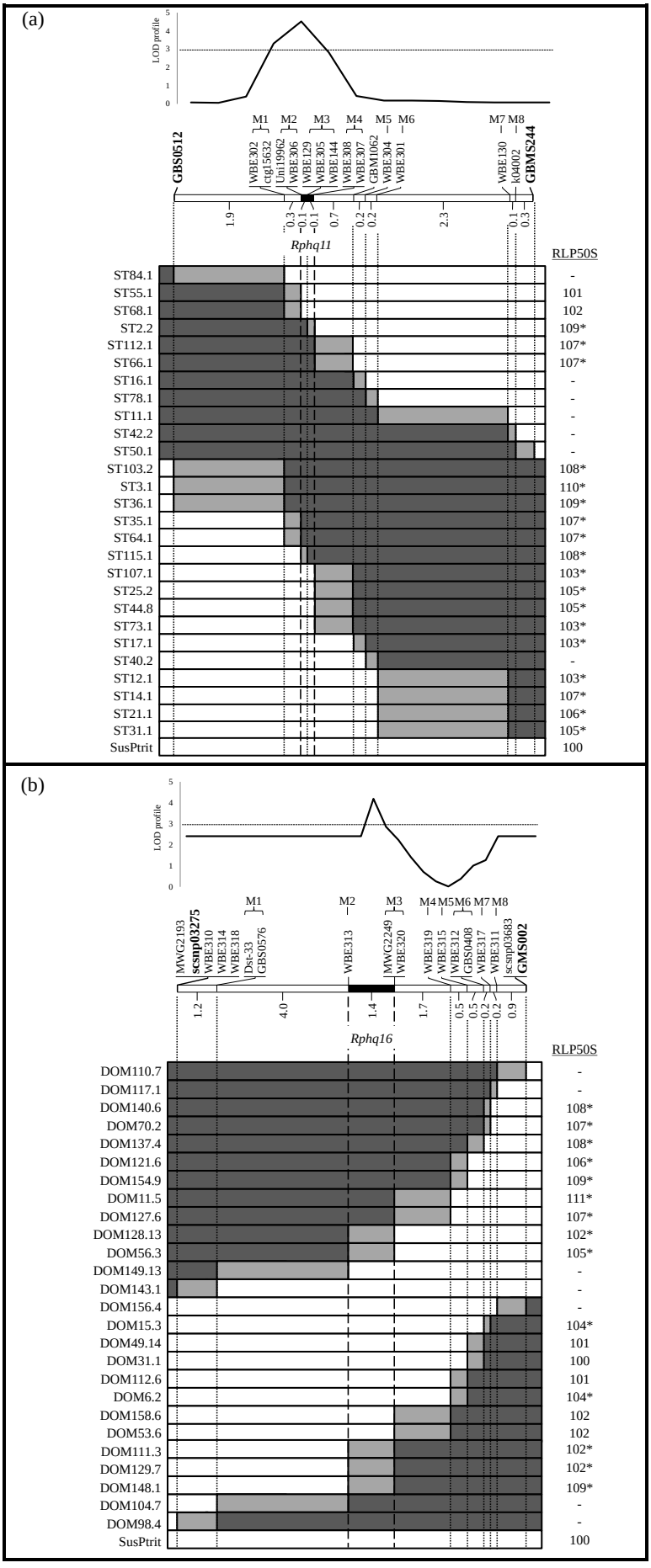

Figure 6: Graphical genotypes and phenotype means (RLP50S) for fixed QTL-recombinants of (a) *Rphq11* and (b) *Rphq16*; the phenotype means were compiled from results of the different rounds of disease test. The white bars represent homozygous SusPtrit. Black bars represent homozygous Steptoe (a) or Dom (b). Grey bars represent intervals where recombination took place. RLP50S values with an asterisk are significantly longer than the RLP50S on SusPtrit. The number between two markers on the chromosome bar indicates the recombination frequency observed. The new genetic window of *Rphq11* and *Rphq16* is indicated between the long dash lines.

Efficient fine-mapping of *Rphq11* and *Rphq16*

A QTL can be fine-mapped without the interference of other QTLs if a NIL were used as starting material. The NIL development is, however, laborious and time consuming. For barley, one generation is approximately four months, thus the development of a NIL with approximately 95% genome of recurrent parent at generation BC_4 will take two years and four months. Another year will be necessary to obtain enough seeds of homozygous recombinant plants (*i.e.* sub-NILs) to allow fine-mapping. In total, about three years and four months are needed to fine-map a QTL to a certain genetic window. One way to shorten this procedure is by exploiting individual lines from recombinant inbred lines (RILs), DH lines, chromosome segment substitution lines (CSSL) and backcross inbred lines (BILS) (Gao et al. 2004; Liu and Bai 2010; Zhang et al. 2011). Effectively, individual line(s) which contain only the targeted donor allele of the QTL can be crossed with a susceptible parent in order to generate recombinant plants that will be used for fine-mapping. However, the construction of RILs, CSSL and BILs are as laborious and time consuming as NIL and sub-NILs development.

The fine-mapping strategy followed in this study aimed at identifying recombinants in early breeding material developed to produce QTL-NILs. The material was first selected to carry the targeted QTL in heterozygous condition and susceptibility alleles at other resistance QTLs in homozygous condition. This strategy took 4-5 generations to obtain fixed QTL-recombinants. This way, it became possible to fine-map a QTL in less than two years. In parallel, the NIL of the targeted QTLs were developed, which allowed the confirmation of the effect of *Rphq11* and *Rphq16* in an isogenic background (data not shown).

The position of *Rphq11* has been narrowed down to a genetic window of 0.2 cM (1460 gametes scored) and *Rphq16* to a genetic window of 1.4 cM (1310 gametes scored). The fixed QTL-recombinants were not monitored for the presence of donor genome outside the regions of the targeted QTLs. Theoretically, the plant materials used to fine-map *Rphq11* should have approximately 50% of donor genome (i.e. F_3). For *Rphq16*, the plant materials may still have approximately 13% of donor genome (i.e. BC_2S_1). Consequently, previously undetected minor effect QTLs for resistance may still be present and even segregate in the material used to fine-map *Rphq11* and *Rphq16*. As a result of this heterogeneous genetic background, we may expect genetic and hence phenotypic noise in determining the QTL position and assessment of its effect. This may explain that several of the fixed QTL-recombinants carrying the susceptible allele at the target QTL gives a significantly longer latency period than the susceptible line SusPtrit used for crossing.

Nevertheless, it was still possible to dissect the position of *Rphq11* and *Rphq16* as the RLP50 range between fixed QTL-recombinants carrying the susceptibility allele and fixed QTL-recombinants carrying the resistance allele at the corresponding QTL were nearly distinct (i.e. RLP50S was 101-107 *versus* 107-110 for *Rphq11*, and 100-105 *versus* 106-111 for *Rphq16*). Moreover, the positions of *Rphq11* and *Rphq16* were supported by mapping QTLs on the high-resolution map. The peak marker of *Rphq11* became WBE129. The peak marker for *Rphq16* became MWG2249/WBE320, which is one of the markers flanking the window. Note that MWG2249/WBE320 has the second highest LOD score. The peak LOD score for *Rphq16* was in a marker interval. The new small genetic window of *Rphq11* and *Rphq16* remains consistent with the position of their peak marker originally identified (Figure 3 and 4). The position of *Rphq11* is further supported by an eQTL mapping performed in the S/M population by Chen et al. (2010). The authors mapped eQTL with measures of transcript abundance obtained in the S/M population 18 hours after inoculation with *P. hordei* isolate 1.2.1. They analysed the correlation between the identified eQTL and the pQTL including *Rphq11*. They identified 54 eQTL located in the confidence interval of *Rphq11* and six genes were proposed as candidate genes for *Rphq11*. Of these six, 'Unigene2453' encoding a phospholipid hydroperoxide glutathione peroxidase (PHGPx) was considered the strongest candidate for *Rphq11*. Interestingly, the marker developed on 'Unigene2453', WBE129, indeed was the peak marker of *Rphq11* located within the refined 0.2 cM position of this pQTL.

Rphq11 and *Rphq16* are the second and third fine-mapped QTLs for barley partial resistance to leaf rust. The fine-mapping strategy followed in this study has proven efficient to fine-map these two QTLs with a slightly smaller effect on the resistance level than of *Rphq2*. The latter gene explained 50% of the phenotypic variance, and was fine-mapped in another study (Marcel et al. 2007a). However, in order to fine-map smaller effect QTLs it would probably be necessary to reduce the noise caused by genetic background by starting the fine-mapping process at BC_3 or at even later backcross generations as suggested by Yang et al. (2012).

Disrupted synteny between barley, rice and *B. distachyon* at *Rphq11* and *Rphq16*

The evolutionary history of grasses – Poaceae reveals that *Oryza* (rice), *Hordeum* (barley) and *Brachypodium* (purple false brome) are split into sister taxa Ehrhartoideae (*Oryza*) and Pooideae (*Hordeum* and *Brachypodium*) (GPWG 2001; Kellogg 2001). Due to their evolutionary history, genomes of grasses are highly collinear. Collinearity can be defined as a specific form of synteny between two or more organisms with conserved gene order (Tang et al. 2008). Disruption of genome collinearity can occur during the evolution of

grasses. Comparative genomics between rice, barley and *B. distachyon* shows that barley has higher genome colinearity with *B. distachyon* in comparison with rice (Mayer et al. 2011). Disruption of genome collinearity is observed in the vicinity of *Rphq11* and *Rphq16*.

The genetic window of *Rphq11* is syntenic with rice chromosome 4, in agreement with Pourkheirandish et al. (2007), and with *B. distachyon* chromosome Bd5 (Mayer et al. 2011). The orientation of the syntenic region corresponding to *Rphq11* is conserved between rice and *B. distachyon* but inverted in barley, which leads to a disruption in genome collinearity. Despite this reordering in gene order, the synteny was useful to saturate the *Rphq11* region with new molecular markers. The size of the *Rphq11* syntenic region in rice is approximately 161 kb, with 18 annotated genes. In *B. distachyon*, the size is approximately 79 kb with nine annotated genes. None of the genes found in this region are of the NBS-LRR type. However, several of them belong to gene families previously shown to be involved in resistance in other plant-pathogen systems. These genes include an actin-depolymerizing factor (Tian et al. 2009), a glutathione peroxidase (Lamb and Dixon 1997) and glucosyltransferases (Langlois-Meurinne et al. 2005; von Saint Paul et al. 2011). All of them are conserved between rice and *B. distachyon.*

Glutathione peroxidase is the best candidate gene for *Rphq11*. The peak marker for *Rphq11* on the high-resolution genetic map is WBE129, which has been developed on the candidate gene Unigene2453 encoding for the phospholipid hydroperoxide glutathione peroxidase (PHGPx). This PHGPx gene has also been identified as the strongest candidate to explain *Rphq11* by Chen et al. (2010), because it was detected as a high-LOD *cis*-regulated expressed-QTL with significantly different transcript abundances between Steptoe and Morex.

The PHGPx gene corresponding to Unigene2453 is the strongest candidate gene for *Rphq11*. As it is conserved across rice, *B. distachyon* and barley, it may also have a conserved function in defence against pathogens across plant species. In rice, the expression of rice PHGPx homolog – OsPHGPx - is induced by infection by *Magnaporthe grisea* (Agrawal et al. 2002). The tomato PHGPx homolog – LePHGPx - also confers resistance, *viz*., against *Botrytis cinerea*, when stably expressed in tobacco (Chen et al. 2004).

The genetic window of *Rphq16* is syntenic with rice chromosome 3, in agreement with Close et al. (2009), and *B. distachyon* chromosome Bd1 (Mayer et al. 2011). The orientation of the syntenic region corresponding to *Rphq16* is conserved between barley and rice, but it is inverted in *B. distachyon*. The telomeric region, where *Rphq16* is

mapped, is indeed prone to dynamic chromosomal evolution (See et al. 2006). The size of the syntenic region is approximately 118 kb with 20 annotated genes in rice, and 188 kb with nine annotated genes in *B. distachyon*. Several of these genes belong to gene families involved in resistance in other plant-pathogen systems, including an oxidoreductase (Montesano et al. 2003), an aspartokinase (Stuttmann et al. 2011), and a proteasome subunit (Yao et al. 2012) which are conserved between rice and *B. distachyon*. There is also a glutathione S-transferase (Dean et al. 2005) and a transcription factor BTF3 (Huh et al. 2012) found only in the rice syntenic region, as well as a protein kinase C (Subramaniam et al. 1997), protein tyrosine phosphatases (He et al. 2012), glycosyltransferases (Langlois-Meurinne et al. 2005) and an NBS-LRR gene (Loutre et al. 2009) found only in the *B. distachyon* syntenic region. There is no favourite candidate gene in the interval for the moment.

The genome comparison between barley, rice and *B. distachyon* showed that the observed inversion of gene order can be specific to one of the three genomes or shared between two genomes (Mayer et al. 2011). If synteny remains a powerful tool to saturate a region of interest with molecular markers for high-resolution mapping, it should always be used with caution, notably regarding the transferability of candidate genes from one species to the other. Besides that, the genetic distance is not a precise estimation of physical size. *Rphq11* has a smaller genetic interval than *Rphq16*. The comparison of their physical size in rice and *B. distachyon* does not tally to their genetic interval. The *Rphq11* syntenic region in rice has approximately 1 gene in every 9 kb. In *B. distachyon*, there is approximately 1 gene in every 6 kb. The *Rphq16* syntenic region in rice and *B. distachyon* has approximately 1 gene in every 6 kb.

Feasibility of map-based cloning *Rphq11* and *Rphq16*

A map-based cloning approach starts with fine-mapping the QTL in a genetic window sufficiently narrow to make physical mapping feasible. The QTL phenotypic effect has also to be strong enough to be unambiguously recognised. The fixed-QTL recombinants (obtained from F_4/F_5 and BC_2S_1/BC_2S_2 recombinant screen for *Rphq11* and *Rphq16,* respectively) which carry the QTL allele *Rphq11* or *Rphq16* prolong the latency period by 12 hours in comparison to SusPtrit, which is sufficient to differentiate plants with the resistance allele QTL from those with the susceptibility allele. In agreement with this observation, the effect of *Rphq11* and of *Rphq16* was confirmed in their NIL (data not shown). Consequently, the phenotypic effect of *Rphq11* and *Rphq16* in their respective genetic background is high enough to pursue fine-mapping.

Rphq11 and *Rphq16* were fine-mapped to barley regions of 0.2 cM and 1.4 cM, respectively, following a time-efficient strategy. Even though there is phenotypic noise, it was still possible to dissect the position of *Rphq11* and *Rphq16*. *Rphq11* mapped in a high recombination rate region (1.1Mb/cM) of barley chromosome 2H, and *Rphq16* in a very high recombination rate region (0.2-0.9 Mb/cM) of barley chromosome 5H (Künzel et al. 2000). This offers good perspectives for the map-based cloning of the gene(s) underlying these two QTLs. Indeed, according to the given ratios, *Rphq11* is now estimated to be located in a region of approximately 220 kb, and *Rphq16* in a region of approximately 200-900 kb of the barley genome.

Many QTLs for partial resistance were mapped in barley against barley leaf rust but the underlying genes have not been identified so far. *Rphq2* has previously been fine-mapped to a genetic interval of 0.11 cM (Marcel et al. 2007a), encompassing a barley region of approximately 190 kb. Eight candidate genes to explain *Rphq2* have been identified and are currently being tested functionally. The precise mapping of *Rphq11* and *Rphq16* is a new step towards the understanding of the genetic basis of partial resistance to barley leaf rust. The sequenced Morex genome (The International Barley Genome Sequencing Consortium 2012) can be the reference for constructing the physical map and identifying candidate genes for *Rphq11* and *Rphq16.* This is followed by functional studies of those candidate genes. If the gene for *Rphq11* and *Rphq16* is not present in Morex, bacterial artificial chromosomes (BACs) for Steptoe (*Rphq11* donor) and DOM (*Rphq16* donor) should be constructed. These BAC libraries are then used to build physical maps for *Rphq11* and *Rphq16*, respectively. Alignment of *Rphq11* and *Rphq16* sequences from Morex, Steptoe and DOM, respectively, can help to find the candidate genes that cannot be found in Morex. The identified gene(s) for *Rphq2*, *Rphq11* and *Rphq16* will reveal if the genes for barley partial resistance at seedling stage belong to the same gene family or if different types of genes are involved.

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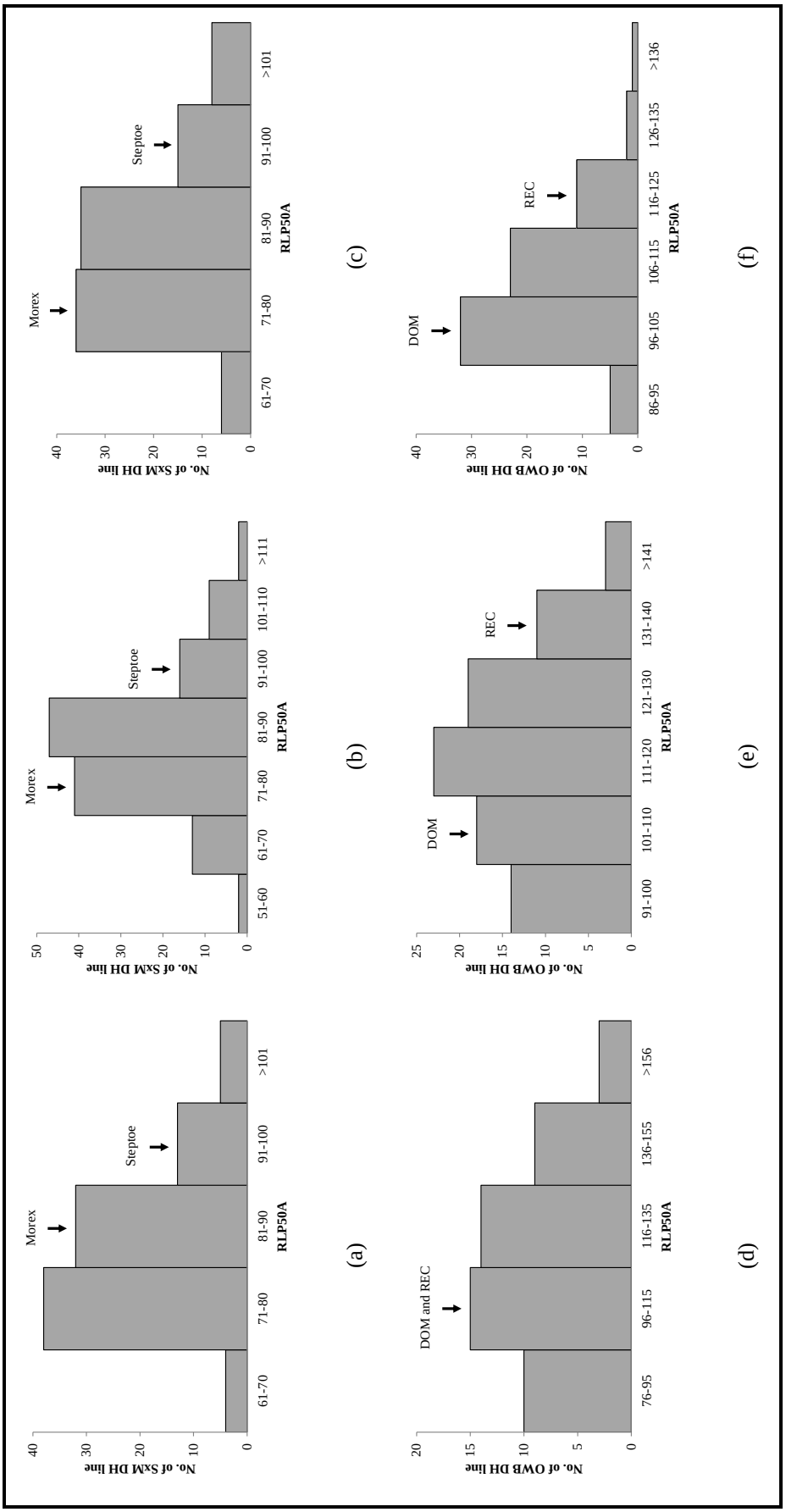
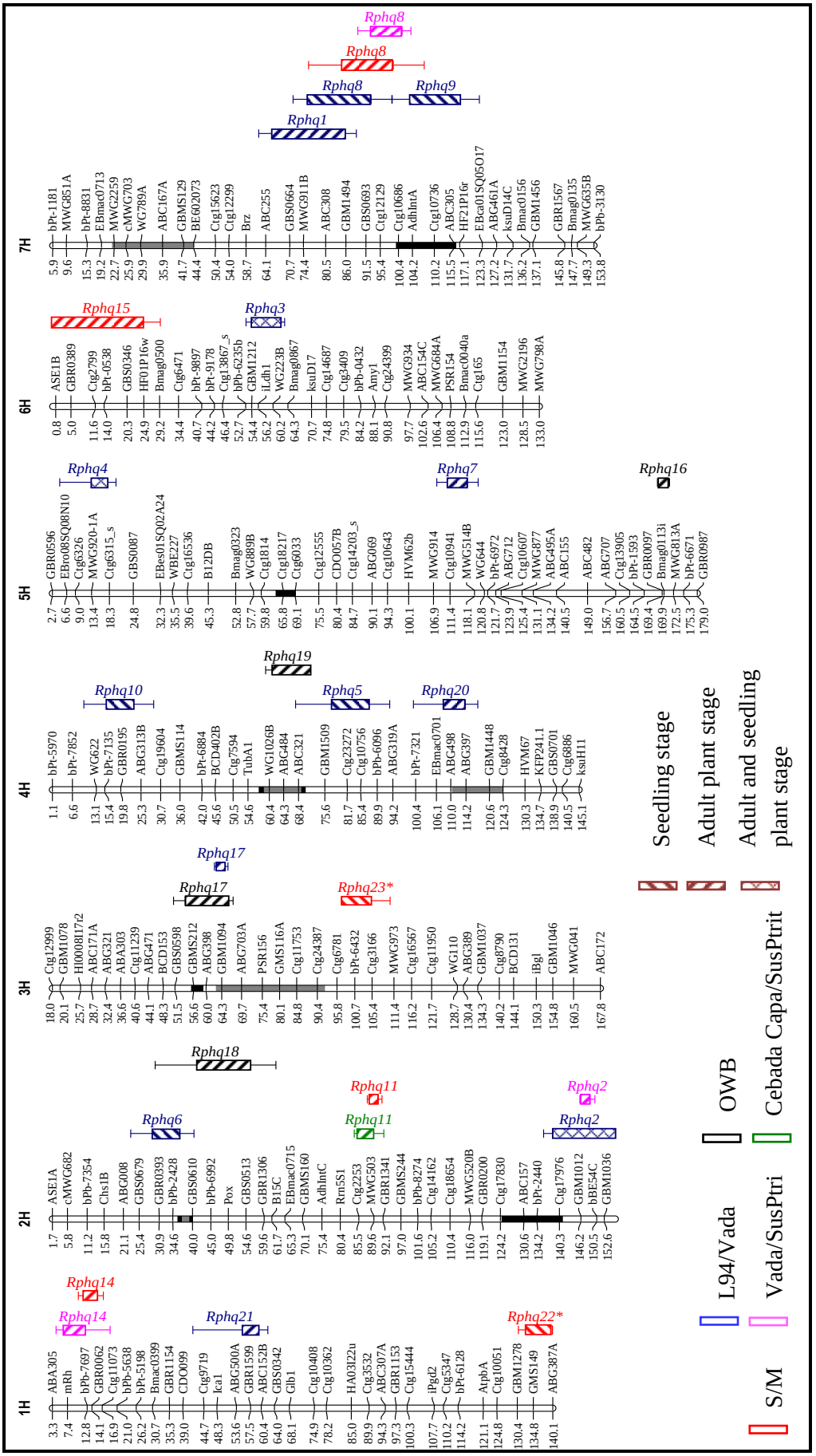
Supplemental Figures



Supplemental Figure 1: Frequency distribution of RLP50A in three series of disease test for S/M and OWB populations. Values of the parental lines are indicated by an arrow. (a) S/M first series (b) S/M second series (c) S/M third series (d) OWB first series (e) OWB second series (f) OWB third series.



Supplemental Figure 2: The map position of QTLs for partial resistance to *P. hordei* mapped in five barley mapping populations (including the QTLs detected in this study) on the integrated map, Marcel 2009. The length of the QTL bars corresponds approximately to the rMQM one LOD confidence interval and the extended lines from the QTL bars corresponds approximately to the rMQM two LOD confidence interval. QTLs with an asterisk are QTLs detected in this study. The black bars within chromosome bars correspond to plant height QTLs and grey bars to heading date QTLs. Numbers on the left side of chromosome bars show the distance in cM (according to Kosambi).

Supplemental Tables

Supplemetal Table 1: The new markers developed for *Rphq11* on chromosome 2H

Name	Type	Restriction enzyme	Tm (°C)	Primer sequences (5'-3')	Source[!]
Ctg15632	CAPS	MboII	58	*F:* TGGCAAATGACGGGGCACTAAAAC *R:* AACCGGCCTACAGATCGCAACCTT	Boyd et al. (2007)
GBM1062	SSR			Confidential	Li et al. (2003)
GBMS244	SSR			Confidential	Thiel et al. (2003)
GBS0512	CAPS	AciI	58	*F:* CCACATGCTGCGGAGGT *R:* CGTTGAGGATGATGCTGAGG	Stein et al. (2007)
k04002	SCAR		60	*F:* GACACAGGACCTGAAGCACA *R:* CGGCAGGCTCTACTATGAGG	Hori et al. (2005)
Uni19962	CAPS	MseI	58	*F:* GTCCCACATCACTGCACATC *R:* CAGTCGCAGAAGTTACTGAAG	Boyd et al. (2007)
WBE129	CAPS	HpyCHY4IV	58	*F:* CCCCCAAACTCCCAACT *R:* CTCCAGCCAGCAGGTCTAA	Rice synteny
WBE130	dCAPS	XapI	58	*F:* CTCGTATGTTGTGTGGAATTGTGA-GCCCAATCTTAATCCTAAGATCTCGAA *R:* GGTCTCCCAGCTAAAGTCTCC	Rice synteny
WBE144	CAPS	BsrI	58	*F:* GAGGCCCTTATCATTCTGTTGTCC *R:* ATGCTGGCGCGTTTTTGGGTATG	Rice synteny
WBE301	SCAR		65	*F:* TCGATGAGCGGATGGGTAAGGTAT *R:* ATTCCCAGCTGCCCAGTGTTTCT	Potokina et al. (2008)
WBE302	CAPS	Tsp4CI	65	*F:* ATGATCTTCGCCCTCGTCTACTGC *R:* TGGTCTTGAATGGGATCGCTCTGA	Potokina et al. (2008)
WBE304	CAPS	SacII	65	*F:* AGCTAGCTGTTGGGCGTGAAAATC *R:* CAAGGGGGTGGAGGAGGAAGAAGT	Potokina et al. (2008)
WBE305	CAPS	MwoI	65	*F:* CCGTCCCGTCACCCGAGTCC *R:* TCAGGCCTTCCAGTAGCGAGTTCC	Rice synteny
WBE306	CAPS	NdeI	65	*F:* CGGGGGCGCCTCCTCTACTC *R:* GTCCGGGTCATCATCTTCCACAAC	Rice synteny
WBE307	CAPS	SduI	65	*F:* GGCGCTCCGTGCAAAGAAGA *R:* GGAGACGAGGAGCAAAAGACACAA	Rice synteny
WBE308	CAPS	ClaI	65	*F:* CTGAGCCTGGGAAACAAAGTCG *R:* CAGCGCTGATGCAACAATAGGAT	Rice synteny
Bmac0216*	SSR			*F:* GTACTATTCTTTGCTTGGGC *R:* ATACACATGTGCAAAACCATA	Ramsay et al. (2000)
Bmag0125*	SSR			*F:* AATTAGCGAGAACAAAATCAC *R:* AGATAACGATGCACCACC	Ramsay et al. (2000)
GBM1440*	SSR			*F:* CTACCGAGCTCCTCCTCCTC *R:* GGCCTCCTTCTTGTCGTAGA	Marcel et al. (2007b)
scsnp06130*	CAPS	HinfI	56	*F:* GACGTCCCTCGCGTAAATGG *R:* TTGGCCGGGAACTTATGGTG	Rostok et al. (2005)

* The markers which were mapped near but outside the flanked QTL interval

[!] The references for CAPS and SCAR markers give the origin of the sequences obtained for marker development.

Supplemetal Table 2: The new markers developed for *Rphq16* on chromosome 5H.

Name	Type	Restriction enzyme	Tm (°C)	Primer sequences (5'-3')	Source[!]
DsT-33	SCAR		45	*F:* GCACACATATTATCATGAAAAAGAGC *R:* ACCCCAAATGAGTTTCGATG	BarleyWorld.org
GBS0408	CAPS	MseI	56	*F:* ATGCCACCCCTTATGAATCCT *R:* TTGCCCGTTGAAAAGTCCA	Stein et al. 2007
GBS0576	CAPS	BspLI	56	*F:* GTCCGGGCACAAGAACCTC *R:* GGCTGGGCATCATCCTCAA	Stein et al. 2007
GMS002	SSR			*F:* CCGACAACATGCTATGAAGC *R:* CTGCAGCAAATACCCATGTG	Struss and Plieske (1998)
MWG2193	CAPS	AluI	56	*F:* CAAACCCTTGAGGTCAGTTGC *R:* TCAGCTCTAAGATGCAGCACG	Graner et al. (1991)
MWG2249	CAPS	DdeI	56	*F:* GGCATGTGAGGGAAGCAATGG *R:* TGGAGAAGAACGTGTGGGTCG	Graner et al. (1991)
scsnp03275	CAPS	BglII	65	*F:* AACGGCCAGGCTATAACCATCACA *R:* CGGCGGCTTCATCAATTTCACTAA	Rostoks et al. (2005)
scsnp03683	CAPS	HpyCHY4IV	56	*F:* CAACGGCGCCACCTTCTACT *R:* CACATACCCCACTGCCATGC	Rostoks et al. (2005)
WBE310	SCAR		65	*F:* GGCGCTTTTGGTTTTCCTGA *R:* CGGCCTGGTATAATTAAGAGTGTG	Potokina et al. (2008)
WBE311	SCAR		65	*F:* CCAGAAAGGCGAGGAAGG *R:* TCGGATTATTGCACACCAGAAAAC	Potokina et al. (2008)
WBE312	CAPS	MseI	58	*F:* TGTGCCGTGTTATAATGGGGAATG *R:* CACAAAATCGGGCCTGCTTATCTT	Potokina et al. (2008)
WBE313	CAPS	MwoI	58	*F:* TGCCGAGTCGCCTAACCATA *R:* TCAACAACTACCTGCCAAATACCA	Potokina et al. (2008)
WBE314	CAPS	SphI	65	*F:* CCAGGGAATTACCAGGGAGACA *R:* TGAAGCCGACAACAAAAACAGG	Potokina et al. (2008)
WBE315	CAPS	HinfI	65	*F:* CCCCCTTCGCCGGCTTCTCAACC *R:* ATTCACAAAGCGCCGGCACACCAG	Potokina et al. (2008)
WBE317	CAPS	AcyI	65	*F:* ATCCCAGCCGACAGCATCC *R:* GAGAGCAGGCACCCGCATAG	Rice synteny
WBE318	CAPS	Hin1II	65	*F:* ACGGTGGTGGTGGTGGTCA *R:* GCCCGCAGCGTCTCGTAG	Rice synteny
WBE319	CAPS	HhaI	65	*F:* GATGGGTAGGCTTAAGCAGAAACT *R:* AACGCGCCTAACACAAACTCCTAC	Rice synteny
WBE320	CAPS	MseI	58	*F:* CCCCCGGCTGGTGTGGA *R:* CAGCTGTGGCGTGATGTATTTGTA	Potokina et al. (2008)
ABC622*	CAPs	AluI	65	*F:* AGGGAAGGGCTGCAAACTGTA *R:* ACCAACTGATCGCTGCCTGTGTAT	Rostoks et al. (2005)
ABG390*	CAPs	AluI	56	*F:* TGTTCCCAGCATTTGAACAG *R:* CGGCAATCCTAATTTTTGGA	Rostoks et al. (2005)
ABG391*	CAPs	AluI	56	*F:* GCAAGTGCACTGCTGTACAA *R:* TGTTCTCGTACCATGACTTC	Rostoks et al. (2005)
CMWG650*	CAPs	Hin1II	56	*F:* ATGCCTGGGTACAAAAATCAAATG *R:* TCACCCAGCCTACCAAAATAACAG	Stein et al. 2007
GMS001*	SSR			*F:* CTGACCCTTTGCTTAACATGC *R:* TCAGCGTGACAAACAATAAAGG	Struss and Plieske (1998)
scsnp00635*	CAPS	HinfI	65	*F:* TGAGCAGCCGTGTCAGCTTC *R:* AAACATTGGATTGGGCACGC	Rostoks et al. (2005)
scsnp07825*	SCAR		65	*F:* GGCGCGGCGGACTGACAAG *R:* GTGGTGCTGCGACGAGGAGACG	Rostoks et al. (2005)
scssr03907*	SSR			*F:* CTCCCATCACACCATCTGTC *R:* GACATGGTTCCCTTCTTCTTC	Ramsay et al. (2004)
scssr09041*	SSR			*F:* CATGTCAGTGGGGTTCTAGC *R:* TCTACTTGGACCTGCTGACC	Ramsay et al. (2004)

* The markers which were mapped near but outside the flanked QTL interval

[!] The references for CAPS and SCAR markers give the origin of the sequences obtained for marker development.

Supplemetal Table 3: The new markers developed for *Rphq15* and *Rphq17*.

Name	Type	Chrom.	Restriction enzyme	Tm (°C)	Primer sequences (5'-3')	Source[!]
					Rphq15	
Bmag0500	SSR	6H			*F:* GGGAACTTGCTAATGAAGAG *R:* AATGTAAGGGAGTGTCCATAG	Ramsay et al. (2004)
GBM1355	SSR	6H			*F:* ATCCGTCGTATTCGCATCTC *R:* GCTGGTACTGGGAGAAATGG	Varshney et al. (2006)
GBMS033	SSR	6H			Confidential	Li et al. 2003
MWG966	CAPS	6H	BspLI	57	*F:* ATGCGTGCCCTTTGGAACA *R:* TGGCCTGCGATATGGAGACC	Graner et al. (1991)
scssr09398	SSR	6H			*F:* AGAGCGCAAGTTACCAAGC *R:* GTGCACCTCAGCGAAAGG	Ramsay et al. (2004)
					Rphq17	
Bmac0067	SSR	3H			*F:* AACGTACGAGCTCTTTTTCTA *R:* ATGCCAACTGCTTGTTTAG	Ramsay et al. (2004)
Bmag0136	SSR	3H			*F:* GTACGCTTTCAAACCTGG *R:* GTAGGAGGAAGAATAAGGAGG	Ramsay et al. (2004)

* The markers which were mapped near but outside the flanked QTL interval

[!] The references for CAPS and SCAR markers give the origin of the sequences obtained for marker development.

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Chapter 5

Non-Gridded BAC libraries of the barley cultivars Vada and SusPtrit for physical mapping of *Rphq2*, a partial resistance QTL to *Puccinia hordei*

To be submitted

Non-gridded BAC libraries of the barley cultivar Vada and line SusPtrit for physical mapping of *Rphq2*, a partial resistance QTL to *Puccinia hordei*

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Abstract

Evidence suggests partial resistance of barley to powdery mildew and rust fungi to be a weak form of nonhost resistance based on pathogen-associated molecular pattern (PAMP)-triggered immunity. A better understanding of partial resistance may help us to gain more insight into nonhost resistance, and *vice versa*. More than 20 partial resistance quantitative trait loci (QTLs) to *Puccinia hordei* have been mapped at seedling and adult plant stages. In order to determine the genes underlying those QTLs, we need to identify and validate the candidate genes of a selection of QTLs. Two non-gridded BAC libraries were constructed from the partially resistant cultivar Vada and from the very susceptible line SusPtrit. Based on the observed insert sizes of the BAC clones, the estimated genome coverage of the Vada BAC library is 2.6x and of the SusPtrit BAC library 3.7x. Together, the two BAC libraries give more than 99% probability of recovering any specific sequence from the barley genome. Three BAC clones of Vada which cover the *Rphq2* genetic window were sequenced. Three BAC clones of SusPtrit were also sequenced but they did not cover the entire *Rphq2* genetic window. The obtained sequences between markers flanking *Rphq2* encompassed 195 Kbp in Vada and 226 Kbp in SusPtrit. This difference in size is linked to the lack of homology between the Vada sequence derived from an introgression from the exotic *Hordeum laevigatum* and the SusPtrit sequence homologous to other barley genotypes such as Morex and L94. The TriAnnot pipeline predicted 12 genes on both Vada and SusPtrit contigs. Among the predicted genes, only five were common between Vada and SusPtrit. No nucleotide-binding-site-leucine-rich repeat resistance gene was annotated in the *Rphq2* region. The possible candidate genes for *Rphq2* code for peroxidases, kinases and a member of Seven in absentia protein family.

Keywords: Barley, Bacterial Artificial Chromosome (BAC) Library, Partial Resistance, Quantitative Trait Locus (QTL), *Puccinia*

Introduction

Partial resistance is an incomplete host resistance which retards epidemic development despite a compatible infection type (Niks et al. 2011; Parlevliet and van Ommeren 1975). Partially resistant barley to *Puccinia hordei* has lower infection frequency, lower sporulation rate and longer latency period of the pathogen, and such effects are not associated with hypersensitivity. Evidence suggests partial resistance of barley to powdery mildew and rust fungi to be a weak form of nonhost resistance based on pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Jones and Dangl 2006; Niks and Marcel 2009; Niks et al. 2011; Trujillo et al. 2004). Nonhost resistance is, by definition, a resistance observed in all genotypes of a nonhost plant species to all genotypes of a potential pathogen species (Niks et al. 2011). However, plant species may turn out to have a marginal host or near-nonhost status to some pathogen species (Niks 1987). Barley (*Hordeum vulgare*) is such a marginal host or near-nonhost to *Puccinia triticina*, *Puccinia hordei-murini* and some other non-adapted rust fungi because a small proportion of barley accessions (less than 10%) are susceptible (> 10 pustules on the first leaf) at seedling stage when inoculum is applied at high density (Atienza et al. 2004). In the context of this paper, non-adapted rust fungi are rust fungi that are poorly or not adapted to barley, but primarily to one or few other plant species. We try to investigate nonhost resistance by dissecting the genetics of near-nonhost resistance with the assumption that we can extrapolate the findings to explain also the basis of full nonhost resistance. SusPtrit was developed for such purpose by accumulating susceptibility genes from rare barley accessions which were somewhat susceptible to *P. triticina* at seedling stage. SusPtrit is, at seedling stage, highly susceptible to *P. triticina* and to at least nine other non-adapted rust fungi (Atienza et al. 2004). This experimental line was used to develop mapping populations Vada/SusPtrit (Jafary et al. 2006), Cebada Capa/SusPtrit (Jafary et al. 2008) and SusPtrit/Golden Promise (Chapter 2, this thesis), and near isogenic lines (Chapter 3, this thesis). Resistance of barley to non-adapted rust fungi inherits polygenically just like partial resistance to *P. hordei*, the barley leaf rust fungus (Jafary et al. 2006; 2008; Marcel et al. 2007b; Niks et al. 2000; Qi et al. 1998).

The locations of genes for resistance to non-adapted rust fungi tend to overlap with those for partial resistance to *P. hordei* (González et al. 2010; Jafary et al. 2008; Niks and Marcel 2009) and they share the same prehaustorial resistance mechanism, viz. a high rate of failed attempt to haustorium formation (Niks 1983a, b). If both the partial resistance to adapted fungi and nonhost resistance to non-adapted fungi are mainly based on PTI, a better understanding of partial resistance will help us to gain more insight into nonhost resistance, and *vice versa*. More than 20 partial resistance quantitative trait loci (QTLs) to

P. hordei have been mapped at seedling and adult plant stages (Jafary et al. 2006; Marcel et al. 2007b; 2008; Niks et al. 2000; Qi et al. 1998; 1999). In order to determine the underlying genes of those QTLs, we need to identify and validate the candidate genes of a selection of QTLs. To date, five large-effect resistance QTLs have been cloned (Fu et al. 2009; Fukuoka et al. 2009; Hayashi et al. 2010; Krattinger et al. 2009; Manosalva et al. 2009). The cloned QTL are different from each other in gene structure and function, which implies a diversity of mechanisms underlying partial resistance. The genes that have been cloned thus far do not have the typical modular NB-LRR structure of *R*-genes. *Rphq2* is one of the partial resistance QTLs to *P. hordei* mapped in L94/Vada (Qi et al. 1998), Vada/SusPtrit (Jafary et al. 2006) and in an association mapping study comprising 146 barley genotypes (Kraakman et al. 2006). In L94/Vada and Vada/SusPtrit populations, Vada is the donor of *Rphq2*. Vada is a cultivar developed from *Hordeum laevigatum*/Gold (Dros 1957). Near isogenic lines (NILs) are available for *Rphq2* in L94 genetic background (L94-*Rphq2*) (Marcel et al. 2007a; van Berloo et al. 2001) and for *rphq2* of L94 in Vada background (Vada-*rphq2*) (Marcel et al. 2007a). L94 is an Ethiopian landrace-derived line that has some level of susceptibility to some non-adapted rust fungi (e.g *Puccinia hordei-secalini* and *P. hordei-murini*) (Atienza et al. 2004). When L94-*Rphq2* was inoculated with *P. hordei-secalini* and *P. hordei-murini,* it had a significantly lower infection level compared to L94 (Chapter 3, this thesis). This suggests that the postulated *Rphq2* gene explaining the resistance to *P. hordei* also affects the resistance to some non-adapted rust species. Substitution mapping by using sub-NILs generated from Vada-*rphq2* allowed pinpointing *Rphq2* to an interval of about 0.1 cM, corresponding to about 121 to 198 kb (Marcel et al. 2007a). This estimated physical interval is sufficiently small to justify the development of a Bacterial Artificial Chromosomes (BAC) library in order to pin down *Rphq2* to one or few more candidate genes.

Construction and organization of BAC libraries remains laborious and costly, especially from organisms with large and complex genomes like barley [5.1 Gb (Doležel et al. 1998); 4.6 Gb (Jones and Pašakinskienė 2005); 4.98 Gb (The International Barley Genome Sequencing Consortium 2012)]. In barley, about 200,000 clones with an average insert size of 120 kb would be required to achieve a genome coverage of five genome-equivalents, which is needed for a more than 99 % probability of recovering any specific sequence of interest. To date, gridded BAC libraries are available for barley cv. Morex (Schulte et al. 2011; Yu et al. 2000), Haruna Nijo (Saisho et al. 2007) and a doubled haploid barley line CS134 derived from Clipper/Sahara-3771 (Shi et al. 2010). The inconveniences linked to the gridding, storage and maintenance of such a quantity of clones can be circumvented by the pooled library approach described by Ma et al. (2000) for wheat and Isidore et al. (2005) for barley. This approach consists of pooling several hundreds of clones together without the need of picking and storing individual clones. The

first pooled BAC library of barley developed from cv. Cebada Capa was successfully used to establish a single contig of six BAC clones spanning 230 kb at the *Rph7* locus on chromosome 3H (Isidore et al. 2005). The BAC libraries from the four mentioned barley genotypes could help in the construction of physical maps around any target gene, but to isolate genes of interest in plants, it is often essential to construct BAC libraries from specific genotypes. Indeed, the gene content may vary between individuals of the same species and the gene of interest may not be present in the genomic libraries of related genotypes. This consideration is especially relevant concerning the genes involved in resistance to pathogens, which are known to be under strong selective pressure (Meyers et al. 2005; Salvaudon et al. 2008; Shen et al. 2006).

The current study aimed to develop two non-gridded BAC libraries from cultivar Vada and line SusPtrit which will allow the isolation of genes for partial and nonhost resistances. Having a BAC library from the resistant parent as well as from a susceptible parent is required because genes involved in such resistances can either be a resistance or susceptibility factor. After screening the newly developed BAC libraries, we identified and sequenced BAC clones in the *Rphq2* region of both genotypes. The assembly and annotation of BAC sequences revealed several genes that might be responsible for the phenotypic contrast between Vada and SusPtrit for partial resistance due to *Rphq2*.

Materials and Methods

The methodology followed to construct the two BAC libraries has been described in detail by Peterson et al. (2000), with several modifications proposed in subsequent papers (Allouis et al. 2003; Chalhoub et al. 2004; Isidore et al. 2005).

Preparation of high-molecular-weight DNA

About 500 seeds from Vada and from SusPtrit were sown in plastic trays and placed in a greenhouse compartment. Leaves were harvested twice from the same plants between two and four-weeks after sowing, flash-frozen in liquid nitrogen and stored at -80 °C. Before each harvest the plants were kept in the dark during 48 hours to promote the burst of chloroplasts and limit chloroplast DNA in the BAC libraries. About 40-50 g of leaf were used to extract nuclei and prepare high molecular weight (HMW) DNA following the protocol described by Peterson et al. (2000), with modifications (Allouis et al. 2003; Chalhoub et al. 2004). The modifications consisted mainly in the omission of polyvinyl

pyrolidone 40000 (PVP-40) and ascorbic acid from the sucrose-based extraction buffer (SEB) and from the lysis buffer. The quantity of PVP-40 in the wash buffers (WB-A, -B, -C) was lowered to 0.25% instead of 2%.

Partial digestion and size fractionation

Plugs of HMW DNA were prepared in 0.75% InCert® agarose (BMA) as described by Peterson et al. (2000). Twelve plugs macerated in the *Hind*III modified restriction (H3M) buffer were digested at different enzyme concentrations: 0.2, 0.5, 1.0, 2.0, 5.0, 7.5, 10.0, 15.0, 20.0 and 40.0 units. The partial digestion was performed in a 37 °C water bath during exactly 20 minutes. Then, the plugs were migrated together on a 1.0 % SeaKem® Gold agarose gel (Cambrex) in 0.25x TBE in a CHEF-Mapper apparatus (Bio-Rad) with the following conditions: pulse linear ramping from 1 to 40 sec, angle 120°, current 6.0 V/cm and 21 hours run time at 14 °C. After electrophoresis, the partially digested DNA was subjected to a single size selection. The flanking lanes loaded with the lambda ladder PFGE marker (New England Biolabs) were removed from the gel and stained with ethidium bromide to indicate the location of the size ranges. For each library, five slices of agarose-containing DNA in the ranges 50-100 kb (H0 fraction), 100-150 kb (H1 fraction), 150-200 kb (H2 fraction), 200-250 kb (H3 fraction) and 250-300 (H4 fraction) were excised from the gel and stored at 4 °C in 1x TAE buffer.

The HMW DNA was isolated by electro-elution using a BioRad Electroelution system run one hour at 60 mA direct current and 90 V alternating current. From each agarose slice, 40 to 80 µl was recovered with a wide-bored tip.

Ligation and transformation

The insert DNA from H0 to H4 fractions was ligated separately into the pIndigoBAC vector (CalTech) prepared for high efficiency cloning with *Hind*III as described by Chalhoub et al. (2004) or into the commercial pIndigoBAC-5 vector (Epicentre Biotechnologies). Ligations were performed in a 50 µl reaction volume with 33 µl insert DNA (50-100 ng), 50 ng of vector DNA, 10 µl of 5x reaction buffer and 5 units of T4 DNA ligase (Invitrogen). Ligation mixtures were incubated at 16 °C overnight and dialyzed 90 min at 4 °C as described by Chalhoub et al. (2004). Sixteen microliters of de-salted ligation were mixed with 110 µl ElectroMax DH10B electrocompetent cells (Invitrogen). Seventeen microliters of the mixture were electroporated at 330 V and the

electroporations were pooled in a tube containing 2 ml SOC medium (Sambrook et al. 1989) with 0.3 ml of 2 M glucose.

Pooling of the BAC clones

Transformed cells diluted with SOC were incubated at 37 °C under gentle agitation (220 rpm) for 60 min and plated on a selective LB medium (Luria-Bertani medium) with 12.5 µg chloramphenicol (CAM), 0.55 mM IPTG (isopropylthio-β-D-galactoside) and 80 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (Sambrook et al. 1989). A test plating of each transformation was performed in order to allow an average of about 1,500 colonies per plate as suggested by Isidore et al. (2005). The plates were incubated at 37 °C for 20 hours. The clones were collected from each plate in 3 ml storage buffer (LB medium supplemented with 50% glycerol) and homogenized for 30 min under gentle agitation (220 rpm). Each of the resulting 3 ml cultures represents a pool. The pools were then aliquot into four tubes, each corresponding to one copy of the library (copies A, B, C and D). Copy A is stored in a -80 °C freezer at Unité de Recherche en Génomique Végétale (URGV – Evry, France), copy B at Institute of Botany, Chinese Academy of Sciences (IBCAS – Beijing, China). Copies C and D are stored at Wageningen UR, Plant Breeding (WUR – the Netherlands).

Characterization of the BAC libraries

Twenty-four BAC clones were randomly selected from the fractions H1, H2 and H3 of each library (i.e. 72 BAC clones per library) and grown for 24 hours at 37 °C in 1.5 ml LB medium containing 12.5 µg CAM. The BAC DNA was extracted following an alkaline lysis procedure (Sambrook et al. 1989) with ready-to-use buffers P1, P2 and P3 (Qiagen) and digested overnight with *Not*I (New England Biolabs). Digested products were separated on a 1% SeaKem® LE agarose gel (BMA) in 0.5x TBE in a CHEF-DR™ II apparatus (BioRad) with the following pulsed field gel electrophoresis parameters: 200 V, 5-15 sec switch time, for 14.3 hours at 10 °C. The insert sizes of selected BAC clones were estimated after comparison with the CHEF DNA size standard lambda ladder (Bio-Rad) run in the same gel.

BAC-pool DNA was isolated from 250 µl aliquot per pool from the copy D of the libraries as described previously. The two barley BAC libraries were characterized for genome representation by PCR-screening of 46 pools per library with one microsatellite marker from each of the 14 barley chromosome arms. The markers were selected from the barley

microsatellite consensus map of Varshney et al. (2007). The reverse primer of each microsatellite was labeled with IRDye-700 or IRDye-800 and the PCR-product visualized on a LICOR 4200 DNA sequencer (LICOR® Biosciences).

Screening the library for BAC clones spanning the *Rphq2* locus

The work flow for identifying Vada and SusPtrit BAC clones spanning the *Rphq2* locus is presented in Figure 1. The solid and liquid selective LB media (here onwards LBA and LB, respectively) used were supplemented with 20 μg/ml of CAM unless indicated otherwise. The BAC libraries were screened following a PCR based method (Figure 1a). In a first step, we used molecular markers known to be closely linked to *Rphq2* to screen the BAC pools and to identify positive BAC clones. In a second step, after the identification and sequencing of several positive BAC clones, we used primers designed to amplify the BAC-end-sequences (bes), the genes annotated in these clones and newly developed markers. We also used primers to amplify sequences at the edges of gaps in the already assembled BAC sequences (Table 1).

For each BAC pool that was positive in the PCR screen, test plating was performed in order to allow an average of about 2,000 to 3000 colonies on a large square Petri dish (506.25 cm^2) which is used for robotic picking of BAC clones (Figure 1b). Each positive BAC pool was then diluted accordingly and 2 ml of the diluted positive BAC pools were plated on large Petri dishes containing LBA (Figure 1c). The BAC clones were grown at 37 °C for about 14 hours, and afterwards kept at 4 °C until use. The BAC clones on the large Petri dishes were picked using a picking robot (Genomic Solutions Flexsys picker) and cultured in 384-well plates containing LB-freeze [LB, 36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM trisodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% (vol/vol) glycerol] with 20 μg/ml CAM. The picked BAC clones were grown at 37 °C for 14 to 16 hours and stored at -80 °C (Figure 1d). Each 384-well plate was replicated onto a small square Petri dish (144 cm^2) containing LBA. The replicates were kept at 37 °C overnight. On each plate, 3 ml of LB were added and the clones were scrapped and collected (sub-pool) into a culture tube (Figure 1e). The DNA of each sub-pool was isolated (Sambrook et al. 1989) and PCR-screened to identify the positive sub-pool. The 384-well plate corresponding to the positive sub-pool was replicated again as described above onto three small square Petri dishes (Figure 1f).

In one of the small square Petri dishes, BAC clones from each column were pooled (24 column-pools). In another Petri dish, BAC clones from each row were pooled (16 row-pools) (Figure 1g). The pooling was done by streaking across a column or row using an

autoclaved toothpick and the pooled BAC clones were cultured overnight at 37 °C in culture tube containing 3 ml of LB. The DNA of each column- and row-pool was isolated (Sambrook et al. 1989) and PCR-screened to identify positive pools. The intersection between a positive column-pool and row-pool indicates a positive BAC clone. The positive BAC clone was picked from the third Petri dish using a toothpick and grown in a culture tube containing 3 ml LB at 37 °C overnight. The liquid culture was then diluted 10,000 to 20,000x and 100 µl of the culture was plated on a small round Petri dish (ø 94 x 16 mm) containing LBA and grown at 37 °C overnight. Five single-colonies were picked using a toothpick and grown as described above (labeled as 'a' to 'e'). Their DNA was isolated (Sambrook et al. 1989) and the colonies validated after PCR-amplification with the markers and primers used during the screening process. The positive single-colonies were maintained in glycerol stock (LB supplemented with 25% glycerol). The insert size of the BAC clones was determined as described in the previous section before further analyses (see below). The BAC clones were named as follow: "BAC pool number; Sub-pool number (384-well plate number); row letter; column number; single-colony letter" (eg. V41 P7 L 3 A).

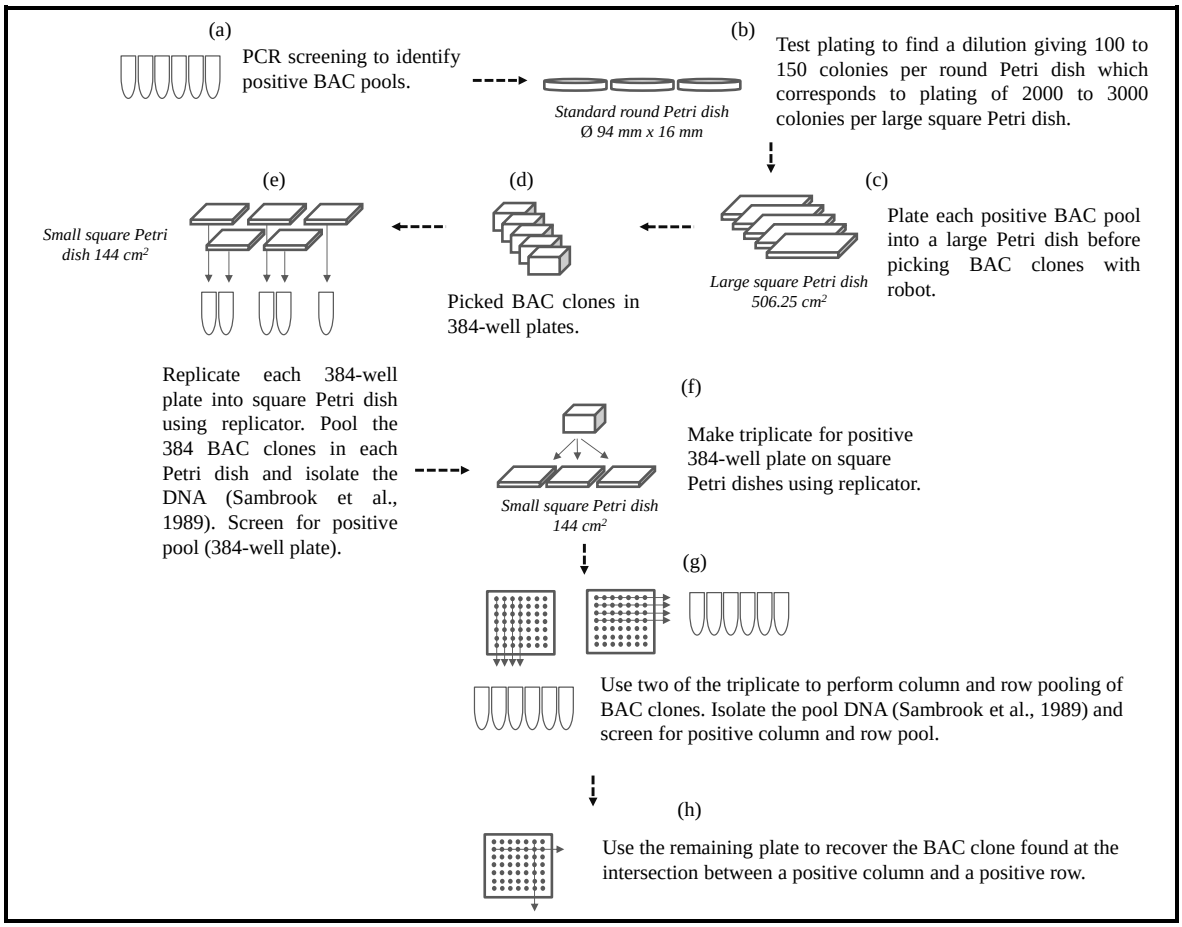

Figure 1: Work flow for the identification of BAC clones spanning the *Rphq2* locus in Vada and in SusPtrit.

Table 1: Primer pairs used to identify BAC clones of Vada and SusPtrit at the *Rphq2* locus

Name	Primers sequences (5'- 3')	Ta °C
[M]WBE114 [VS]	*F*: GGCGACCTCCAGCGTATC *R*: GTGGTTCGGTCCTTGATGAG	58
[M]WBE115 [VS]	*F*: GGCGGTCGGCATCGTCCAGT *R*: ATGCGTCCACAAAACCAATCTTCA	61
[M]P15M51-204 [V]	*F*: CGGAGGAAACATGGACAACGAA *R*: AGCGAGCTCACTGCCAATCTACC	56
[M]P14M54-252 [S]	*F*: AGACCAGCATTACCTAAGCAGAGA *R*: AGAGGAGAGTGAGTGTAGGTGTCG	56
[M]besV76P5D5AR [V]	*F*: GAGGAGCCGTGTCGTCTTGT *R*: CCGTTTCCGTTCACTGGTTAT	56
[M]besS35P2K14EF [S]	*F*: TTGAAACAGCTGGGGTCTT *R*: TGGTACACAAATATTCGTCTGC	58
[MG]Rphq2.S01 [S]	*F*: TGAAGGCGGGTTTGGTGTGGTGTA *R*: CCCGCGTATGATTCTCTGCCTCTT	58
[MG]Rphq2.V30 [S]	*F*: CGGCGGTGCGATCATAGAAT *R*: TCCCCGGCCGTAGAGTCC	65
[G]Rphq2.V32 [S]	*F*: GGGGCCCCGGCTATCGTGTA *R*: AACTTTCCGCGGCAATCCTTCTTCT	65
[*]S35P100001F4 [S]	*F:* CCTCGCTAGTCAAGGAGGTG *R:* GTGGCTGTTGTAGGGACGAT	65
[*]S35P100004F2 [S]	*F:* TTAATTTCTGCTCGCGTGTG *R:* TGCATGCACTCCTCGTTTAG	65
[M]S7300002F [S]	*F:* GACGTTGAGGAGAGCAAAGG *R:* GCCGTTTATCACGAGGTTGT	65

[M] A molecular marker

[G] An annotated gene

[MG] An annotated gene converted into a marker

[*] A short DNA fragment at the edge of a gap in assembled BAC sequences.

[S] Primer pair used to screen only the BAC library of SusPtrit

[V] Primer pair used to screen only the BAC library of Vada

[VS] Primer pair used to screen the BAC library of Vada as well as SusPtrit

Ta is the annealing temperature

BAC clones fingerprinting

All the confirmed positive BAC clones from the Vada and SusPtrit libraries were fingerprinted following the AFLP procedure from Brugmans et al. (2006) using the H*ind*III/*Taq*I restriction enzyme combination. The generated fragments were separated on a LICOR 4200 DNA sequencer (LI-COR Biosciences, Lincoln, NE, USA). The fingerprints were scored manually. Shared bands between BAC clones indicated sequence overlaps between the clones.

BAC-end sequencing

The extremities of all the confirmed positive BAC clones were sequenced (i.e. BAC-ends sequencing). The clones were digested individually with 12 different blunt-end restriction endonucleases (*Alu*I, *Bsh*1236I, *BspL*I, *Bsu*RI, *Dpn*I, *Dra*I, *Exo*32I, *Hin*cII, *KspA*I, *Rsa*I, *Sca*I, *Sma*I and *Ssp*I), and ligated with non-specific blunt adapters (genome walker adapter). The restriction-ligations were performed in 50 µl reaction volume comprising 50-100 ng DNA template, 1x restriction and ligation buffer (RL buffer), 0.02 unit T4 DNA ligase, 0.1 unit restriction enzyme, 0.5 µM genome walker adapter (GWadp; top: 5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGA-3'; bottom: 5'-PO_4-TCCAGCCC-NH_2-3'), and 0.2 mM adenosine triphosphate (ATP). The reactions were incubated overnight at 37 °C and diluted 20x with MilliQ water (RL-DNA). For each sample, a nested PCR approach with two rounds of amplification were carried out on the 12 RL-DNA in order to obtain PCR products as specific as possible before sequencing. The first PCR was performed in 20 µl reaction volume comprising 5 µl RL-DNA, 1x PCR buffer, 0.2 mM dNTPs, 0.15 mM forward-1 pIndigoBAC/ pIndigoBAC-5 primer (5'-GGATGTGCTGCAAGGCGATTAAGTTGG-3'), 0.15 mM adapter primer-1 (5'-TAATACGACTCACTATAGGGC-3'), and 0.02 unit Taq DNA polymerase. A separate PCR reaction was performed using the reverse-1 pIndigoBAC/ pIndigoBAC-5 primer (5'-CTCGTATGTTGTGTGGAATTGTGAGC-3'). The first-PCR product was diluted 50x in MilliQ water (Merck Millipore) and 5 µl of the diluted PCR product was used as template for the second PCR. The second PCR was performed in 20 µl reaction volume. The PCR reaction mixture was similar to the first PCR, except for the primers that were used. They were the forward-2 pIndigoBAC/ pIndigoBAC-5 primer (5'-ACGGCCAGTGAATTGTAATA-3') and adapter primer-2 (5'-ACTATAGGGCACG CGTGGT-3'). A separate PCR reaction was performed using the reverse-2 pIndigoBAC/ pIndigoBAC-5 primer (5'-GGAAACAGCTATGA CCATGA-3').

The first PCR reaction was carried out with 24 cycles of 30 seconds denaturation at 94 °C, 30 seconds annealing at 56 °C and 90 seconds extension at 72 °C (Profile A). The second PCR reaction was carried out with 5 minutes of initial denaturation at 94 °C, 35 cycles of Profile A and 7 minutes of final extension at 72 °C. The final PCR product was visualized on 1% agarose gel. For each BAC clone, the largest fragment (depending on the initial restriction endonuclease) obtained in the final PCR product of the forward and of the reverse PCR reactions was selected for sequencing. Primers which amplify the BAC-ends were designed by using Lasergene software (DNASTAR® 8 Inc., Madison, WI, USA).

BAC clone sequencing and annotation

The *Rphq2* genetic window is delimited by proximal (WBE114) and distal (WBE115) markers for which at least one recombination event has been found with *Rphq2*. Three BAC clones which fully covered the *Rphq2* genetic window (V41P7L3A, V48P5B18A and V76P5D5A) were sequenced following a shotgun approach at Macrogen. One SusPtrit BAC clone was sequenced via a shotgun approach as well, S35P2K14E. The number of reads obtained for the BAC clones of Vada and SusPtrit ranges from 1000 to 1740 reads with an average length of 867 bp, corresponding to 8x sequencing depths. For each BAC clone, the short shotgun sequences were assembled in contigs and ordered by Macrogen. Several gaps remained within the BAC clones with five to 14 contigs per clone. Therefore, the three BAC clones of Vada and S35P2K14E of SusPtrit were resequenced following a 454 sequencing approach at Greenomics™ in order to bridge the gaps. Two additional BAC clones of SusPtrit (S35P1J10A and S73P5N20A) were also sequenced following this 454 sequencing approach. The three SusPtrit BAC clones, S35P2K14E with S35P1J10A and S73P5N20A, together cover only partially the targeted *Rphq2* genetic window. The number of reads per BAC clone obtained from 454 sequencing ranges from 15570 to 38120 reads with an average length of 350 bp, correponding to 20x sequencing depths for each BAC clone. For each BAC clone, the short sequences of 454 sequencing were assembled in contigs by Greenomics™.

The obtained 454 sequence contigs were aligned to the previously assembled shotgun sequence contigs [Lasergene software (DNASTAR® 8 Inc., Madison, WI, USA)]. No discrepancy was observed between the assemblies obtained via both approaches. Based on the AFLP fingerprints of the BAC clones of Vada, V76P5D5A overlaps proximally with V41P7L3A and distally with V48P5B18A. We identified the overlapping sequences and confirmed the fingerprints. According to the AFLP fingerprints of the BAC clones of SusPtrit, S73P5N20A overlaps with S35P2K14E, and S35P1J10A is located proximally and not overlapping. We identified the overlapping sequences between S73P5N20A and S35P2K14E which also helped us to order the 454 sequence contigs. The 454 sequence contigs obtained for clone S35P1J10A were ordered according to a dot plot analysis (MUMMER; https://bacregistry.potatogenome.net) against V41P7L3A which share the same marker (WBE114).

The consensus sequence corresponding to the *Rphq2* genetic window of Vada, flanked by markers WBE114 and WBE115, was generated and annotated using the TriAnnot pipeline (Leroy et al. 2012) following the architecture for barley (http://wheat-urgi.versailles.inra.fr/Tools/Triannot-Pipeline/Architecture). The consensus sequence corresponding to the

Rphq2 genetic window of SusPtrit were annotated separately using the same TriAnnot pipeline.

Marker saturation of the regions containing *Rphq2*

Between the flanking markers (WBE114 and WBE115), BAC-end sequences and annotated gene sequences were used to develop sequence characterized amplified regions (SCAR) and cleaved amplified polymorphic sequence (CAPS) markers, polymorphic between Vada, SusPtrit and L94. Markers polymorphic between Vada and L94 was genetically mapped using homozygous recombinant plants from Marcel et al. (2007a) in order to confirm their position and order. SCAR and CAPS markers were also developed from the AFLP fingerprints by converting polymorphic bands to single locus PCR markers following the strategy proposed by Brugmans et al. (2003). Primers were designed using Lasergene software (DNASTAR® 8 Inc., Madison, WI, USA). The markers developed were used to assist in ordering of BAC clones.

WBE114 and WBE115 (Table 1) together with two newly developed markers, viz. Rphq2.S01 (Table 1) and Rphq2.V09; F: 5'-GCCTCTACTTCCACGACTGC-3', R: 5'-CCGGAGATGAC GATGATGT-3') were used to screen the Morex BAC library (Nils Stein, Leibniz Institute of Plant Genetic and Crop Plant Research, IPK) to identify the homologous *Rphq2* region in the Morex genomic sequence.

Comparative mapping in barley, rice and *Brachypodium*

The sequence of annotated genes in the physical window of *Rphq2* were used for blast searches of rice and *Brachypodium distachyon* homologous genes, respectively, in the Rice Genome Annotation Project blast search (http://rice.plantbiology. msu.edu/analyses_ search_blast.shtml) and in the *B. distachyon* blast portal (http://blast. brachypodium.org/). For each annotated gene in barley, the best blast hit was retained above a threshold e-value $\leq$ 1.0E-15.

Results

We have constructed two non-gridded BAC libraries of barley from the cv. Vada and from the experimental line SusPtrit, respectively. Vada not only carries the resistance allele of our target QTL for map-based cloning, *Rphq2*, but also for many other QTLs for partial and nonhost resistances to adapted and non-adapted rust species (Jafary et al., 2006; 2008), for which SusPtrit contains the susceptibility allele.

Construction and characterization of the BAC libraries

The Vada BAC library was generated from 6 different ligation reactions with pIndigoBAC vector and 8 different ligation reactions with pIndigoBAC-5 vector. The Vada library was organized in 116 pools named V1 to V116 (Supplemental Table 1), containing an average of 1,435 clones per pool. The percentage of (white) recombinant clones was estimated to be 96.8% based on the count of blue (non-recombinant) and white (recombinant) colonies per plate. Thus, the library consists of approximately 161,000 recombinant clones. The average size of inserts ranges from 67 Kbp to 98 Kbp. The observed insert sizes for each fraction do not correspond to the expectations based on the size selection (Table 2).

Table 2: Composition of the Vada BAC library

Size selection range (Kbp)	Number of pools	White colonies	Blue colonies [1]	Insert size [2] (Kbp)	Coverage [3] (Mbp)
H0: 50-100	3	225	0	- *	-
H1: 100-150	41	1636	45	98.3	6,593.6
H2: 150-200	35	1724	79	67.4	4,066.9
H3: 200-250	34	963	20	71.9	2,354.1
H4: 250-300	3	97	1	- *	-
Total	**116**	**1389**	**46**	**81.2**	**13,014.6**

[1] Estimation of the average number of colonies per pool

[2] Average insert size estimated with 24 randomly selected BAC clones per fraction

* because of their low number, the clones from fractions H0 and H4 have been neglected

[3] Calculated with the estimated number of white colonies and their average insert size.

The SusPtrit BAC library was generated from six different ligation reactions with pIndigoBAC vector and seven different ligation reactions with pIndigoBAC-5 vector. The SusPtrit library was organized in 110 pools named S1 to S110 (Supplemental Table 2),

containing an average of 1,606 clones per pool. The percentage of recombinant clones was estimated to be 97.9 % based on the count of blue and white colonies per plate. Thus, the library consists of approximately 173,000 recombinants clones. The average size of SusPtrit inserts ranged from 107 Kbp for selected fraction H1 to 141 Kbp for selected fraction H3 (Table 3).

Table 3: Composition of the SusPtrit BAC library

Size selection range (Kbp)	Number of pools	White colonies	Blue colonies [1]	Insert size [2] (Kbp)	Coverage [3] (Mbp)
H0: 50-100	9	3,316	3	83.0 *	2,477.1
H1: 100-150	37	1,716	43	106.7	6,775.8
H2: 150-200	38	1,704	48	110.5	7,152.9
H3: 200-250	26	571	12	140.6	2,087.7
H4: 250-300	-	-	-	-	-
Total	**110**	**1,572**	**34**	**108.0**	**18,493.5**

[1] Estimation of the average number of colonies per pool
[2] Average insert size estimated with 24 randomly selected BAC clones per fraction
* corresponds to the insert size of a single BAC clone isolated from fraction H0
[3] Calculated with the estimated number of white colonies and their average insert size.

Based on a haploid barley genome size of 4.98 Gb (The International Barley Genome Sequencing Consortium 2012) and on the genome coverage of each fraction of the libraries (Tables 2 and 3), we estimated that the coverage of the Vada and SusPtrit BAC libraries are approximately 2.6 and 3.7 genome-equivalents, respectively. Together, the libraries cover 6.4 genome-equivalents that allow for a probability greater than 99% of recovering any specific sequence from the barley genome (Clarke and Carbon 1976).

Genome representation of the BAC libraries

To verify the genome representation of the libraries, we screened 46 pools of Vada (V1 to V46) and 46 pools of SusPtrit (S1 to S46) corresponding approximately to 1.4 and 1.9 genome-equivalents, respectively, with 14 microsatellite markers, each representing a chromosome arm of barley (Table 4). The microsatellite markers were mapped on a consensus map of barley (Varshney et al. 2007). We selected in priority the most robust markers that were also polymorphic between Vada and SusPtrit. For only two microsatellites, GBMS062 and GBM1482, Vada and SusPtrit had the same allele (Table

4). The number of positive pools was determined (Table 4) by counting the number of pools displaying a band of similar size as the one of the parental genomic DNA run on the same gel. None of the bands amplified in a BAC pool from one genotype had the size of the allele from the other genotype, indicating that the contamination of one library with clones from the other library is unlikely.

An average of 2.7 positive pools per microsatellite marker was obtained for the Vada library and an average of 3.5 positive pools for the SusPtrit library (Table 4). All markers were represented at least once in the 46 pools of the SusPtrit library and only two markers were not represented in the 46 pools of the Vada library, indicating that the overall barley genome is well represented in our BAC libraries. Based on the average representation of the 14 microsatellite markers in 46 pools per library, we estimated that the total coverage of the Vada and SusPtrit BAC libraries are 5.0 and 6.8 genome-equivalents, respectively.

Table 4: PCR-based screening of the Vada and SusPtrit BAC libraries with microsatellite markers representing each chromosome arm of barley on a subset of 46 pools per library (representing approximately 1.4 and 1.9 barley genome-equivalents)

Chrom.	Position (cM) [1]	Microsatellite	Vada allele [2] (bp)	SusPtrit allele [2] (bp)	Nr. Pools Vada [3]	Nr. Pools SusPtrit [3]
1HS	25.0	GBMS062	127	127	2	8
1HL	73.4	Bmac0032	-	220	1	2
2HS	28.6	HVM36	126	108	6	4
2HL	90.9	GBM1062	215	219	3	2
3HS	35.4	scssr10559	210	216	3	4
3HL	150.5	HVM62	260	250	2	4
4HS	47.1	GBM1482	210	210	12	5
4HL	125.5	GBM1015	232	220	0	1
5HS	21.2	GBM1176	-	300	1	4
5HL	88.0	Bmag0223	174	170	1	5
6HS	4.6	Bmac0316	169	165	1	3
6HL	129.2	GBM1087	201	198	4	2
7HS	18.6	Bmag0007	200	204	2	4
7HL	87.7	GBM1359	151	145	0	1

[1] Position of the corresponding microsatellite marker on the barley integrated map, Marcel 2009 available at http://wheat.pw.usda.gov/GG2/index.shtml (Aghnoum et al. 2010)

[2] Approximate size of the allele amplified on Vada or SusPtrit genomic DNA

[3] Number of positive pools per library

Generation of Vada and SusPtrit sequences at *Rphq2*

We used four pairs of primers for the BAC library of Vada and ten primer pairs for the BAC library of SusPtrit (Table 1) to screen for BAC clones spanning the Vada or SusPtrit allele at *Rphq2*. For Vada, the four primer pairs detected 16 positive BAC pools in total from the Vada BAC library and for SusPtrit the ten primer pairs detected a total of 21 positive BAC pools in the SusPtrit BAC library (Supplemental Table 3). The BAC pools positive for two or more primer pairs were prioritized for BAC clones picking. We picked around 1900 (five 384-well plates) to 5760 (15 384-well plates) colonies per positive pool. Then, we identified the 384-well plates (sub-pools) positive for the primer pairs. Consecutively, we confirmed the amplification with primer pairs on row and column pools of the positive sub-pool. Finally, we validated seven Vada BAC clones originating from four Vada BAC pools and 17 SusPtrit BAC clones originating from nine SusPtrit BAC pools. All BAC clones were fingerprinted. The order of the BAC clones was not only based on the BAC fingerprint, but also on the primer amplification.

From the seven BAC clones of Vada, the AFLP fingerprint revealed a minimum tiling path of only three clones [V41P7L3A (120 Kbp), V76P5D5A (87 Kbp) and V48P5B18A (150 Kbp)], which were overlapping each other to cover the *Rphq2* genetic window between markers WBE114 and WBE115. Based on the primer amplification, the three Vada BAC clones were bridged together by V76P5D5A. This BAC clone overlapped with V41P7L3A harboring WBE114 and V48P5B18A harboring WBE115 (Table 5).

From the 17 BAC clones of SusPtrit, six had an identical AFLP fingerprint with S35P1J10A (105 Kbp) which harbored WBE114, but were not overlapping with any of the other identified SusPtrit BAC clones. On the other side, three BAC clones positive for WBE115 were overlapping with each other [S35P2K14E (140 Kbp), S7P2C21A (80 Kpb) and S81P2C6A (135 Kbp)], and with a fourth BAC clone [S73P5N20A (165 Kbp)] (Table 5). S35P2K14E was selected for sequencing because it was positive not only for WBE115 but also for P14M54-252, a marker mapped closer to *Rphq2* (Marcel et al. 2007a; Figure 2). S73P5N20A was also selected for sequencing because it overlapped with S35P2K14E distal to WBE115, and was expected to extend further into the *Rphq2* genetic window.

The three BAC clones of Vada were sequenced following both shotgun and 454 sequencing approaches. The sequences obtained with one or the other approaches were assembled independently. The sequence assembly obtained from shotgun sequencing had ten gaps between WBE114 and WBE115, while the sequence assembly obtained from 454 sequencing had 12 gaps. Aligning both assemblies led to a consensus sequence with only two gaps left. One of the gaps was between two sequence contigs from V76P5D5A and

the other gap was between two sequence contigs of V48P5B18A. Together, the two gaps represent approximately 7 Kbp, estimated by comparing the estimated insert size of the BAC clone with the size of the consensus sequence. The physical length of the three contigs covering the Vada BAC sequence from WBE114 to WBE115 is approximately 195 kb not including the two gaps.

Three BAC clones were sequenced from the SusPtrit library. The BAC clone S35P2K14E was sequenced following both shotgun and 454 sequencing approaches while the other two BAC clones S73P5N20A and S35P1J10A were sequenced following a 454 sequencing approach only. The sequences obtained with one or the other approaches were assembled independently. The sequence assembly of S35P2K14E from shotgun sequencing had six gaps, while the sequence assembly obtained from 454 sequencing for this clone had four gaps. Aligning both assemblies led to a consensus sequence of 122 Kbp with no gap for S35P2K14E. The sequence assemblies for the other two SusPtrit clones were composed of four contigs each (three gaps for each clone). The consensus sequence of S73P5N20A and S35P1J10A contigs resulted in three contigs of sizes 139 Kbp, 563 bp, and 60 Kbp. Based on the available sequences, we estimated a 226 Kbp length for the complete SusPtrit consensus sequence between WBE114 and WBE115.

Table 5: Ordering of the BAC clones from Vada (A) and SusPtrit (B) libraries according to positive PCR amplification with developed primers at the *Rphq2* locus.

(a)	besV41P7L3AR	WBE114	besV76P5D5AF	besV41P7L3AF	P51M51-204	besV48P5B18AR	besV76P5D5AR	WBE115	besV48P5B18AF
V41P7L3A	+	+	+	+	-	-	-	-	-
V76P5D5A	-	-	+	+	+	+	+	-	-
V48P5B18A	-	-	-	-	-	+	+	+	+

(b)	besS35P1J10AR	WBE114	besS35P1J10AF	besS35P2K14EF	Rphq2.S01	P14M54-252	besS81P2C6AF	bfsS35P2K14EF-267	bfsS35P2K14EF-283	WBE115	besS7P2C21EF	bfsS35P2K14EF-468	besS35P2K14ER	besS81P2C6AR
S35P1J10A	+	+	+	-	-	-	-	-	-	-	-	-	-	-
S73P5N20A*	-	-	-	+	+	NT	NT	+	+	NT	NT	-	-	NT
S35P2K14E	-	-	-	+	NT	+	+	NT	+	+	+	+	+	-
S7P2C21A	-	-	-	-	NT	-	-	NT	-	+	+	-	-	-
S81P2C6A	-	-	-	-	NT	-	+	NT	+	+	+	+	+	+

Grey boxes indicate the positive amplification of the primers on the respective BAC clones.
* We failed to obtained primers which can amplify the BAC ends.
a "+" indicates the positive amplification.
NT, Not tested.
Marker name "bes…" refers to marker developed from bac end sequence, and "bfs…" refers to marker developed from bac AFLP-fingerprint sequence
Marker information is available in supplemental Table 4

Sequence annotation

The TriAnnot pipeline (Leroy et al. 2012) predicted 12 genes on both Vada and SusPtrit contigs. On Vada contigs, the predicted genes encoded three peroxidases (V.Perox-1, -2, -3), two kinases (V.Kin-1, -2), one Seven in absentia protein family (V.SINA), one protein legume lectin domain (V.Leg-Lec-D), and five unknown function/hypothetical/ uncharacterized proteins (V.UF/HP/UP. On SusPtrit contigs, the predicted genes encoded three kinases (S.Kin-1, -2, -3), two peroxidases (S.Perox-1, -2), and seven unknown function/hypothetical/uncharacterized proteins (S.UF/HP/UP). Among the predicted genes, V.Perox-1, V.Perox-2, V.UP, V.Kin-1 and V.Kin-2 from Vada were positioned at the same locus (allelic) as S.Perox-1, S.Perox-2, S.UP, S.Kin-2 and S.Kin-3 from SusPtrit, respectively. Their positions were supported by co-dominant markers except the locus of V.Perox-2 and S.Perox-2 (Figure 2). The common genes between Vada and SusPtrit shared at least 99% similarity except for V.Kin-1 and S.Kin-2 which had similarity of at least 90%. The other annotated genes were not shared between Vada and SusPtrit (Figure 2). The V.UF and V.HP from Vada were not aligning to the S.UF and S.HP from SusPtrit, respectively, and were regarded as different loci. The likelihood of genes listed in Figure 2 to be candidates for *Rphq2* will be discussed in the Discussion section.

Using the sequence information between WBE114 and WBE115, we developed 38 new markers that were polymorphic between Vada, SusPtrit and L94. Interestingly, SusPtrit and L94 have the same genotype for all the markers obtained. Together with two AFLP-converted single locus PCR markers developed by Marcel et al. (2007a), 40 markers were mapped between the flanking markers WBE114 and WBE115 (Supplemental Table 5). The genetic positions of the 40 markers were resolved based on their physical order and position on the sequence between WBE114 and WBE115. Among the 40 markers, 20 were dominant markers amplifying Vada DNA and 17 were dominant markers amplifying SusPtrit DNA (Figure 2). There were three co-dominant markers; two were developed based on the common annotated genes between Vada and SusPtrit and one based on a random sequence from one of the sequence contigs of S73P5N20A. These three co-dominant markers were located close to each other near WBE115. Based on the sequence annotation and the dominant nature of most of the newly developed markers located between WBE114 and WBE115, there seems to be a lack of homology between Vada and SusPtrit for the region containing *Rphq2*. This is further supported by a dot plot analysis (MUMMER; https://bacregistry.potatogenome.net) which compares the sequence of Vada and SusPtrit between WBE114 and WBE115 (Figure 3). Sequence similarity is only observed on approximately the first 36 Kbp of S35P1J10A which included WBE114.

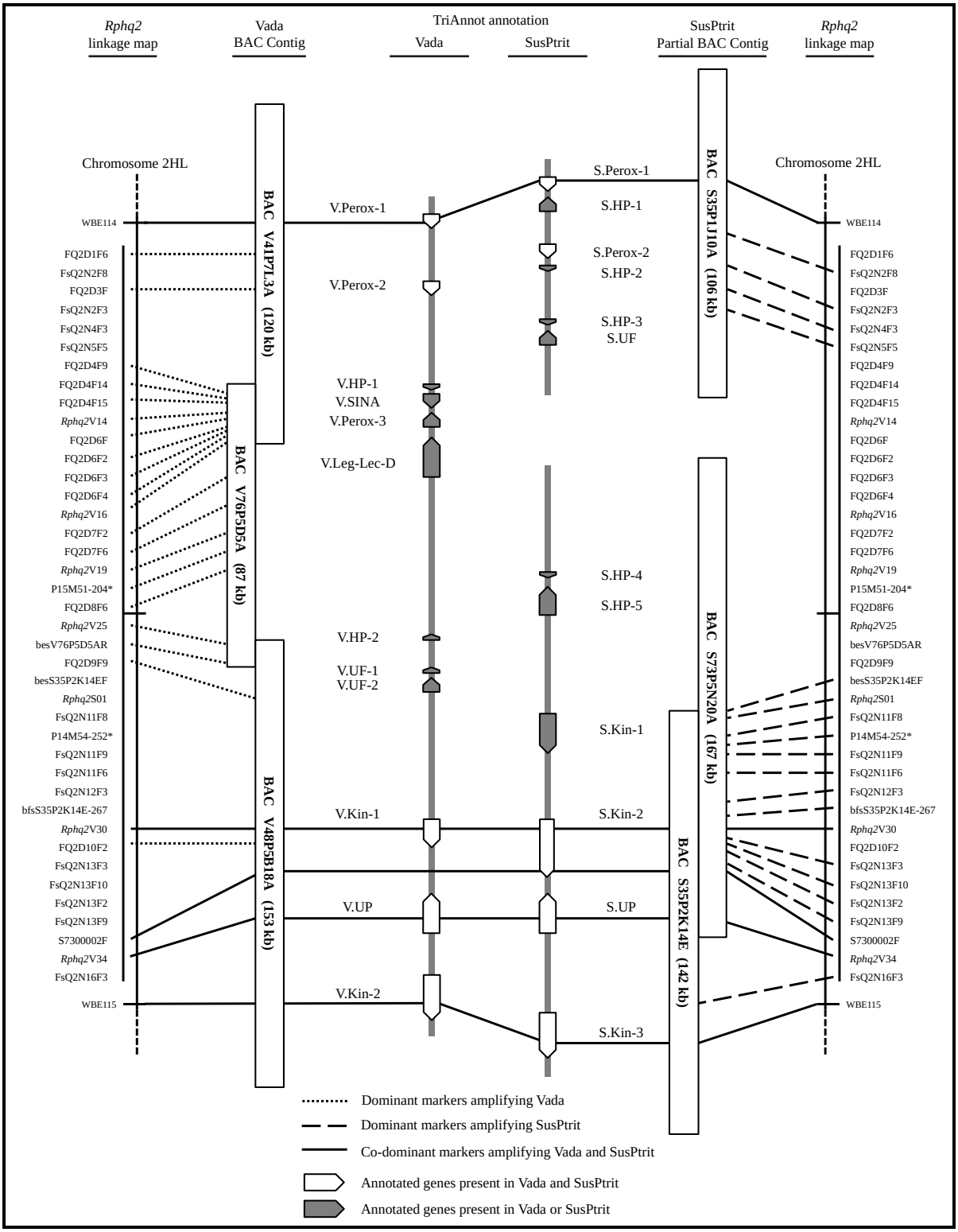

Figure 2: A schematic presentation of the 0.1 cM marker interval between WBE114 and WBE115 containing *Rphq2*: linkage map, the BAC contig of Vada and SusPtrit, and the Triannot annotated genes in the contigs. Markers with * are AFLP-converted single locus PCR markers developed by Marcel et al. (2007a).

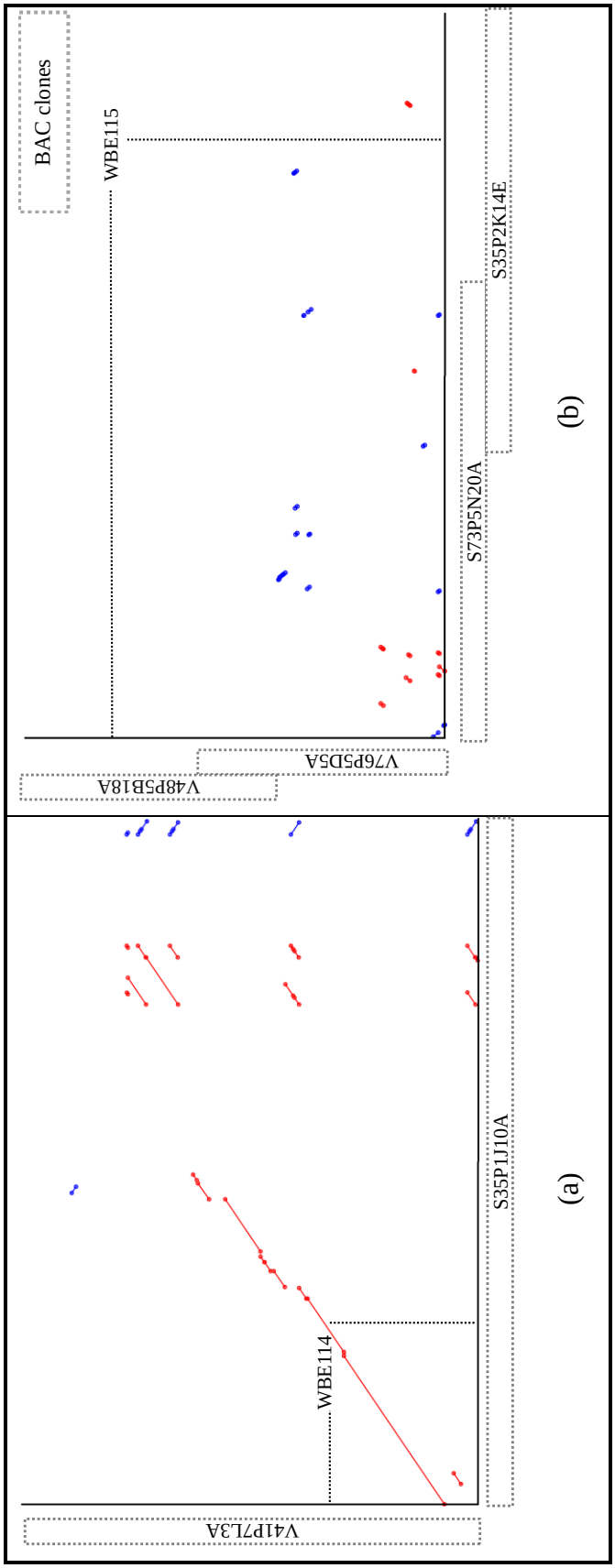

Figure 3: Dot plots suggest low homology between the *Rphq2* sequence of Vada and SusPtrit. (a) Alignment between sequences of Vada and SusPtrit proximal to the gap in SusPtrit BAC contig. (b) Alignment between sequences of Vada and SusPtrit distal from the gap in SusPtrit BAC contig

We searched for the homologous *Rphq2* region in the Morex genomic sequence (The International Barley Genome Sequencing Consortium 2012). A pair of primers which amplifies an annotated gene in Vada, Peroxidase 2 (Rphq2.V09) detected four BAC clones of Morex (HVVMRXALLEA0011E05, HVVMRXALLEA0179K22, HVVMRXALLe A0269J17 and HVVMRXALLeA0278A17). These four BAC clones are overlapping with each other and positioned between 806 to 981 kb of contig 44195 (1.9Mb) on chromosome 2HL (http://mips.helmholtz-muenchen.de/plant/barley/index.jsp). Another pair of primers which amplifies an annotated gene in SusPtrit, Kinase 1 (Rphq2.S01; Table 1) detected one BAC clone, HVVMRXALLeA0299N24 which also belongs to contig 44195, positioned between 961 and 1101 kb of the contig. A total of 118 Morex sequence contigs (Mor_cont) from the BAC contig 44195 (kindly provided by Nils Stein, Leibniz Institute of Plant Genetic and Crop Plant Research, IPK) were aligned to the sequence between WBE114 and WBE115 of Vada and SusPtrit using Lasergene software with a minimum sequence match of 80% (DNASTAR® 8 Inc., Madison, WI, USA). Eleven of the 118 Mor_cont from BAC contig 44195 aligned to the Vada sequences and 17 to the SusPtrit sequences (Table 6). We found that Mor_cont 43090 was approximately 300 bp distal from WBE114. Another one, the Mor_cont 2546833, aligned to a region including WBE115. We estimated the physical distance between WBE114 and WBE115 of Morex to be of approximately 254 kb which is very similar to the estimated physical distance in SusPtrit. More sequences from SusPtrit could be aligned on the Morex sequences indicating that SusPtrit and Morex are likely to be more similar than Vada and Morex at the *Rphq2* locus.

The synteny between barley, rice and *Brachypodium*

The *Rphq2* region is located in the barley 2L1.0 region, which has a syntenic relationship with a region on rice Chromosome 4 (Marcel et al. 2007a) and a region on *Brachypodium* Chromosome 5 (Mayer et al. 2011). Homologs of the two genes used to develop markers WBE114 and WBE115 have indeed been identified on rice Chromosome 4 and on *Brachypodium* Chromosome 5 (Table 7). The interval between the rice homologs Loc_Os04g59260 and Loc_Os04g59320 contains five annotated genes encoding a hypothetical protein, two retrotransposon proteins, a strictosidine synthase, and a phospholipase C. The interval between the *Brachypodium* homologs Bradi5g27210 and Bradi5g27240 contains 2 genes encoding a peroxidase and a phospholipase C. The gene used to develop the marker WBE114 encodes a peroxidase precursor. Interestingly, this gene is present in a single copy in rice, in two copies in *Brachypodium* and in barley line SusPtrit, and in three copies in barley cultivar Vada (Table 7). In addition, only two additional genes seem to be shared between the three species and the two barley genotypes

including a phospholipase C and a protein kinase used to develop marker WBE115. The remaining 7 genes predicted only from the Vada sequence and 7 genes predicted only from the SusPtrit sequence have no homolog in the identified synthetic regions of rice and *Brachypodium*. The best blast hits (threshold ≤ 1.0E-15) obtained for some of those genes are located outside of the *Rphq2* syntenic interval (Table 7).

Table 6: Alignment of 18 Morex sequence contigs on Vada and SusPtrit sequence assemblies between WBE114 and WBE115 (corresponding to Morex BAC contig 44195)

Morex contigs; (sequence size, bp)	Align to Vada	Align to SusPtrit
Mor_cont 43090; (5993)/WBE114[*]	Yes[$]	Yes
Mor_cont 53633; (3569)	Yes	No
Mor_cont 2267159; (240)	No	Yes
Mor_cont 88428; (1710)	No	Yes
Mor_cont 2550490; (9192)	No	Yes
Mor_cont 2343918; (327)	No	Yes
Mor_cont 41082; (7623)	No	Yes
Mor_cont 442275; (1129)	No	Yes
Mor_cont 280043; (1061)	No	Yes
Mor_cont 1590687; (2251)	Yes	Yes
Mor_cont 287733; (1989)	Yes	Yes
Mor_cont 224400; (1060)	Yes	Yes
Mor_cont 321571; (308)	Yes	Yes
Mor_cont 60124; (3413)	Yes	Yes
Mor_cont 1588307; (1769)	Yes	Yes
Mor_cont 1572547; (2594)	Yes	Yes
Mor_cont 8886; (1904)	Yes	Yes
Mor_cont 2546833; (3163)/WBE115[!]	Yes	Yes

[*] WBE114 is 301 bp distal from Mor_cont 43090

[$] Yes means that the Morex sequence aligned to the subject with a minimum sequence match of 80%

[!] The WBE115 sequence of Vada aligned to Mor_cont 2546833 with one single nucleotide polymorphism and two for the WBE115 sequence of SusPtrit.

Table 7: Best blast hits (≤ 1.0E-15) of the predicted genes in Vada and SusPtrit sequences with the rice and the *Brachypodium* gene catalogues

Vada	Rice	*Brachypodium*	SusPtrit	Rice	Brachypodium
* Peroxidase 1	Loc_Os04g59260 (5.6E-127)	Bradi5g27210 (1.3E-134)	* Peroxidase 1	Loc_Os04g59260 (3.4E-128)	Bradi5g27210 (2.7E-134)
Na			Hypothetical protein	x	x
Peroxidase 2	Loc_Os04g59260 (3.9E-103)	Bradi5g27220 (9.7E-119)	Peroxidase 2	Loc_Os04g59260 (1.0E-103)	Bradi5g27220 (3.0E-119)
Na			Hypothetical protein	x	x
Na			Hypothetical protein	x	x
Na			Unknown function	LOC_Os07g35310 (4.2E-61)	Bradi1g25552 (3.0E-73)
Hypothetical protein	x	x	Na		
Seven in Absentia protein	LOC_Os05g06070 (1.7E-32)	Bradi2g01770 (7.7E-43)	Na		
Peroxidase 3	Loc_Os04g59260 (3.8E-108)	Bradi5g27220 (4.1E-120)	Na		
Protein containing legume lectin domain	LOC_Os08g03002 (4.4E-60)	Bradi1g28320 (7.2E-63)	Na		
Na			Hypothetical protein	LOC_Os11g30140 (1.7E-24)	x
Na			Hypothetical protein	x	x
Hypothetical protein	x	x	Na		
Unknown function	LOC_Os10g37260 (1.4E-66)	Bradi3g30820 (4.4E-63)	Na		
Unknown function	LOC_Os10g37260 (4.0E-28)	Bradi3g30820 (1.2E-20)	Na		
Na			Kinase 1	LOC_Os07g35310 (1.4E-136)	Bradi1g25552 (5.0E-194)
Kinase 1	LOC_Os08g03020 (5.4E-140)	Bradi1g28320 (8.7E-225)	Na		
Na			Kinase 2	LOC_Os08g02996 (4.6E-145)	Bradi1g28320 (3.5E-221)
Uncharacterizaed Protein	Loc_Os04g59310 (5.3E-138)	Bradi5g27230 (7.0E-160)	Uncharacterizaed Protein	Loc_Os04g59310 (2.8E-137)	Bradi5g27230 (1.9E-159)
! Kinase 2	Loc_Os04g59320 (1.5E-201)	Bradi5g27240 (2.4E-222)	! Kinase 3	Loc_Os04g59320 (1.5E-201)	Bradi5g27240 (2.4E-222)

Na; Not annotated

x; No hit or no significant hit

Grey boxes indicate the syntenic interval of *Rphq2* with rice chromosome 4 (indicated by Loc_Os**04**) and *Brachypodium* chromosome 5 (indicated by Bradi**5**).

* WBE114 is located within this annotated gene.

! WBE115 is located within this annotated gene.

Discussion

Map-based cloning is one of several approaches to isolate the gene(s) responsible for a QTL with an effect explaining more than 10 % of the phenotypic variation (Kou and Wang 2012). For rice, map-based cloning was successful to isolate the genes responsible for two resistance QTLs against *Magnaporthe oryzae* (Fukuoka et al. 2009; Hayashi et al. 2010). For wheat, genes were isolated for one resistance QTL against *P. striiformis* (Fu et al. 2009) and one wide-spectrum QTL against *P. triticina*, *P. striiformis* and *Blumeria graminis* (Krattinger et al. 2009) following a map-based cloning approach. The partial resistance QTL *Rphq2* explains 35% of the phenotypic variation in L94/Vada recombinant inbred lines population (Qi et al. 1998) and therefore, map-based cloning of *Rphq2* should be possible.

Vada and SusPtrit BAC libraries

Two non-gridded BAC libraries were constructed for Vada and SusPtrit barley genotypes. We observed in the Vada BAC library that the average size of inserts decreased from 98.3 Kbp for selected fraction H1 to 71.9 Kbp for selected fraction H3 (Table 2), while fractions H1 to H3 correspond to size selected fragments increasing from 100 to 250 kb, respectively. This could be due to a bias in the size selection procedure during library construction. Indeed, only one size selection was performed instead of two or three as recommended by different authors (Chalhoub et al. 2004; Peterson et al. 2000). If the first size selection allows selection of mostly DNA fragments longer than 100 Kbp, some small DNA fragments may remain trapped within the longer ones; and this may be especially true when the DNA concentration in the plugs is relatively high (Peterson et al. 2000). The average insert size over the complete Vada BAC library was 81 Kbp, with individual clones ranging from 18 to 209 Kbp (Supplemental Figure 1a; Table 2). In contrast to the Vada BAC library, the average size of inserts in the SusPtrit BAC library logically increased from 107 Kbp for selected fraction H1 to 140.6 Kbp for selected fraction H3. We presume that a lower concentration of SusPtrit DNA in the size fraction gel resulted in a lower amount of small DNA fragments being trapped than for Vada DNA. Consequently, the average insert size over the complete SusPtrit BAC library was 108 Kbp, with individual clones ranging from 33 to 274 Kbp (Supplemental Figure 1b; Table 3). On average SusPtrit inserts are 27 Kbp longer than Vada inserts. The average insert size of the Vada BAC library is the smallest among the BAC libraries available for barley. Such small average insert size is also observed in BAC libraries from other plant species such as wheat (Janda et al. 2004; Nilmalgoda et al. 2003) and soy bean (Xia et al. 2014).

The average insert size of the Vada and SusPtrit BAC libraries is still comparable to three of the Morex BAC libraries (HVVMRXALLrA, HVVMRXALLhB and HVVMRXALLh C) recently made available by Schulte et al. (2011). However, the average insert size of the constructed BAC libraries is smaller than the one reported for the Cebada Capa BAC library (140 Kbp) which was constructed using the same protocol (Isidore et al. 2005).

Repeated size selections can increase the average insert size but may also be followed by a severe drop in transformation efficiency (Cai et al. 1995; Chalhoub et al. 2004). The balance to find between those two parameters depends on the final use of the BAC library. If a BAC library is to be used for genome-wide physical mapping and genome sequencing, then maximizing the average size of inserts is essential to limit walking. However, if a BAC library is to be used for positional cloning of genes that have already been confined to a very small interval, then having a large number of clones is more important in order to increase the chance to find the gene of interest. The principal aim of our BAC libraries is to isolate genes involved in basal resistance to cereal rust fungi after their high-resolution genetic mapping. Consequently, our priority was to obtain a large number of clones at lower cost.

Mostly, DNA fractions H1, H2 and H3 were used to construct the libraries. The percentage of non-recombinant clones (blue colonies) was below 4%. Such a high proportion of recombinant clones over the non-recombinant clones reduce the problem of a possible bias in the libraries caused by faster growth of non-recombinant clones during the short amplification step (Isidore et al. 2005).

Genome representation of the BAC libraries

Based on the observed insert sizes of the BAC clones, the estimated genome coverage of the Vada BAC library is 2.6x and of the SusPtrit BAC library 3.7x. The genome coverage of the Vada library is comparable to the genome coverage of the Morex HVVMRXALLhB library, and SusPtrit to HVVMRXALLeA (Schulte et al. 2011). The estimation of the genome coverage based on microsatellite markers indicates, however, coverages of 5.0x and 6.8x for the Vada and the SusPtrit BAC libraries, respectively. The discrepancy between both estimations may be due to an underestimation of the average size of the BAC clones. As it is often observed in monocots (Peterson et al. 2000), several bands of identical sizes may be obtained after *Not*I restriction of BAC clones, which may result in the underestimation of the insert size from some clones. On the other hand, half of the microsatellite markers used for screening the BAC pools were derived from barley ESTs/genes (i.e. EST-SSR markers), implying that a marker amplifying a member from a

gene family may in some cases amplify other genes from the same family as well unless the sequences of the primer pairs are unique (Thiel et al. 2003). Indeed, the pressure of a primer to anneal on a similar but not identical sequence is much stronger on BAC DNA than it is on full genomic DNA. Therefore, the genome coverage of the two libraries remains uncertain, but is probably slightly higher for SusPtrit than for Vada. Based on insert sizes, the BAC library of Vada gives at least 93% probability of identifying a clone corresponding to any sequence of Vada and for BAC library of SusPtrit a probability of 98% is expected (Clarke and Carbon 1976). Together, the two BAC libraries give more than 99% probability of recovering any specific sequence from the barley genome.

Physical map of the *Rphq2* locus

Rphq2 is positioned at the telomeric region of chromosome 2HL which is known to be a gene rich region containing genes for different agronomic traits, including flowering time and disease resistance (Chen et al. 2009). *Rphq2* is a partial resistance QTL against *P. hordei*, but it seems also to be effective to some non-adapted rust species (Jafary et al. 2006; Chapter 3, this thesis). Recently, Johnston et al. (2013) suggested that *Rphq2* is possibly a weaker allelic form of a novel leaf rust resistance gene *Rph22* (or *Rph22.ak*) found in *H. bulbosum*, a nonhost species for *P. hordei*. Similar to *Rphq2*, *Rph22* confers a non-hypersensitive reaction resistance. Therefore, it is interesting to clone *Rphq2* which will provide molecular information to further study partial and nonhost resistances, and their possible association.

The donor of *Rphq2* is Vada (Jafary et al. 2006; Qi et al. 1998), which is a cultivar developed from *Hordeum laevigatum*/Gold (Dros 1957). The *Rphq2* locus of Vada was donated by *H. laevigatum* (Arru et al. 2003; Giese et al. 1993). The name of the latter accession is taxonomically invalid, since it suggests a different (wild) species in the *Hordeum* genus. However, it is perfectly crossable with *H. vulgare* accessions and also has the *H. vulgare* general morphology, including non-shattering spikes. Therefore it should be regarded as *H. vulgare.* It occurs in the ancestry of many West-european cultivars, including Emir, Delta and Minerva [(Hickey et al. 2012); Germplasm Resources Information Network (GRIN) http://www.ars-grin.gov/npgs/holdings. html]. SusPtrit was bred from a double cross; Menelik/L100//Trigo Biasa/Nigrinudum [GRIN; (Atienza et al. 2004)]. The very low degree (or even absence) of homology in the *Rphq2* region is mainly due to the West-european cultivar Vada and hence due to the donor line *H. laevigatum* that contributed this chromosome section (Marcel et al. 2007a). The *Rphq2*-introgression from *H. laevigatum* is approximately 188 Kbp starting from approximately three Kbp distal from the WBE114 to WBE115. Other barley lines, viz the Ethiopian L94, the American

cultivar Morex and SusPtrit as a descendant from various exotic barley accessions, seem to have maintained their homology in this area, as it appears from the alignment of the Morex genome sequences with the SusPtrit sequences and not with the Vada sequences (Table 6). Accordingly, the primer pairs designed on SusPtrit sequences did amplify DNA of L94 while primer pairs designed on Vada sequences did not. A practical consequence is that we do not expect further recombination between WBE114 and WBE115, as in heterozygous material the chromosome regions will probably hardly pair. Indeed we failed to obtain recombinant plants in this area after screening more than 3000 plants (data not shown). Suppression of recombination prevents further fine-mapping of *Rphq2*. This lack of homology implies that having a BAC library from the parent donor of the resistance allele (Vada) is a necessity, and that if we would have used BAC libraries from other genotypes we might have had great difficulties to acquire sequence information in the region and to identify the candidate genes from Vada.

Gene annotation at the *Rphq2* locus

The annotation of the *Rphq2* region using Triannot with the architecture for barley identified 12 genes in Vada and 12 genes in SusPtrit, but only five of the annotated genes are shared between Vada and SusPtrit. These four annotated genes were also conserved in rice and *B. distachyon*. No nucleotide-binding-site-leucine-rich repeat (NBS-LRR) resistance gene was annotated in the *Rphq2* region. The candidate genes for *Rphq2* might be a resistance factor in Vada or a susceptibility factor in SusPtrit. A study of differentially expressed genes between L94 and L94-*Rphq2* NIL suggested seven candidates for *Rphq2* (Chen et al. 2010). Among the candidates, only one gene, unigene2111 (encoding a peroxidase), was similar to an annotated gene at *Rphq2*. Unigene2111 has 99% identity with the coding sequence of V.Perox-2 of Vada and S.Perox-2 of SusPtrit. This suggests peroxidase 2 as a good candidate to explain *Rphq2*. This is also supported by the fact that peroxidases are known to be involved in defense reactions i.e. cell wall reinforcement and hypersensitive reaction [reviewed in Hückelhoven and Kogel (2003); (Almagro et al. 2009)]. Furthermore, González et al. (2010) found 61% of the QTLs for partial resistance to adapted rust fungi (including *Rphq2*) co-localize with the peroxidase based markers. The same phenomenon is true for resistance QTLs for powdery mildew fungi (Schweizer and Stein, 2011). The kinases as well are good candidates to explain *Rphq2*. Kinases are involved in various signaling pathways including plant defense system against pathogens [Reviewed in Rodriguez et al. (2010); (Antolín-Llovera et al. 2012)]. It is possible that one of the peroxidase or kinase genes identified in the physical window of *Rphq2* affects the resistance phenotype observed. Another possibility is that peroxidase or kinase gene members function as a complex QTL as observed in rice where the resistance effect of a

QTL on chromosome 8 was shown to be contributed by a cluster of germin-like protein genes (Manosalva et al. 2009).

A gene from the Seven in absentia protein family (SINA) was annotated only on the *Rphq2* sequence of Vada. This might be a candidate as well. SINA proteins are E3 ligases with a RING finger domain at the N-terminal followed by a conserved SINA domain which has a function in substrate binding and dimerization (Hu and Fearon 1999). One particular SINA protein is found, in a symbiotic interaction, to impair the rhizobial infection in *Medicago truncatula* (Mbengue et al. 2010) and *Lotus japonicus* (Den Herder et al. 2012). The candidate genes for *Rphq2* resemble to none of the partial resistance genes cloned previously (Fu et al. 2009; Fukuoka et al. 2009; Hayashi et al. 2010; Krattinger et al. 2009; Manosalva et al. 2009). Genes for partial resistance can be resistance factors, such as ABC transporter gene of *Lr34* (Krattinger et al. 2009), or susceptibility factors, such as a proline-rich protein of Pi21 (Fukuoka et al. 2009). Therefore, the candidate genes of *Rphq2* might be either a resistance factor from Vada or a susceptibility factor from SusPtrit.

Stable transformation of candidate genes for resistance in a susceptible barley genotype can be performed using Golden SusPtrit, a new genetically well transformable barley line (Chapter 2, this thesis). Golden SusPtrit inherited the susceptibility of SusPtrit to *P. hordei* and to non-adapted rust fungi, as well as the transformability of Golden Promise. The transformants in the genetic background of Golden SusPtrit will allow the testing of *Rphq2* candidate genes affecting resistance to adapted and non-adapted rust fungi. This will be valuable information to understand partial resistance in barley and its possible association with nonhost resistance.

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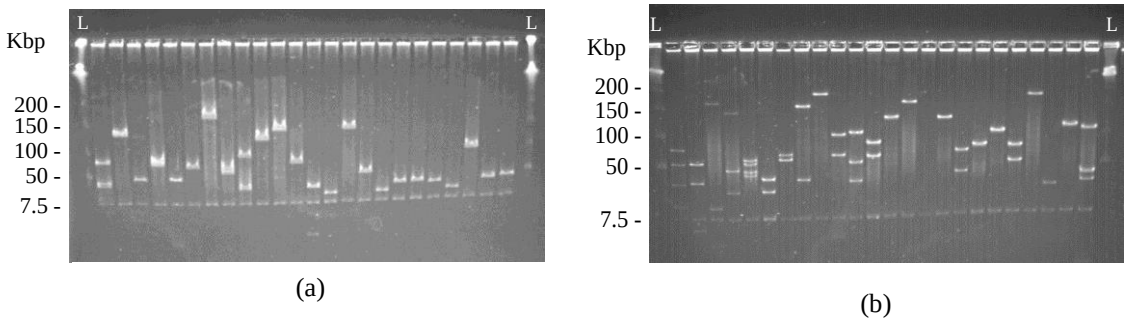
Supplemental Figures



Supplemental Figure 1: Analysis of 24 randomly selected barley BAC clones from Vada, fraction VH2 (a), and from SusPtrit, fraction SH3 (b), on an ethidium bromide-stained CHEF gel (5-15 sec switch time, 14.3 hours) showing insert DNA above and below the common 7.5 Kbp pIndigoBAC or pIndigoBAC-5 vector band.

Supplemental Tables

Supplemental Table 1: The descriptions of the 116 BAC pools of Vada.

Pool	White *	Blue *	Fraction	Vector	Insert size	Individual pool genome contribution !
V1	20	0	H0	pIndigoBAC-5	83	1660
V2	411	0	H0	pIndigoBAC-5	83	34113
V3	244	0	H0	pIndigoBAC-5	83	20252
V4	864	30	H1	pIndigoBAC	99	85536
V5	1871	39	H1	pIndigoBAC	99	185229
V6	1871	39	H1	pIndigoBAC	99	185229
V7	1871	39	H1	pIndigoBAC	99	185229
V8	1871	39	H1	pIndigoBAC	99	185229
V9	1871	39	H1	pIndigoBAC	99	185229
V10	1871	39	H1	pIndigoBAC	99	185229
V11	1871	39	H1	pIndigoBAC	99	185229
V12	966	12	H1	pIndigoBAC-5	83	80178
V13	156	4	H1	pIndigoBAC	111	17316
V14	2604	76	H1	pIndigoBAC	111	289044
V15	2604	76	H1	pIndigoBAC	111	289044
V16	2604	76	H1	pIndigoBAC	111	289044
V17	2604	76	H1	pIndigoBAC	111	289044
V18	2604	76	H1	pIndigoBAC	111	289044
V19	2604	76	H1	pIndigoBAC	111	289044
V20	2604	76	H1	pIndigoBAC	111	289044
V21	1160	50	H1	pIndigoBAC-5	83	96280
V22	966	12	H1	pIndigoBAC-5	83	80178
V23	1871	39	H1	pIndigoBAC	83	155293
V24	966	12	H1	pIndigoBAC-5	83	80178
V25	966	12	H1	pIndigoBAC-5	83	80178
V26	966	12	H1	pIndigoBAC-5	83	80178
V27	966	12	H1	pIndigoBAC-5	83	80178
V28	966	12	H1	pIndigoBAC-5	83	80178
V29	966	12	H1	pIndigoBAC-5	83	80178
V30	966	12	H1	pIndigoBAC-5	83	80178
V31	966	12	H1	pIndigoBAC-5	83	80178
V32	606	12	H1	pIndigoBAC-5	96	58176
V33	1786	67	H1	pIndigoBAC-5	96	171456
V34	1786	67	H1	pIndigoBAC-5	96	171456
V35	1786	67	H1	pIndigoBAC-5	96	171456
V36	1786	67	H1	pIndigoBAC-5	96	171456
V37	1786	67	H1	pIndigoBAC-5	96	171456
V38	1786	67	H1	pIndigoBAC-5	96	171456
V39	1786	67	H1	pIndigoBAC-5	96	171456
V40	1786	67	H1	pIndigoBAC-5	96	171456
V41	1786	67	H1	pIndigoBAC-5	96	171456
V42	1786	67	H1	pIndigoBAC-5	96	171456
V43	1786	67	H1	pIndigoBAC-5	96	171456
V44	1786	67	H1	pIndigoBAC-5	96	171456
V45	319	1	H2	pIndigoBAC	94	29986
V46	2136	34	H2	pIndigoBAC	94	200784
V47	2136	34	H2	pIndigoBAC	94	200784
V48	2136	34	H2	pIndigoBAC	94	200784
V49	2136	34	H2	pIndigoBAC	94	200784
V50	2136	34	H2	pIndigoBAC	94	200784
V51	2136	34	H2	pIndigoBAC	94	200784
V52	84	0	H2	pIndigoBAC	129	10836
V53	995	8	H2	pIndigoBAC	129	128355
V54	995	8	H2	pIndigoBAC	129	128355
V55	995	8	H2	pIndigoBAC	129	128355

Supplemental Table 1: Cont…

Pool	White *	Blue *	Fraction	Vector	Insert size	Individual pool genome contribution !
V56	995	8	H2	pIndigoBAC	129	128355
V57	995	8	H2	pIndigoBAC	129	128355
V58	725	31	H2	pIndigoBAC-5	55	39875
V59	1947	57	H2	pIndigoBAC-5	55	107085
V60	1947	57	H2	pIndigoBAC-5	55	107085
V61	1947	57	H2	pIndigoBAC-5	55	107085
V62	1947	57	H2	pIndigoBAC-5	55	107085
V63	1947	57	H2	pIndigoBAC-5	55	107085
V64	1947	57	H2	pIndigoBAC-5	55	107085
V65	1947	57	H2	pIndigoBAC-5	55	107085
V66	508	12	H2	pIndigoBAC-5	50	25400
V67	2051	178	H2	pIndigoBAC-5	50	102550
V68	2149	144	H2	pIndigoBAC-5	50	107450
V69	2100	161	H2	pIndigoBAC-5	50	105000
V70	2100	161	H2	pIndigoBAC-5	50	105000
V71	2100	161	H2	pIndigoBAC-5	50	105000
V72	2100	161	H2	pIndigoBAC-5	50	105000
V73	2100	161	H2	pIndigoBAC-5	50	105000
V74	2100	161	H2	pIndigoBAC-5	50	105000
V75	2100	161	H2	pIndigoBAC-5	50	105000
V76	2100	161	H2	pIndigoBAC-5	50	105000
V77	2100	161	H2	pIndigoBAC-5	50	105000
V78	2100	161	H2	pIndigoBAC-5	50	105000
V79	2100	161	H2	pIndigoBAC-5	50	105000
V80	168	5	H3	pIndigoBAC	104	17472
V81	377	11	H3	pIndigoBAC	104	39208
V82	377	11	H3	pIndigoBAC	104	39208
V83	377	11	H3	pIndigoBAC	104	39208
V84	377	11	H3	pIndigoBAC	104	39208
V85	46	3	H3	pIndigoBAC	40	1840
V86	515	8	H3	pIndigoBAC	40	20600
V87	515	8	H3	pIndigoBAC	40	20600
V88	1322	34	H3	pIndigoBAC-5	92	121624
V89	1024	5	H3	pIndigoBAC-5	92	94208
V90	1024	5	H3	pIndigoBAC-5	92	94208
V91	1024	5	H3	pIndigoBAC-5	92	94208
V92	1024	5	H3	pIndigoBAC-5	92	94208
V93	1024	5	H3	pIndigoBAC-5	92	94208
V94	1024	5	H3	pIndigoBAC-5	92	94208
V95	1024	5	H3	pIndigoBAC-5	92	94208
V96	1024	5	H3	pIndigoBAC-5	92	94208
V97	1024	5	H3	pIndigoBAC-5	92	94208
V98	1024	5	H3	pIndigoBAC-5	92	94208
V99	1024	5	H3	pIndigoBAC-5	92	94208
V100	1024	5	H3	pIndigoBAC-5	92	94208
V101	870	10	H3	pIndigoBAC-5	54	46980
V102	1294	42	H3	pIndigoBAC-5	54	69876
V103	1294	42	H3	pIndigoBAC-5	54	69876
V104	1294	42	H3	pIndigoBAC-5	54	69876
V105	1294	42	H3	pIndigoBAC-5	54	69876
V106	1294	42	H3	pIndigoBAC-5	54	69876
V107	1294	42	H3	pIndigoBAC-5	54	69876
V108	1294	42	H3	pIndigoBAC-5	54	69876
V109	1294	42	H3	pIndigoBAC-5	54	69876
V110	1294	42	H3	pIndigoBAC-5	54	69876
V111	1294	42	H3	pIndigoBAC-5	54	69876
V112	1294	42	H3	pIndigoBAC-5	54	69876

Supplemental Table 1: Cont…

Pool	White *	Blue *	Fraction	Vector	Insert size	Individual pool genome contribution !
V113	1294	42	H3	pIndigoBAC-5	54	69876
V114	20	1	H4	pIndigoBAC-5	83	1660
V115	112	0	H4	pIndigoBAC-5	83	9296
V116	158	0	H4	pIndigoBAC-5	83	13114

* The estimated number of white and blue colonies

! The genome contribution of the individual pool calculated by multiplying the number of white colonies with the estimated insert size

Supplemental Table 2: The descriptions of the 110 BAC pools of SusPtrit.

Pool	White *	Blue *	Fraction	Vector	Insert size	Individual pool genome contribution !
S1	218	11	H0	pIndigoBAC-5	89	19402
S2	3703	2	H0	pIndigoBAC-5	89	329567
S3	3703	2	H0	pIndigoBAC-5	89	329567
S4	3703	2	H0	pIndigoBAC-5	89	329567
S5	3703	2	H0	pIndigoBAC-5	89	329567
S6	3703	2	H0	pIndigoBAC-5	89	329567
S7	3703	2	H0	pIndigoBAC-5	89	329567
S8	3703	2	H0	pIndigoBAC-5	89	329567
S9	3703	2	H0	pIndigoBAC-5	89	329567
S11	790	14	H1	pIndigoBAC	95	75050
S10	1957	40	H1	pIndigoBAC	95	185915
S12	1957	40	H1	pIndigoBAC	95	185915
S13	1957	40	H1	pIndigoBAC	95	185915
S14	1957	40	H1	pIndigoBAC	95	185915
S15	1957	40	H1	pIndigoBAC	95	185915
S16	1957	40	H1	pIndigoBAC	95	185915
S17	1957	40	H1	pIndigoBAC	95	185915
S18	276	0	H1	pIndigoBAC	124	34224
S19	2609	61	H1	pIndigoBAC	124	323516
S20	2609	61	H1	pIndigoBAC	124	323516
S21	2609	61	H1	pIndigoBAC	124	323516
S22	2609	61	H1	pIndigoBAC	124	323516
S23	2609	61	H1	pIndigoBAC	124	323516
S24	1702	86	H1	pIndigoBAC-5	106	180412
S25	1126	23	H1	pIndigoBAC-5	106	119356
S26	1126	23	H1	pIndigoBAC-5	106	119356
S27	1126	23	H1	pIndigoBAC-5	106	119356
S28	1126	23	H1	pIndigoBAC-5	106	119356
S29	1126	23	H1	pIndigoBAC-5	106	119356
S30	1126	23	H1	pIndigoBAC-5	106	119356
S31	1126	23	H1	pIndigoBAC-5	106	119356
S32	1126	23	H1	pIndigoBAC-5	106	119356
S33	1126	23	H1	pIndigoBAC-5	106	119356
S34	1126	23	H1	pIndigoBAC-5	106	119356
S35	1126	23	H1	pIndigoBAC-5	106	119356
S36	1126	23	H1	pIndigoBAC-5	106	119356
S37	1126	23	H1	pIndigoBAC-5	106	119356
S38	1126	23	H1	pIndigoBAC-5	106	119356
S39	578	4	H1	pIndigoBAC-5	104	60112
S40	2522	82	H1	pIndigoBAC-5	104	262288
S41	2522	82	H1	pIndigoBAC-5	104	262288
S42	2522	82	H1	pIndigoBAC-5	104	262288
S43	2522	82	H1	pIndigoBAC-5	104	262288
S44	2522	82	H1	pIndigoBAC-5	104	262288
S45	2522	82	H1	pIndigoBAC-5	104	262288
S46	2522	82	H1	pIndigoBAC-5	104	262288
S47	280	9	H2	pIndigoBAC	108	30240
S48	1954	32	H2	pIndigoBAC	108	211032

Supplemental Table 2: Cont…

Pool	White *	Blue *	Fraction	Vector	Insert size	Individual pool genome contribution !
S49	1954	32	H2	pIndigoBAC	108	211032
S50	1954	32	H2	pIndigoBAC	108	211032
S51	1954	32	H2	pIndigoBAC	108	211032
S52	1954	32	H2	pIndigoBAC	108	211032
S53	27	0	H2	pIndigoBAC	161	4347
S54	609	6	H2	pIndigoBAC	161	98049
S55	609	6	H2	pIndigoBAC	161	98049
S56	609	6	H2	pIndigoBAC	161	98049
S57	609	6	H2	pIndigoBAC	161	98049
S58	609	6	H2	pIndigoBAC	161	98049
S59	1464	54	H2	pIndigoBAC-5	116	169824
S60	1029	28	H2	pIndigoBAC-5	116	119364
S61	1029	28	H2	pIndigoBAC-5	116	119364
S62	1029	28	H2	pIndigoBAC-5	116	119364
S63	1029	28	H2	pIndigoBAC-5	116	119364
S64	1029	28	H2	pIndigoBAC-5	116	119364
S65	1029	28	H2	pIndigoBAC-5	116	119364
S66	1029	28	H2	pIndigoBAC-5	116	119364
S67	1029	28	H2	pIndigoBAC-5	116	119364
S68	1029	28	H2	pIndigoBAC-5	116	119364
S69	1029	28	H2	pIndigoBAC-5	116	119364
S70	1029	28	H2	pIndigoBAC-5	116	119364
S71	1029	28	H2	pIndigoBAC-5	116	119364
S72	460	2	H2	pIndigoBAC-5	105	48300
S73	3113	102	H2	pIndigoBAC-5	105	326865
S74	3113	102	H2	pIndigoBAC-5	105	326865
S75	3113	102	H2	pIndigoBAC-5	105	326865
S76	3113	102	H2	pIndigoBAC-5	105	326865
S77	3113	102	H2	pIndigoBAC-5	105	326865
S78	3113	102	H2	pIndigoBAC-5	105	326865
S79	3113	102	H2	pIndigoBAC-5	105	326865
S80	3113	102	H2	pIndigoBAC-5	105	326865
S81	3113	102	H2	pIndigoBAC-5	105	326865
S82	3113	102	H2	pIndigoBAC-5	105	326865
S83	3113	102	H2	pIndigoBAC-5	105	326865
S84	3113	102	H2	pIndigoBAC-5	105	326865
S85	209	5	H3	pIndigoBAC	154	32186
S86	949	9	H3	pIndigoBAC	154	146146
S87	949	9	H3	pIndigoBAC	154	146146
S88	949	9	H3	pIndigoBAC	154	146146
S89	949	9	H3	pIndigoBAC	154	146146
S90	949	9	H3	pIndigoBAC	154	146146
S91	949	9	H3	pIndigoBAC	154	146146
S92	25	0	H3	pIndigoBAC	168	4200
S93	199	1	H3	pIndigoBAC	168	33432
S94	303	0	H3	pIndigoBAC	168	50904
S95	251	1	H3	pIndigoBAC	168	42168
S96	251	1	H3	pIndigoBAC	168	42168
S97	251	1	H3	pIndigoBAC	168	42168
S98	346	13	H3	pIndigoBAC-5	124	42904
S99	491	21	H3	pIndigoBAC-5	124	60884
S100	491	21	H3	pIndigoBAC-5	124	60884
S101	491	21	H3	pIndigoBAC-5	124	60884
S102	491	21	H3	pIndigoBAC-5	124	60884
S103	491	21	H3	pIndigoBAC-5	124	60884
S104	491	21	H3	pIndigoBAC-5	124	60884
S105	128	4	H3	pIndigoBAC-5	127	16256

Supplemental Table 2: Cont...

Pool	White *	Blue *	Fraction	Vector	Insert size	Individual pool genome contribution !
S106	849	24	H3	pIndigoBAC-5	127	107823
S107	849	24	H3	pIndigoBAC-5	127	107823
S108	849	24	H3	pIndigoBAC-5	127	107823
S109	849	24	H3	pIndigoBAC-5	127	107823
S110	849	24	H3	pIndigoBAC-5	127	107823

* The estimated number of white and blue colonies

! The genome contribution of the individual pool calculated by multiplying the number of white colonies with the estimated insert size

Supplemental Table 3: The positive BAC pools from Vada and SusPtrit BAC libraries detected using 12 PCR primers.

	WBE114	P15M51-204	besV76P5D5AR	S35P100001F4	S35P100004F2	besS35P2K14EF	Rphq2.S01	S7300002F	Rphq2.V30	Rphq2.V32	P14M54-252	WBE115
Vada BAC library	V11				NT				NT	NT		
		V17			NT				NT	NT		
	V21				NT				NT	NT		
			V35		NT				NT	NT		
	V38				NT				NT	NT		
	V41				NT				NT	NT		V41
	V45				NT				NT	NT		
	V48		V48		NT				NT	NT		V48
		V51			NT				NT	NT		
		V69			NT				NT	NT		
		V76	V76		NT				NT	NT		
		V77			NT				NT	NT		
	V84				NT				NT	NT		
	V89				NT				NT	NT		
					NT				NT	NT		V104
	V113				NT				NT	NT		
SusPtrit BAC library	S7					S7	S7				S7	S7
	S8											
							S13				S13	
	S22											
	S23											
	S30											
	S35			S35	S35	S35	S35		S35		S35	S35
										S36		
	S39											
	S40			S40	S40	S40				S40		
	S43											S43
	S45			S45	S45		S45	S45			S45	
						S51	S51				S51	S51
	S56											
									S58		S58	
							S73		S73		S73	
	S75			S75						S75		
									S77			
									S81			S81
	S82											
							S109		S109		S109	S109

The shaded areas indicate no amplification was expected from the primers on the respective BAC library.
NT; Not tested

Supplemental Table 4: The primers used for determining the order of BAC clones in Table 5

Name	Primers sequences (5'- 3')	Ta (°C)	Source
WBE114	Refer Table 1		Marcel et al. (2007a)
WBE115	Refer Table 1		Marcel et al. (2007a)
besV41P7L3AF	*F:* GTTGCTTCATGTATACTTCTTCTT *R:* ATCTTCCCAACGTCAACAAATC	56	BAC end
besV41P7L3AR	*F:* ATAATCTTAGCCCTCACATCACCA *R:* AGTTCCAAGCAAAGCGTCGTAG	56	BAC end
besV76P5D5AF	*F:* ATAGGGATGCTTACCACTGAA *R:* AAATTACTAGCTAGACTCCCACTC	56	BAC end
besV76P5D5AR	Refer Supplemental Table 5		BAC end
besV48P5B18AR	Refer Supplemental Table 5		BAC end
besV48P5B18AF	*F:* TACTATCCTTCCGCTCACAACTCA *R:* GGGACCCCTATTACCACCAG	58	BAC end
Rphq2.S01	Refer Supplemental Table 5		SusPtrit annotated gene
P14M54-252	Refer Supplemental Table 5		Marcel et al. (2007a)
besS35P1J10AF	*F:* CTGCCACTCTTTATCTTTTTG *R:* TAGTATCGGGGAGTATTAGC	56	BAC end
besS35P1J10AR	*F:* TCCGGTATGCACGAAAAC *R:* CCTGCCGGTAAACGAGAT	58	BAC end
besS35P2K14EF	Refer Supplemental Table 5		BAC end
besS35P2K14ER	*F:* TGTTCCGTTCATACTCACCTT *R:* ACCATAGAACGACCCTCACA	56	BAC end
besS7P2C21EF	*F:* GAAGTATATGCCGACAACCAAATG *R:* GAAAACCGTCCAACCTCTACAAGT	58	BAC end
besS81P2C6AF	*F:* CCCTAGGGAAAGCCATCATACG *R:* GGGTTTGCCTCATCCATAGC	58	BAC end
besS81P2C6AR	*F:* CGCCGTTTTGACATCCATCTG *R:* TCAAATCCGAGGGCAAAGTGTT	58	BAC end
bfsS35P2K14EF-267	Refer Supplemental Table 5		BAC AFLP
bfsS35P2K14EF-283	*F:* ATGCGACCTATTGCATGTCT *R:* TGACGGTAAACAAGCCTTTC	56	BAC AFLP
bfsS35P2K14EF-468	*F:* CTCATGGAAGCAGCAAAACTA *R:* GCCGGCATACTCACCACT	58	BAC AFLP

Supplemental Table 5: The 40 molecular markers developed and mapped between flanking markers WBE114 and WBE115 of *Rphq2*

Name	Tm (°C)	Restriction Enzyme	Primers sequences (5'- 3')	Source
Dominant markers amplifying Vada				
besV76P5D5AR	56		*F:* GAGGAGCCGTGTCGTCTTGT *R:* CCGTTTCCGTTCACTGGTTAT	BAC end
bfsS35P2K14E-267	56		*F: CGCCGTATACCAAGGCTATT* *R: ATGAGCTCGTAGACCAGCAG*	BAC AFLP
FQ2D1F6	65		*F:* ATGTGGGCCAACGGTGCAAATCAGG *R:* CAATACGGAGGTGTCGCCCATAAC	Vada BAC sequence
FQ2D3F	65		*F:* CGTCTGCGGCCCCGTCGTCTCC *R:* GATGGGCGCGGTGGTCTTGTTCTTG	Vada BAC sequence
FQ2D4F9	65		*F:* GCCCCGTGCATCCGTTCGT *R:* TCCGCAGATTTCATAGGCAGGTGT	Vada BAC sequence
FQ2D4F14	65		*F:* TTTGATGCGCAGGGTTTGGAGAGGT *R:* GGGAGGGGTGAGGGGGCTGGAG	Vada BAC sequence
FQ2D4F15	65		*F:* TCCTCCACGGCACCTACCAAGACG *R:* CCCGGACGGACGCCTGAAG	Vada BAC sequence
FQ2D6F	65		*F:* TAGGGGCGATAGAACCAGAAAGT *R:* CTCCCCAAGGCCAAGATAAGA	Vada BAC sequence
FQ2D6F2	65		*F:* CCCGCCGGAATAGCAGAATCAGG *R:* GCATCCGGCCACGTCCAGTCAG	Vada BAC sequence
FQ2D6F3	65		*F:* CATCCGCGCAGCCACACCTTTCATA *R:* ACATTTCCCGCCATTTCCGACAACT	Vada BAC sequence
FQ2D6F4	65		*F:* TCTTGCATCTGGCGGAGGAACTG *R:* TTTGGCACCGTATACCGAGGCTGAG	Vada BAC sequence
FQ2D7F2	65		*F:* AGAAACTCCAACTCCTCGGCTCCAT *R:* TGTCGACGCAATCTTAACCTTCTGA	Vada BAC sequence
FQ2D7F6	65		*F:* GGATGCCATATTTCACGTAGACAGG *R:* TCGTGGGAGGCATTGAGATTTGAGG	Vada BAC sequence
FQ2D8F6	65		*F:* GTGACAACCGACCAACGAC *R:* GCGAGCGCCTTATCCATTAG	Vada BAC sequence
FQ2D9F9	65		*F:* GCGGGTAGGCCTTGGTCTGTTC *R:* GGGAGGTGCATGCCAAAAAGTCAAT	Vada BAC sequence
FQ2D10F2	60		*F:* CATGGCGGATTATTGGTGTTAGTAG *R:* CAGTGCGGTGGGGTGCTC	Vada BAC sequence
P15M51-204	56		*F:* CGGAGGAAACATGGACAACGAA *R:* AGCGAGCTCACTGCCAATCTACC	Marcel et al. (2007a)
Rphq2V14	58		*F:* CGCCGCCAACTGCAGCAAGAATCC *R:* CAACGTCGACGGCAGTCCCGATG	Vada annotated gene
Rphq2V16	58		*F:* TTGCGGTGGAGTTCGACATCTTCA *R:* GTCATCGGGTCCACTTTGCCTTCC	Vada annotated gene
Rphq2V19	65		*F:* CCCCGCGGTCTCATTCCTT *R:* TCTTTTTATCTTGGGCAACCGTGTA	Vada annotated gene
Rphq2V25	65		*F:* TGTCTTCCTTCGGTTCCTTCC *R:* TCCGCCATGGCCACGATACG	Vada annotated gene

Supplemental Table 5: Cont…

Dominant markers amplifying SusPtrit				
Name	Tm (°C)	Restriction Enzyme	Primers sequences (5'- 3')	Source
besS35P2K14EF	58		*F:* TTGAAACAGCTGGGGTCTT *R:* TGGTACACAAATATTCGTCTGC	BAC end
FsQ2N2F3	56		*F:* GCACGGGCGGCCACAGAGGAG *R:* TGTCGCCCAGCAGCTACGGAACC	SusPtrit BAC sequence
FsQ2N2F8	62		*F:* TGGCGGAGTCAAAATCAAGAGTT *R:* TCGTGGATATAGCGGCAGAGGTC	SusPtrit BAC sequence
FsQ2N4F3	58		*F:* GCTGATCCCACCCGCCATTC *R:* CATTCCTACCGCCCGCTTTCTTACG	SusPtrit BAC sequence
FsQ2N5F5	63		*F:* CCGCCGAGGACTGATACTT *R:* GCAACCAAACGCACCCTTAGA	SusPtrit BAC sequence
FsQ2N11F6	62		*F:* CACTTCTCCAATGACTGCCCTTATG *R:* ATCGCCTTTACGTGAACTATCCAG	SusPtrit BAC sequence
FsQ2N11F8	58		*F:* GAAATAATCAACTTGTGGCATAC *R:* CTTAGGGCAGCGAGGTTAG	SusPtrit BAC sequence
FsQ2N11F9	62		*F:* CATCATATTGGCAGCAGTGG *R:* AATCCCGAGCCTTCTTGACATA	SusPtrit BAC sequence
FsQ2N12F3	63		*F:* ACTGGTGGGTCCCCTTCTGGTA *R:* GCTTTGCCGGTCTTGTTCGTATT	SusPtrit BAC sequence
FsQ2N13F2	63		*F:* AGCCCCTCGACAGTTCCAGCATAGA *R:* CAGCCCGACCACATACCTCCACAGT	SusPtrit BAC sequence
FsQ2N13F3	63		*F:* AAAGAGGAGGGTGGCGGTGGTAGGA *R:* GGGGTGCTCGCGTCTGAACTCTGAA	SusPtrit BAC sequence
FsQ2N13F9	60		*F:* AGCGGTCTTAGTCTGGTCGTTGTA *R:* TCTTCAGGGCCATTTTCTATTTATC	SusPtrit BAC sequence
FsQ2N13F10	62		*F:* GGCCTCACTAACCAAAACGCAGAC *R:* ATGATTTTCCGACCACGACAACGAT	SusPtrit BAC sequence
FsQ2N16F3	63		*F:* GGGTGCTTGTGCCATGGGAGTAGG *R:* GGGGGTGGAGTGCGGAGGAAGAC	SusPtrit BAC sequence
P14M54-252	56		*F:* AGACCAGCATTACCTAAGCAGAGA *R:* AGAGGAGAGTGAGTGTAGGTGTCG	Marcel et al. (2007a)
Rphq2S01	58		*F:* TGAAGGCGGGTTTGGTGTGGTGTA *R:* CCCGCGTATGATTCTCTGCCTCTT	SusPtrit annotated gene
Co-dominant markers				
Rphq2V30	65	MboII	*F:* CGGCGGTGCGATCATAGAAT *R:* TCCCCGGCCGTAGAGTCC	Vada annotated gene
Rphq2V34	58	*Taq*I	*F:* ACCCCGGCTCCCTCGTCCTC *R:* CTTTTGCCGCAGCGCCTTCATCT	Vada annotated gene
S7300002F	65	*Sdu*I	*F:* GACGTTGAGGAGAGCAAAGG *R:* GCCGTTTATCACGAGGTTGT	SusPtrit BAC sequence

Supplemental Reference: Literature cited in Supplemental Table 4 and 5

Marcel TC, Aghnoum R, Durand J, Varshney RK, Niks RE (2007a) Dissection of the barley 2L1. 0 region carrying the '*Laevigatum*' quantitative resistance gene to leaf rust using near-isogenic lines (NIL) and subNIL. Molecular Plant-Microbe Interactions 20:1604-1615

Chapter 6

General Discussion

Introduction

Partial resistance results in reduced epidemic development, despite a compatible infection type (Niks et al. 2011). Partial resistance inherits polygenically as was demonstrated for various pathosystems: (1) barley-rust (Marcel et al. 2007b; Qi et al. 1998) (2) barley-powdery mildew (Aghnoum et al. 2010) (3) wheat-rust (Singh et al. 2005; Lowe et al. 2011) and sunflower-*Phoma macdonaldii* (Darvishzadeh et al. 2007). Partial resistance is supposed to act on a minor-gene-for-minor-gene model (González et al. 2012; Marcel et al. 2008; Darvishzadeh et al. 2007; Qamar and Niks 2007). In the barley-rust pathosystem, the quantitative trait loci (QTLs) for partial resistance against barley leaf rust are abundantly available for breeders, viz. per cultivar a different set, per developmental stage a different set, per rust species a different set. Even to some extent per isolate a different set (González et al. 2012; Marcel et al. 2007b; 2008; Qi et al. 1998; 1999). Stacking of QTLs for partial resistance has been shown to increase the disease resistance in plants and even achieving near immunity (Castro et al. 2003a,b; Richardson et al. 2006; Singh et al. 2000; 2005).

Nonhost resistance provides immunity to all members of a plant species (nonhost) against a potential pathogen species (non-adapted/heterologous pathogen species). Different approaches can be used to investigate the inheritance of nonhost resistance: (1) interspecific crosses between the host and nonhost plant species (2) crossing individuals within a nonhost species with different levels of resistance to a particular non-adapted pathogen and (3) within a near-nonhost/marginal host species, crossing a rare susceptible individual with the one of common nonhost resistance (Niks and Marcel 2009). Nonhost resistance inherits polygenically as was demonstrated in various pathosystems; Based on approach (1), *Lactuca-Bremia* (Jeuken et al. 2008), approach (2), *Arabidopsis*-wheat leaf rust (Shafiei et al. 2007), approach (3) barley-powdery mildew (Aghnoum and Niks 2010) and barley-rust (Jafary et al. 2006; 2008). In barley-rust pathosystem, the QTLs for nonhost resistance against various rust fungi are abundantly available (Jafary et al. 2006; 2008).

Nonhost resistance can be an attractive source of resistance for breeding. Zhang et al (2009) stacked different combinations of nonhost resistance QTLs to *Bremia lactucae*, from a nonhost species (*Lactuca saligna*) into a host species (*L. sativa*) which is susceptible to *B. lactucae*. The stacking of a particular combination of QTLs increased the level of resistance for *L. sativa* against *B. lactucae* and one particular QTL combination even led to a complete resistance. For barley (*Hordeum vulgare*), which is a host to *Puccinia hordei*, an introgression of a resistance gene *Rph22* from a nonhost species *Hordeum bulbosum* into cultivar Golden Promise gave a very high level of partial

resistance to *P. hordei* on adult plants and seedlings (Johnston et al. 2013). Nonhost resistance can be used in breeding through a pre-breeding program to avoid introducing undesirable traits from the nonhost species into the new variety or elite cultivars of host species. Other approaches are by identifying the homologs of nonhost resistance genes in the host species which give resistance and be used for breeding.

In the barley-rust pathosystem, evidence suggests partial and nonhost resistance to be probably associated. At microscopic level, the mechanism of partial resistance is shared with, but less complete than, nonhost resistance of barley to non-adapted rust fungi (Niks 1983a, b). Based on genetic studies, partial and nonhost resistance of barley may share some genes for resistance (Jafary et al. 2006; 2008). Partial resistance seems to be a weak form of nonhost resistance (Niks and Marcel 2009; Niks et al. 2011) which probably rests on similar principles. One can understand nonhost resistance by studying partial resistance and *vice versa*.

The adapted and non-adapted rust fungal species plausibly share similar pathogen-associated molecular patterns (PAMPs). Consequently, both the adapted and non-adapted rust fungal species will activate PAMP-triggered immunity (PTI). The adapted and non-adapted rust fungal species will try to suppress PTI by secreting effectors. The genes explaining the effects of QTLs (or their corresponding transcripts or proteins) for partial and nonhost resistance are probably the operative targets of pathogen effectors. The ability of effectors to manipulate these operative targets will determine the suppression level of the defense response. Adapted pathogens should generally be much more successful in suppressing defense than non-adapted pathogens in a particular plant species. Stacking of resistance QTLs/operative targets would result in a resistance phenotype as a result of unsuppressed defense response. In contrast, stacking the variants of operative targets which can be manipulated by the effectors would result in a susceptible phenotype. Accumulation of such operative targets may explain the susceptibility of barley experimental lines such as SusPtrit and SusPmur against some non-adapted rust fungi (Atienza et al. 2004). It is probably also true for barley experimental lines, viz. $SusBgt_{SC}$ and $SusBgt_{DC}$, which are somewhat susceptible to certain non-adapted powdery mildew fungi (Aghnoum and Niks 2010)

In order to understand the molecular basis of partial and nonhost resistance, the genes explaining the QTLs need to be identified, cloned and validated for their function in resistance. Knowing the principles underlying nonhost resistance may allow the development of methods to engineer new nonhost-like types of resistance in hosts to their adapted pathogens/and or to select partial resistance more effectively.

Map-based cloning of minor genes for partial and nonhost resistance of barley

Map-based cloning or positional cloning is a step-by-step procedure to position a QTL into the smallest possible genetic interval and then anchoring it to a corresponding physical interval where candidate genes can be identified and validated. In the previous chapters, we presented work in progress towards cloning minor genes for partial and nonhost resistance and we have developed different tools which are necessary assets to clone them.

QTL validation

After QTLs have been identified in mapping populations, they need to be confirmed. Such a validation of QTLs is usually performed using NILs. These isogenic lines provide a uniform genetic background to test the effect of a single QTL without the interference of other QTLs influencing the same trait. In Chapter 3, we describe the development of NILs in SusPtrit genetic background for four partial resistance QTLs (*Rphq2*, *Rphq3*, *Rphq11* and *Rphq16*) and two alleles of one nonhost resistance QTL viz. Vada and L94 alleles (*Rnhq.V* and *Rnhq.L*). Marker assisted selection enabled the backcrossing procedure. These newly developed NILs together with the NILs in L94 genetic background containing *Rphq2*, *Rphq3*, *Rphq4* (Marcel et al. 2007a; van Berloo et al. 2001) and *Rnhq.V* (Niks, unpublished) were inoculated with different rust fungal species/isolates.

All the QTLs selected for validation had a proportion of the explained phenotypic variance ranges from 15 to 35%. In the NILs, the effect of all the QTLs was confirmed. The effect of QTLs can possibly be isolate- and species-specific when tested against different rust fungal species/isolates. For example, the *Rphq16* introgression in SusPtrit affects resistance only to *P. hordei* and was effective against all three isolates of *P. hordei*. In contrast, the *Rphq11* introgression in SusPtrit not only conferred resistance to *P. hordei* in an isolate-specific fashion, but the introgression was also effective to other rust fungal species, such as *P. hordei-bulbosi* isolate Iran where the *Rphq11* introgression resulted in near immunity. Based on the disease tests on NILs, it seems that partial and nonhost resistance indeed share some genes. We cannot rule out the possibility that we may have overestimated the resistance spectrum of the QTL introgressions because the wide resistance spectrum conferred by some introgressions may be due to presence of: (1) additional resistance genes to the same or other rust isolate/species introgressed from the donor away from the introgression, and (2) several resistance genes in the QTL introgression, each with a narrow spectrum of effectiveness, but together conferring a broader resistance spectrum against different rust fungal species/rust or (3) resistance conferred by an introgression may be the result of several infection reducing and even

promoting genes within the introgression, as was reported in *Lactuca saligna*-derived nonhost resistance in lettuce (*L. sativa*) to *Bremia lacticae* (den Boer et al. 2013).

Rphq2, *Rphq3* and *Rnhq.V* seemed to affect the resistance differently in different genetic backgrounds. For example, *Rphq2* introgression in L94 was effective against *P. hordei* and two non-adapted rust species, but in SusPtrit, the *Rphq2* introgression conferred isolate-specific resistance to *Ph*.1.2.1 and not to other rust species. In contrast, *Rnhq.V* introgression in L94 was effective against four non-adapted rust species but not to adapted rust fungi. In SusPtrit, *Rnhq.V* introgression was effective to *P. hordei* isolate Uppsala. The different resistance phenotypes conferred by a QTL in different genetic backgrounds can result from the interactions between the QTL introgression with other gene(s) elsewhere in the genome (Holland 2007; Lagudah 2011) or with cytoplasmic factor(s) (Akula et al. 2012; Levings and Siedow 1992; Mazouz et al. 2002). Another explanation would be difference in the size of the introgressions in SusPtrit and L94 which may lead to difference in resistance genes present in the NILs.

In the future, these NILs can serve as parents to accumulate different combinations of resistance QTLs. We can later test the effect of different combinations of QTLs against different rust fungi. For example, the combination of *Rphq2* and *Rphq3* in L94 resulted in higher level of resistance compared to NILs with a single QTL introgression (Marcel et al. 2007a). Combinations of QTLs may or may not result in additive effects on the level of resistance (Zhang et al. 2009). Studying the combinational effect of different QTLs is crucial for breeders to plan the QTL pyramiding strategy.

Fine-mapping

The NILs can also be used as the starting materials to delimit QTLs to the smallest possible genetic interval as described in Marcel et al. (2007a) for fine mapping of *Rphq2*. Development of NILs followed by fine-mapping of QTLs into a certain genetic interval is time consuming. We suggest to develop NILs and to fine-map QTLs in parallel to reduce the time. In Chapter 4, fine-mapping of *Rphq11* and *Rphq16* was initiated by identifying plants from the early backcrossing generation. Molecular markers were used to select plants which carry recombinations at the targeted QTL region. Since the donor of *Rphq11* (Steptoe) and *Rphq16* (DOM) also carry one additional QTL (Marcel et al. 2007b), molecular marker assisted selection was required against the additional QTL, i.e. the SusPtrit susceptibility allele was selected for those QTLs. In less than two years, i.e. before the NILs are ready, we managed to perform two rounds of fine-mapping and positioned *Rphq11* into a 0.2 cM genetic interval and 1.4 cM for *Rphq16*. Further fine-

mapping of the QTLs, if needed, can be done by backcrossing a strategic recombinant to the recurrent parent or obtain heterozygous plant materials from the backcross program and search for additional recombinations to further reduce the genetic interval. The drawback of this approach is the heterogeneous genetic background of the recombinants which may interfere the accuracy of phenotyping the QTL effect during fine-mapping.

Fine-mapping of *Rphq11* and *Rphq16* using this "dirty" procedure demonstrated that there was still a substantial noise by segregating genetic background but this did not hamper the fine-mapping. We suggest that, to follow this "dirty" fine-mapping approach, it is particularly important that the targeted QTL effect is relatively large, stable and that we know whether additional QTLs are contributed by the same donor parent. If so, such QTLs should be selected against. This fine-mapping approach can be further improved by initiating fine-mapping at a later stage of backcrossing such as BC_3 or subsequent generations (Figure 1) as suggested by Yang et al. (2012). At BC_3, we expect that only six percent of the donor genome outside the regions of the targeted QTLs remains in the recurrent parent. Such a low proportion of donor genome in the background will cause little, if any, phenotypic noise during fine-mapping.

Fine-mapping requires the QTL region to be saturated with markers. A higher resolution of the genetic map in the QTL region helps to identify more informative recombinations in the region to effectively delimit a new and smaller marker interval around the responsible gene. The high-density barley integrated map which contains 6,990 markers [Barley, Integrated, Marcel 2009 available at http://wheat.pw.usda.gov/GG2/index.shtml; (Aghnoum et al. 2010)] is a great tool to recruit polymorphic markers for fine-mapping. In this integrated map 43% of the markers are based on EST or gene sequences. Another integrated map based on SNPs of ILLUMINA iSelect 9k barley infinium chip was made available recently by A. Martín-Sanz, R. Niks and P. Schweizer (ERA-PG "TritNONHOST" project, ERAPG08.053; unpublished). These integrated maps are useful to identify candidate genes as well as to search for synteny in grass species such as rice and *Brachypodium* in which whole genome sequences are available (Mayer et al. 2011; Schmidt 2000). Synteny between barley, rice and *Brachypodium* can be used for dissecting a QTL region provided there is no disrupted collinearity and the gene of interest is conserved in the syntenic interval of rice and *Brachypodium* (Chapter 4 and Chapter 5). Successful usage of synteny for map-based cloning of genes is demonstrated for *ror2*, *rym4/5* and *Ppd-H1* genes in barley. However, map-based cloning using synteny for *Rpg1*, *Rpg5*, *rpg4*, and *Vrs1* genes in barley was not effective, because either the orthologs were located at non-syntenous positions or were absent (Graner et al. 2010).

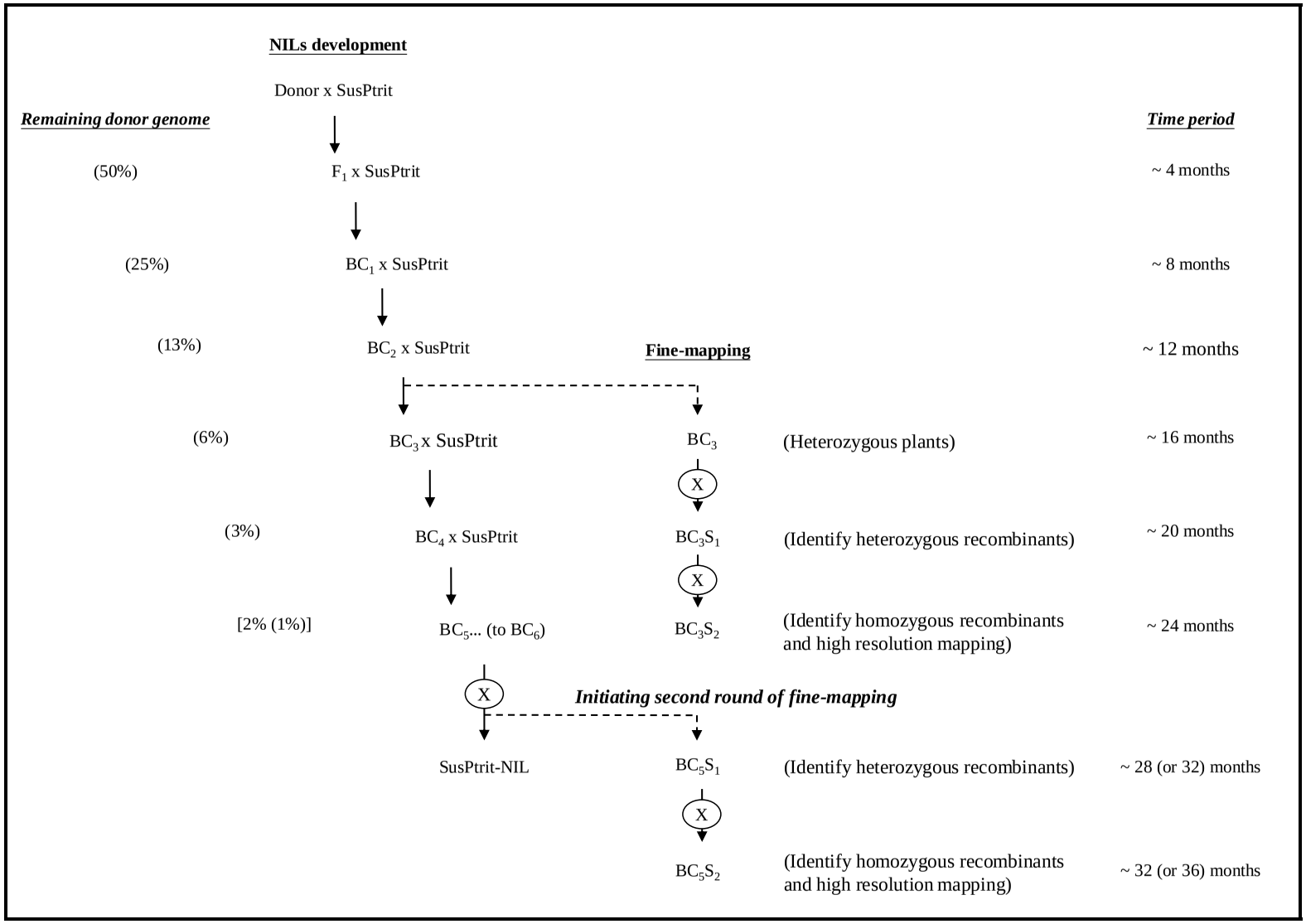


Figure 1: The procedure of fine mapping runs in parallel with NILs development.

The synteny between barley, rice and *Brachypodium* was successfully exploited to saturate the region of *Rphq2* (Marcel et al. 2007a). The physical map of *Rphq2* (Chapter 5) revealed three conserved genes between the three species. One of the conserved genes, a peroxidase, is present in a single copy in rice, in two copies in *Brachypodium* and in barley line SusPtrit, and in three copies in barley cultivar Vada. The other two conserved genes are present in a single copy in all three species. The remaining seven genes predicted only from the Vada sequence and 8 genes predicted only from the SusPtrit sequence have no homolog in the identified syntenic regions of rice and *Brachypodium*. Since the microsynteny is not conserved between barley, rice and *Brachypodium*, we may not be successful in isolating the gene(s) for *Rphq2* if based solely on synteny, and therefore we have to resort to physical mapping.

Physical mapping

A physical map of a genome, chromosome or a chromosome region shows the physical locations of loci. Generating a physical map requires the construction of a genomic library. Such a library consists of a large number of DNA fragments inserted into a cloning vector, and together representing a few times the genome of the organism. A genomic library is preferably composed of clones with large average insert size and can be kept stably for a long term. Large insert cloning vectors with different insert capacity such as yeast artificial chromosomes (YAC), bacterial artificial chromosomes (BAC), P1 phage-derived artificial chromosomes (PAC), among others (Gibson and Muse 2004; Monaco and Larin 1994) are used to construct genomic libraries. YAC has the capacity to accommodate insert sizes up to 100 - 2000 Kb which implies that fewer YAC clones are required to cover a genome or a targeted chromosome region (Burke et al. 1987; Monaco and Larin 1994). This insert capacity is desirable especially for constructing a genomic library for a large-genome species. Unfortunately, YAC has problems with the stability and chimerism of the inserts as well as with the efficiency of purifying the inserted DNA from the YAC DNA (Monaco and Larin 1994; Zhang and Wu 2001). YAC libraries are available for barley (Kleine et al. 1993; 1997; Schmidt et al. 2001) and problems with the stability and chimerism of DNA inserts were observed when used for map-based cloning (Lahaye et al. 1998; Schmidt et al. 2001).

Bacterial-based cloning vectors such as BAC (Shizuya et al. 1992) and PAC (Ioannou et al. 1994) are developed to circumvent the problems with YAC. Although BAC and PAC have relatively smaller insert capacity compared to YAC [insert sizes are up to 300 Kb (Monaco and Larin 1994)], they have a better stability and low frequency of chimerism of inserts. Additionally, purifying the DNA of BAC and PAC from the bacterial host DNA is

much easier than purifying the DNA of YAC from the yeast host DNA (Copeland and Jenkins 2001; Giraldo and Montoliu 2001; Zhang and Wu 2001). The isolation of BAC is done using the existing plasmid isolation protocols. In contrast, isolation of YAC requires tedious methods (Giraldo and Montoliu 2001; Monaco and Larin 1994). In Chapter 5, we decided to construct one BAC library from Vada and another one from SusPtrit.

The BAC cloning vectors had fertility factor (F factor) genes (parA and parB). These genes maintain a low copy number of BAC (one to two copies) in a bacterium (Ioannou et al. 1994) which reduces the chances of recombination between DNA inserts. The BAC libraries constructed for barley so far, have an average insert size ranging from 80 to 140 Kb [Chapter 5, this thesis; (Isidore et al. 2005; Saisho et al. 2007; Schulte et al. 2011; Yu et al. 2000)]. Fine-mapping of QTLs should result in a sufficiently small genetic interval to ease the construction of a physical map using a minimum number of BAC clones and to contain a manageable number of candidate genes in the region. It is difficult, but possible, to fine-map a QTL into a genetic interval containing only one candidate gene (Ashikari et al. 2005; Fridman et al. 2000; Zhang et al. 2012) provided there are sufficient recombination events in the QTL region.

Construction and organization of a gridded BAC library from an organism, especially those with a large genome, is not only laborious and costly but also requires robotic technology as well as large freezer space for storage and maintenance. If the BAC library is only used for map-based cloning or generating markers at regions of interest, construction of a gridded BAC library is not necessary for such applications (Ma et al. 2000). Constructing a gridded BAC library from multiple cultivars is irrational and not cost-effective. Still, one may need to construct BAC libraries from the genotypes of interest. This is, because the targeted gene might be absent in the genomic libraries available for other genotypes due to disrupted intraspecific collinearity (Springer and Stupar 2007). A non-gridded BAC library which can be constructed rapidly and less costly is a good alternative. The BAC libraries of Vada and SusPtrit that we developed (Chapter 5) are non-gridded where the clones are organized in pools instead of individual clones in separate wells. Pooling clones together may create a competition for detection among the clones, but pooling of less than 2000 clones per pool will not hamper the detection efficiency appreciably (Ma et al. 2000). For barley, a BAC library with five genome-equivalents needs about 200,000 clones with an average insert size of 120 Kb. In order to organize the BAC clones to have a gridded BAC library, 520 384-well plates are required, against only 100 pools of BAC clones (2000 clones per pool) kept in 100 microcentrifuge tubes for a non-gridded BAC library. To date, BAC libraries (gridded or non-gridded) are available for six barley genotypes (Table 1).

Table 1: List of BAC libraries currently available for barley.

Barley genotype	BAC library	References
Morex	Six gridded BAC libraries • Constructed by partial digestion with *Hin*dIII (three), with *Eco*RI (one) and with *Mbo*I (one) • One constructed by mechanical shearing	Yu et al. 2000; Schulte et al. 2011
Haruna Nijo	One gridded BAC library • Constructed by partial digestion with *Hin*dIII	Saisho et al. 2007
CS134*	One gridded BAC library • Constructed by partial digestion with *Hin*dIII	Shi et al. 2010
Cebada Capa	One non-gridded BAC library • Constructed by partial digestion with *Hin*dIII	Isidore et al. 2005
Vada	One non-gridded BAC library • Constructed by partial digestion with *Hin*dIII	This thesis, Chapter 5
SusPtrit	One non-gridded BAC library • Constructed by partial digestion with *Hin*dIII	This thesis, Chapter 5

* a doubled haploid line derived from a cross between Clipper and Sahara 3771

In Chapter 5, we screened the non-gridded BAC library of Vada and SusPtrit and constructed a contig spanning *Rphq2* of Vada and a contig covering partially the *rphq2* window of SusPtrit. The study in Chapter 5 together with that of Isidore et al. (2005) shows the usefulness of a non-gridded BAC library for map-based cloning. The screening strategy suggested in both studies was efficient in retrieving specific BAC clones of interest. One part of the screening procedure used in Chapter 5 and Isidore et al. (2005) involves robotic picking of BAC clones which may not be affordable for most laboratories. Recently, Xia et al. (2014) constructed a non-gridded BAC library for soybean and suggested an efficient screening method without resorting to robotic picking of BAC clones.

The non-gridded BAC library of Vada and SusPtrit are not only useful for map-based cloning of *Rphq2*, but also for cloning of other minor genes for partial and nonhost resistance to rust fungi such as *Rphq3*, *Rphq4*, *Rnhq*, among others. The BAC libraries also can be used for map-based cloning of other minor genes such as minor genes for resistance to powdery mildew as were mapped by Aghnoum et al. (2010), minor genes for heading date and plant height (Qi et al. 1998), and other genetic traits for which these accessions contrast. The non-gridded BAC library of Vada and SusPtrit are however, not suitable for whole genome physical mapping. The two BAC libraries were constructed by partial digestion with a single restriction enzyme, *Hin*dIII, which will lead to a biased BAC library, i.e., having an under-representation of some genomic regions due to

nonrandom distribution of the enzyme restriction sites in the genome (Ariyadasa and Stein 2012; Schulte et al. 2011).

Once we identified the BAC clones spanning the targeted chromosomal region, the BAC clones were sequenced. One can sequence the BAC clones either by Sanger sequencing or by using the next generation sequencing technology platforms such as the Roche 454 Genome Sequencer FLX system (Zhou et al. 2010). The sequence can be annotated using the annotation pipelines available on the web such as RiceGAAS (Sakata et al. 2002), FPGP (Amano et al. 2010), MAKER (Cantarel et al. 2008), iPlant (http://dnasubway. iplantcollaborative.org/), and TriAnnot (Leroy et al. 2012). TriAnnot was chosen for annotation of *Rphq2* sequence from Vada and SusPtrit because the panels in the annotation pipeline can be customized for annotating barley sequences.

Candidate gene validation

In the barley-rust pathosystem, the rust fungi target mainly the mesophyll cells of barley. As a result, validation of candidate genes via transient (over)expression and silencing of candidate genes by particle bombardment is not suitable for barley-rust pathosystem, because the carriers of the gene constructs cannot reach the mesophyll cells. This is in contrast to the barley-powdery mildew pathosystem, where the pathogen only infects epidermal cells, and transient (over)expression and silencing of candidate genes by particle bombardment are successfully applied (Douchkov et al. 2005; Miklis et al. 2007). Other options are virus-mediated overexpression (VOX) and virus-induced gene silencing (VIGS).

VOX and VIGS are rapid and easy techniques to validate candidate genes. Barley stripe mosaic virus (BSMV) is usually the vector for these two approaches. Until recently, BSMV-VOX only worked efficiently with small inserts (140 to 500 bp) like transient overexpression of effectors (Chapman et al. 2008; Christie et al. 2012; Lee et al. 2012). Larger inserts, such as green fluorescent protein (GFP) with 720 bp did not work well in BSMV-VOX due to a stability problem of the inserts, as in BSMV-VIGS, which then results in patchy expression (Lee et al. 2012). BSMV-VIGS is a well-established and powerful tool for validating candidate genes (Delventhal et al. 2011; Duan et al. 2013; Liang et al. 2012; Várallyay et al. 2012; Wang et al. 2013). Using this system on barley, the candidate genes are silenced at the most extensive level on the fifth leaf about 14 days after the inoculation was made on the fully expanded second leaf (Burch-Smith et al. 2004; Holzberg et al. 2002). This implies that VIGS cannot be used for candidate genes which are expected to express best at an earlier plant development stage, such as first

seedlings leaves as used in the phenotyping for near-nonhost resistance and partial host resistance to rust fungi. For example, the effect of *Rphq2* against *Ph.*1.2.1 is most effective on the first leaf of seedlings and gradually become less effective starting from the third leaf onward (Wang et al. 2010). Evaluating the phenotype of *Rphq2* on the fifth leaf will not be informative. The same problem may also be true for *Rphq11* and *Rphq16* which were mapped only at the seedling stage, on the first leaf (Chapter 4). Another possible limitation is the genotype-dependent efficiency of BSMV-VIGS (Bruun-Rasmussen et al. 2007). As an example, the pairs of NILs of QTLs in L94 and SusPtrit genetic background (Chapter 3) are available for validating candidate genes through BSMV-VIGS. Unfortunately, BSMV-VIGS is not effective on L94. The silencing of phytoene desaturase (PDS) in L94 only resulted 10% of photobleaching on the fourth leaf, 14 days after inoculation on the first leaf. For SusPtrit, 40% of photobleaching was observed which suggests incomplete silencing of candidate genes. Such incomplete silencing would in case of quantitative resistance genes lead to inconclusive genotypes (T.C. Marcel, Wagenigen UR Plant Breeding, unpublished data). Besides that, if the candidate genes introgressed in SusPtrit are for nonhost resistance, the SusPtrit NILs are also not suitable for validating the candidate genes through BSMV-VIGS. The susceptible phenotype conferred by the alleles of SusPtrit to non-adapted rust fungi is particularly clear in first leaves of seedlings, and not at adult plant stage (Atienza et al. 2004) or intermediate development stages (R.E. Niks, Wageningen UR Plant Breeding, unpublished data). So, the fourth leaf is phenotypically not so contrasting between SusPtrit and regular immune barley accessions. Silencing the candidate genes, even if the genes are expressing at the fourth leaf, will not give a sufficiently contrasting phenotype between SusPtrit NILs and SusPtrit.

For polygenic nonhost resistance there is one more reason that makes transient or stable gene silencing not a suitable approach for analyzing the candidate genes. Barley accessions, such as Vada that are immune to non-adapted rust fungi probably have a high gene dose to confer this resistance (Jafary et al. 2006). Silencing one of those genes might be insufficient to alter the immunity to some level of susceptibility.

Another strategy to validate candidate genes is by targeting induced local lesions in genomes (TILLING). TILLING is particularly applied on species or genotypes that are not well amenable to genetic transformation, such as barley. TILLING aims to produce a large range of mutant phenotypes by creating mutations, such as missense changes, truncation and mutations in splice junction sequences (Kurowska et al. 2011). Several TILLING populations are available for barley, such as Optic (Caldwell et al. 2004), Morex (Talamè et al. 2008), Barke (Gottwald et al. 2009), Lux (Lababidi et al. 2009), among others (Kurowska et al. 2011). Recently, a TILLING population was created for Vada (Y. Wang

and X. Qi, Institute of Botany, Chinese Academy of Sciences, Beijing, China, unpublished), the donor of partial resistance QTLs, *Rphq2*, *Rphq3*, *Rphq4* (Qi et al. 1998) and nonhost resistance QTL, *Rnhq* (Niks et al. 2000). By creating a TILLING population, we circumvent the problems which may arise from validating candidate genes by using BSMV-VIGS. However, for the same reason as explained for the BSMV-VIGS, also for TILLING it applies that a loss-of-function of one gene contributing to nonhost resistance might not alter the phenotype to susceptibility to non-adapted rust fungi, since several more genes remain to confer immunity.

A better approach for testing the candidate genes for resistance would be expressing them in a susceptible barley line by using *Agrobacterium*-mediated stable transformation. In Chapter 2, we developed a new experimental line called Golden SusPtrit to test candidate genes for partial and nonhost resistance. Golden SusPtrit was developed to replace SusPtrit for the studies of partial and nonhost resistance. Golden SusPtrit is about as susceptible as SusPtrit to *Ph.1.2.1* and non-adapted rust fungi tested in Chapter 2, but, in contrast to SusPtrit, it is also amenable to *Agrobacterium*-mediated stable transformation. Stable transformation with a candidate gene allows the transgene to be transmitted to the offspring through grains. As a result, we can obtain multiple identical plants with the transgene inserted at the same chromosomal position and consequently, the phenotype of a candidate gene can be tested with replications. This is not possible for transient assays, because each individually treated plant is unique. Stably expressed candidate genes for partial and nonhost resistance in Golden SusPtrit also provide the possibility to test the gene effect against adapted and non-adapted rust fungi on the same material, as we did for the NILs (Chapter 3). Such disease tests to multiple pathogens can show whether a gene for partial resistance plays a role in nonhost resistance and *vice versa* (Jafary et al. 2008; Chapter 3).

Molecular basis of partial resistance

Various postulations were made on the molecular basis of partial resistance based on evidences such as the association of resistance phenotypes with developmental phenotype, co-localization of partial resistance QTLs with resistance and defense gene analogues, among others (Poland et al. 2009). These postulations are speculative unless the genes explaining the QTLs are isolated and functionally tested. Only recently scientists have started to harvest their efforts of cloning the minor genes for partial resistance.

The minor genes for partial resistance with large effect cloned thus far belong to different types of genes or gene families. They can be categorized into three different classes as

described by Poland et al. (2009): (1) QTLs are minor genes involved in basal defense and defense signal transduction, (2) QTLs are weak form of R-genes, and (3) QTLs are unique genes that were previously unknown to contribute to disease resistance.

QTLs are genes involved in basal defense and defense signal transduction

There are three cloned minor genes for partial resistance belonging to this category. One of them is *Yr36*, a temperature dependent race non-specific stripe rust resistance gene (Fu et al. 2009). *Yr36* confers partial resistance to adult plants (Uauy et al. 2005) and seedlings (Fu et al. 2009) against *P. striiformis* f. sp. *tritici* (*Pst*) effectively at high temperature (25 to 35°C). The gene of *Yr36* encodes a protein with an N terminal kinase domain and a predicted steroidogenic acute regulatory protein-related lipid transfer domain (START) at the C terminal. This gene has been validated using a TILLING population of 1536 mutagenized lines and was confirmed through stable transformation of the gene into a susceptible wheat variety. The combination of kinase and START domains is unique and not present in other organisms. The START domain is postulated to bind lipids from *Pst* (Fu et al. 2009) and to trigger the kinase domain to send out signals for defense response. Further studies are needed to characterize this gene.

Lr34, previously known as *LrT2* (Dyck 1977, 1987), is another cloned minor gene for resistance in wheat (Krattinger et al. 2009). *Lr34* not only confers partial resistance of adult plants against leaf rust, *Puccinia triticina* (*Pt*) (Dyck et al. 1966), it also co-segregates with the adult plant resistance genes *Yr18* against stripe rust (Pst) (McIntosh 1992; Singh 1992) and *Pm38* against powdery mildew (*Blumeria graminis* f. sp. *tritici*) (Spielmeyer et al. 2005). Depending on the genetic background, *Lr34* also is effective against stem rust (*Puccinia graminis* f. sp. *tritici*) (Dyck 1987). *Lr34* has been fine-mapped into a 0.15 cM genetic interval by using three high-resolution backcross populations developed from three pairs of parents (-*Lr34*/+*Lr34*): (1) Arina/Fomo, (2) Thatcher/PI5848, and (3) Avocet/Parula. The 0.15 cM genetic interval has been sequenced from *Lr34*-containing wheat cultivar Chinese Spring and revealed six candidate genes. The coding sequences of the six candidate genes from the three pairs of parental lines of the high-resolution backcross populations were compared. Among the candidate genes, only the coding sequence of ATP-binding cassette (ABC) transporter gene was consistently polymorphic between the alleles of all parental pairs. Then, mutants of the ABC transporter gene were obtained from ɣ-irradiation and sodium azide-induced mutation. The mutants were more susceptible to *Pt*, *Pst*, *B. graminis* f. sp. *tritici* and *P. graminis* f. sp. *tritici*. This strongly suggests *Lr34* to be an ABC transporter gene contributing strongly to the partial resistance against the different pathogens. This gene

belongs to the pleiotropic drug resistance subfamily of ABC transporters, the same family as penetration deficient gene 3 (PEN3) of Arabidopsis. The gene has two cytosolic nucleotide binding domains and two hydrophobic transmembrane domains (Krattinger et al. 2009). *Lr34* may have a similar function as proposed for PEN3 which transports toxic compounds derived from glucosinolates into the plant apoplast at the interaction sites with pathogen (Lipka et al. 2008; 2010). The *Lr34* haplotype of Chinese Spring also occurs in Australian cultivar H45 but this cultivar is highly susceptible to *Pt* and *Pst*. However, H45 recovered its resistance to *Pst* when it was crossed with Avocet which is also susceptible to *Pst*. This implies that the *Lr34* haplotype of Chinese Spring may interact with an unknown factor(s) to confer resistance (Lagudah 2011). It is not known whether the cross between H45 with Avocet recovered the resistance of H45 to *Pt*. In Chapter 3, the *Rphq2*-, *Rphq3*- and the Vada allele of *Rnhq*-introgression in L94 conferred resistance against certain non-adapted rust fungi but not in SusPtrit background. Maybe similar to *Lr34*, the QTL introgressions in L94 confer resistance by interacting with an unknown factor(s) not present in SusPtrit.

The third cloned minor gene for partial resistance that also is involved in basal defense and defense signal transduction is a QTL in rice which contributes to resistance against *M. oryzae* and *Rhizoctonia solani* (Manosalva et al. 2009). The QTL co-localized with oxalate oxidase-like genes now known as germin-like protein (GLP) genes which are candidate defense response genes. Within the QTL interval, there is a cluster of 12 highly conserved GLP gene members. The involvement of these genes in defense response is validated by silencing some to all of these genes through RNA interferences. The transgenic plants became more susceptible to *M. oryzae* as well as to *R. solani* when more of the GLP genes were silenced. This implies that the GLP genes control disease resistance as a complex locus in with each gene contributes a small additive effect. The hypothetic function of the GLP genes in disease resistance involves the production of superoxide dismutase which generates hydrogen peroxide (H_2O_2) that might be involved in the cell wall defense, hypersensitive cell death, signaling in systemic acquired resistance and in the induction of defense response gene expression (Manosalva et al. 2009).

QTLs are weak forms of R-genes

Panicle blast 1 (*Pb1*) is a cloned rice gene explaining a QTL effect against *M. oryzae* (Hayashi et al. 2010) derived from cultivar Modan (Fujii et al. 1999). The candidate genes explaining the QTL were validated through genetic transformation of the genes into the susceptible cultivar Norin 8. *Pb1* is a gene encoding coiled-coil-nucleotide-binding-site-leucine-rich repeat (CC-NBS-LRR) which is the characteristic of *R* genes. The *R* genes

mediate gene-for-gene race specific resistance, but a cultivar carrying *Pb1*, a NIL of *Pb1* and transformants overexpressing *Pb1*, all showed increased resistance to widely distributed blast races compared to cultivars/lines not carrying *Pb1*(Hayashi et al. 2010). *Pb1* encodes an atypical *R* protein-like protein. In the NBS domain of *Pb1*, the prominently conserved P-loop, Walker B, Resistance-NBS-B (RNBS-B) and Gly-Leu-Pro-Leu (GLPL) motifs are diverged from the typical *R* gene (DeYoung and Innes 2006). The pentapeptide EDVID motif located between the CC sequences is another important and conserved motif of *R* genes (Bai et al. 2002) that also is not conserved in *Pb1*. Hayashi et al. (2010) speculated on the implications of the atypical CC-NBS-LRR structure of *Pb1* on the resistance.

QTLs are unique genes previously unknown to be involved in disease resistance

A QTL in rice, *Pi21* encodes a gene which was unknown to be involved in disease resistance (Fukuoka et al. 2009). *Pi21* is a recessive resistance QTL (*pi21*) mapped against rice blast (*Magnaporthe oryzae*) by Fukuoka and Okuno (2001). *Pi21* is fine-mapped into a 1,705 bp region containing only one gene, Os04g0401000, which encodes a protein containing a heavy metal-transport/detoxification protein domain in the N-terminal region. Comparison of the 1,705 bp sequence of a resistant cultivar (Owarihatamochi) with that of two susceptible cultivars (Aichiasahi and Kasalath) revealed seven nucleotide polymorphisms but only two located in the open reading frame associated with the phenotypes. Transformation of the resistance allele *pi21* (from Owarihatamochi) into a susceptible cultivar (Aichiasahi) did not confer resistance. However, transformation of the susceptibility allele *Pi21* (from Aichiasahi) into a NIL carrying *pi21* resulted in an increase of susceptibility to *M. oryzae*. Fukuoka et al. (2009) suggested that the resistance allele *pi21* carries a loss-of-function mutation. The susceptible allele *Pi21* probably regulates the resistance negatively and is confirmed through silencing the expression of *Pi21* which increases the resistance of transformants.

Rphq2 ***and*** **Rphq11** ***differ from the cloned genes explaining resistance QTLs***

The candidate genes explaining *Rphq2* (Chapter 5) and *Rphq11* (Chapter 4) differ from those cloned minor genes for partial resistance described above. For both QTLs, *R*-genes or weak forms of *R*-genes are unlikely candidates. The candidate gene for *Rphq2* can either be a peroxidase or kinase or even a group of peroxidases or kinases as in the case of GLP genes in rice (Manosalva et al. 2009). *Rphq2* may also be a Seven in absentia protein (SINA). For *Rphq11* in Chapter 4, the strongest candidate gene is a phospholipid

hydroperoxide glutathione peroxidase. Other possible candidate genes include an actin-depolymerizing factor and glucosyltransferases. Each of the candidate genes for *Rphq2* and *Rphq11* were previously reported to be involved in disease resistance in one way or another (see Chapter 5 and Chapter 11). Based on the types of candidate gene suggested for *Rphq2* and *Rphq11*, the gene explaining the two partial resistance QTLs are probably involved in basal defense and defense signal transduction. When isolating the candidate genes for *Rphq2* and *Rphq11*, we should isolate the resistance allele as well as the susceptible allele. This is because a gene that explains a QTL can be either a resistance factor or a susceptibility factor (negative regulator) as in the case of *Pi21* (Fukuoka et al. 2009). For *Rphq2*, this may not be possible for some candidate genes which only are present in Vada but not in SusPtrit and *vice versa*.

Other cloned genes explaining resistance QTLs

For QTLs with a small effect, *i.e.* explaining less than 10% of the phenotypic variation, a candidate gene approach is proposed by Hu et al. (2008) to isolate the genes. First, differential expression analysis is performed to identify the candidate defense response genes. Candidate defense response genes should have differential expression either between infected resistant and susceptible plants, or between non-infected and infected plants. Then, the identified genes are mapped onto a linkage map to find genes that co-localize with a resistance QTL. Those genes found to co-localize with QTLs are examined for their expression patterns against different plant-pathogen interactions to identify genes with expression influenced by a wide range of pathogens. Finally, complementary functional analysis is performed to confirm the involvement of the genes in disease resistance. Using this procedure, genes have been cloned that may explain small effect QTLs for disease resistance in rice (Table 2).

Table 2: Genes cloned for small effect QTLs for partial resistance in rice based on candidate gene approach.

Gene name	Gene type	QTL*	Chromosome	Pathogen[!]	References
WRKY13	Transcription factor	3-5%	Chromosome 1	*Mg*, *Xoo*	Hu et al. 2008
DR8	Enzyme-like protein involved in thiamine biosynthesis	2-3%	Chromosome 7	*Mg*, *Xoo*	Hu et al. 2008
GH3-8	Indole-3-acetic acid-amido synthetase	2%	Chromosome 7	*Mg*, *Xoo*	Hu et al. 2008
MPK6	Mitogen-activated protein kinase	4%	Chromosome 10	*Mg*, *Xoo*[a]	Hu et al. 2008
NRR	Unknown protein	6%	Chromosome 1	*Xoo*[a]	Kou et al. 2010
GH3-1	Indole-3-acetic acid-amido synthetase	4-7%	Chromosome 1	*Mg*, *Xoo*	Kou et al. 2010
LSD1	Lesion-simulating disease resistance 1 (LSD-1)-like protein	5%	Chromosome 8	*Mg*[a]	Kou et al. 2010
WRKY53	Transcription factor	6%	Chromosome 5	*Mg*, *Xoo*	Kou et al. 2010
MPK5	Mitogen-activated protein kinase	2%	Chromosome 3	*Bg*[a], *Mg*[a], *Xoo*[a]	Kou et al. 2010
GH3-2	Indole-3-acetic acid-amido synthetase	4-10%	Chromosome 1	*Mg*, *Xoo*, *Xoc*	Fu et al. 2011

* The explained phenotypic variation of the minor effect QTL corresponding to the gene

! The pathogens to which the genes are effective are: *Bg* = *Burkholderia glumae*, *Mg* = *Magnaporthe grisea*, *Xoo* = *Xanthomonas oryzae* pv. *oryzae*, *Xoc* = *X. oryzae* pv. *oryzicola*

[a] The gene is a negative regulator of disease resistance

Specificity and durability of resistance QTLs

We have mapped a great number of QTLs for partial and near-nonhost resistance using the barley-rust pathosystem, using at least six (SusPtrit/Golden Promise, Vada/SusPtrit, Cebada Capa/SusPtrit, Oregon Wolfe Barley, Steptoe/Morex, L94/Vada) mapping populations. The resistance conferred by the QTLs affect resistance either in an isolate specific manner, can be species specific or have a broad resistance spectrum, at least towards the pathogens that were tested [Chapter 3; (González et al. 2012; Jafary et al. 2006; Marcel et al. 2008)].

The isolate specificity of partial resistance deviates from the concept of horizontal resistance (van der Plank 1963, 1968). The specificity of partial resistance has been assumed to be due to a minor-gene-for-minor-gene interaction which is similar to the vertical resistance (Parlevliet and Zadoks 1977). The molecular basis of minor-gene-for-minor-gene interaction would probably be the interaction between the pathogen effectors with their specific operative targets in plant. The operative targets are probably the genes (at the mapped QTLs) or the gene products which are involved in defense response. The more effectors fitting their operative targets, the higher the level of susceptibility is observed on plant.

All the minor genes for partial resistance which have been cloned to date (see above) are reported to have a broad resistance spectrum. The specificity of a QTL against different isolates or even to different related species of a pathogen may depend on the genetic background [Chapter 3; (Lagudah 2011; St.Clair 2010)]. A minor gene at a QTL might interact with another factor in the plant, elsewhere on the genome, to confer resistance. If the QTL is transferred to a genetic background lacking the factor, or possessing a suppressor, a more susceptible phenotype will be observed [Chapter 3; (Lagudah 2011)].

Resistance based on minor-gene-for-minor-genes is believed to be more durable compared to *R* genes mediated resistance which tends to be broken down easily by the pathogen (Pretorius et al. 2012; Rouse et al. 2013; Singh et al. 2011; Wan and Chen 2011). The possible explanations for the durability of partial resistance as proposed by Niks and Marcel (2009) are:

- Developing an effector to silence/neutralize a defense factor requires a particular gain of function mutation by the pathogen, which is rare
- If a pathogen successfully overcomes one minor gene for resistance, the pathogen only gains a marginal advantage due to presence of other resistance genes especially for nonhost resistance.

- A great diversity of QTLs for partial resistance are available, at least in barley for resistance to *Puccinia hordei*. Each gene for partial resistance which is overcome by a mutant pathogen may occur in only a small proportion of the plant population or crop acreage. Therefore, a microbial mutant has limited selective advantage.

Lr34 (Krattinger et al. 2009) and *Sr2* (Ayliffe et al. 2008) in wheat are good examples of durable resistance QTLs where virulence towards these QTLs is not observed after more than 50 years of culture.

Concluding remarks

This thesis has developed tools that are important towards identification of genes for partial and nonhost resistance. First of all, Golden SusPtrit is a valuable experimental line to validate candidate genes for partial and nonhost resistance. Golden SusPtrit also serves as a platform to study the possible association between partial and nonhost resistance. The near isogenic lines of QTLs in SusPtrit background are a good starting material to stack different combinations of minor genes for resistance and to test their effect on partial and nonhost resistance. They are also very useful starting points for fine mapping of the minor genes conferring the QTL effects. The Vada and SusPtrit BAC libraries have been developed as tools to isolate the resistance and susceptible alleles of gene(s) for *Rphq2*, *Rphq3*, *Rphq4* and *Rnhq*. They may also serve for isolation of other genes for which SusPrit and Vada contrast. Some QTLs studied in this thesis have specific resistance effects but others, such as *Rphq11*, have a broader resistance spectrum. However, the specificity seems to depend on the genetic background. Map-based cloning of *Rphq2*, *Rphq3*, *Rphq4*, *Rphq11*, *Rphq16* and *Rnhq* will provide more insight into the diversity of genes and their function in partial and nonhost resistance.

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Summary

Partial resistance of barley to *Puccinia hordei* and near-nonhost resistance to non-adapted rust fungi inherit polygenically. The two types of resistance seem to share some genes and have a similar prehaustorial mechanism of resistance, but partial resistance is less strong than near-nonhost resistance of barley. Partial resistance to adapted, "host", rust fungi seems, therefore, like a weak form of nonhost resistance to non-adapted rust fungi. If partial resistance and nonhost resistance are indeed based on the same principles, one can understand nonhost resistance by studying partial resistance and *vice versa*. To study partial and nonhost resistance, as well as their association, the candidate gene(s) for resistance must be cloned and characterized for their action.

Five resistance quantitative trait loci (QTLs) for partial resistance (*Rphq2*, *Rphq3*, *Rphq4*, *Rphq11* and *Rphq16*) and one nonhost resistance QTL (*Rnhq*) were selected to pursue map-based cloning. First, the effect of the QTLs was verified in near-isogenic lines (NILs). The NILs of *Rphq2*, *Rphq3*, *Rphq4* and *Rnhq* (QTL-NILs) were available in L94 genetic background. L94 is extremely susceptible to *Puccinia hordei*, and, at seedling stage, somewhat susceptible to certain non-adapted rust fungi. The experimental barley line SusPtrit is also susceptible to *P. hordei* but, at seedling stage, also very susceptible to at least nine species of non-adapted rust fungi. In Chapter 3, we developed NILs in SusPtrit background for *Rphq2*, *Rphq3*, *Rphq11*, *Rphq16* and two alleles of *Rnhq*, viz. L94 and Vada alleles. The effect of each QTL in L94 and SusPtrit genetic backgrounds was tested not only against different isolates of *P. hordei* but also against different species and isolates of non-adapted rust fungi. The QTL-NILs suggested that the effects of the partial resistance genes depended on rust species and rust isolates. Some introgressions conferred resistance to a broader spectrum of rust species and isolates than others, the broadest being the *Rphq11*-introgression. The NILs may overestimate the spectrum of effectiveness of the partial resistance genes because some NILs contain inadvertent donor genome in the background and the introgressed QTL region may contain several linked resistance genes, each with a narrow resistance spectrum. The introgression would then confer a resistance spectrum that is the combination of the spectra of several linked resistance genes. Allowing for the possibility of linkage of narrow-spectrum resistance genes, our study suggests that some genes may be involved in partial as well as nonhost resistance. Data also suggest that genetic background may play a role in the resistance conferred by the QTL-introgression.

The NILs also allow fine-mapping of the QTL as was done for *Rphq2* in a previous study. In Chapter 4, we target to fine-map another two partial resistance QTLs of our interest, viz. *Rphq11* and *Rphq16*. We, however, did not use the NILs for fine-mapping of *Rphq11* and *Rphq16*. Instead, after validating the effect of *Rphq11* and *Rphq16* using the early breeding materials for developing NILs of *Rphq11* and *Rphq16*, we developed fixed QTL-recombinants (i.e. homozygous recombinants at the *Rphq11*/*Rphq16* QTL alleles, homozygous susceptible at the non-targeted QTL alleles). The genomic background of fixed QTL-recombinants was still segregating, but expected not to be relevant for the resistance level. *Rphq11* was fine-mapped into a 0.2 cM genetic interval and a 1.4 cM genetic interval for *Rphq16*, before the NILs were ready. The strongest candidate gene for *Rphq11* is a phospholipid hydroperoxide glutathione peroxidase (PHGPx). This gene corresponds to the new *Rphq11* peak marker – WBE129, located within the refined 0.2 cM genetic intervals and was one of the candidate genes for *Rphq11* identified through e-QTL mapping on Steptoe/Morex challenged with the same rust isolate. There was no clear candidate gene identified for *Rphq16*.

A QTL has to be fine-mapped into a sufficiently narrow genetic window to make physical mapping feasible. *Rphq2* with a genetic window of 0.1 cM is ready for physical mapping. In Chapter 5, we have constructed two non-gridded Bacterial Artificial Chromosome (BAC) libraries of barley from Vada and SusPtrit. Based on the insert sizes of the BAC clones, the estimated genome coverage of the Vada BAC library is 2.6x and of the SusPtrit BAC library 3.7x. The genome coverage of Vada is comparable to the BAC library of Morex, HVVMRXALLhB and SusPtrit to HVVMRXALLeA. The estimation of genome coverage based on microsatellite markers indicates, however, Vada and SusPtrit BAC libraries to have 5.0x and 6.8x genome coverage, respectively. Based on genome insert size, the BAC library of Vada gives at least 93% probability of identifying a clone corresponding to any sequence of Vada and for the BAC library of SusPtrit a probability of 98% is expected. Together, the two BAC libraries give more than 99% probability of recovering any specific sequence from the barley genome. A tiling path of three BAC clones was constructed for Vada, which cover the *Rphq2* genetic window. The physical window of *Rphq2* in Vada BAC contig is approximately 195 Kbp. For SusPtrit, the three BAC clones forming the contig did not cover the entire genetic window of *Rphq2*. The physical length bridged by them is approximately 226 Kbp. The TriAnnot pipeline annotated 12 genes in both the Vada and the SusPtrit contig, but only four of the annotated genes are shared between Vada and SusPtrit. The candidate genes for *Rphq2* might be a resistance factor in Vada or a susceptibility factor in SusPtrit. The peroxidases and kinases are good candidates to represent *Rphq2*. It is possible that one of the peroxidase or kinase gene members in the physical window of *Rphq2* explains the resistance phenotype observed. Another possibility is that peroxidase or kinase gene members function as a

complex QTL. A member of the Seven in absentia protein family (SINA) can be a candidate as well. The gene families to which previously cloned genes for partial resistance belong were not found to be represented in the *Rphq2* region.

We propose to perform functional analysis of candidate genes through *Agrobacterium*-mediated stable transformation of the resistance allele into a susceptible genotype, such as SusPtrit. Unfortunately, SusPtrit is, as so many barley accessions, not amenable to *Agrobacterium*-mediated transformation. In Chapter 2, we developed a doubled haploid (DH) mapping population (n=122) by crossing SusPtrit with Golden Promise to develop a 'Golden SusPtrit', i.e., a barley line combining SusPtrit's high susceptibility to non-adapted rust fungi with the high amenability of Golden Promise for transformation. Using the DH population, we identified nine genomic regions occupied by QTLs against four non-adapted rust fungi and *P. hordei* isolate 1.2.1 (*Ph*.1.2.1). From 12 DH lines that were most susceptible to the tested non-adapted rust fungi, we selected four DHs for an *Agrobacterium*-mediated transformation efficiency test. We obtained a DH line (SG062N) with transformation efficiency of 11 to 17 transformants per 100 immature embryos. The level of susceptibility of SG062N to non-adapted rust fungi is either similar to or higher than the level of susceptibility of SusPtrit. Against *P. hordei*, the latency period conferred by SG062N at seedling stage is as short as that conferred by SusPtrit. SG062N, designated 'Golden SusPtrit', will be a valuable experimental line that could replace SusPtrit in future nonhost and partial resistance studies, especially for stable transformation using candidate genes that may determine the differences in resistance levels against adapted and non-adapted rust fungi.

Samenvatting

Partiële resistentie van gerst tegen *Puccinia hordei* en bijna-nietwaard resistentie tegen niet-aangepaste roestschimmels, waarvoor gerst geen waardplant is, hebben een polygene overerving. Sommige genen lijken beide typen resistentie te reguleren, en ook lijken beide resistentietypen voornamelijk te berusten op eenzelfde prehaustoriaal resistentiemechanisme. Partiële resistentie is echter minder sterk dan bijna-nietwaard resistentie van gerst. Partiële resistentie tegen aangepaste roestschimmels, waarvoor gerst waardplant is, lijkt op een zwakke vorm van nietwaard resistentie tegen niet-aangepaste roestschimmels. Als partiële resistentie en nietwaard resistentie inderdaad berusten op dezelfde principes, kan men nietwaard resistentie begrijpen door partiële resistentie te bestuderen en omgekeerd. Om partiële en nietwaard resistentie en hun mogelijke associatie te bestuderen, moeten kandidaat genen voor resistentie worden gekloneerd en gekarakteriseerd wat betreft hun effect op en aandeel in beide resistentietypen.

Vijf kwantitatieve loci (QTLs) voor partiële resistentie (*Rphq2*, *Rphq3*, *Rphq4*, *Rphq11* en *Rphq16*) en een QTL voor nietwaard resistentie (*Rnhq*) werden gekozen om via hun positie op de koppelingskaart gekloneerd te worden. Eerst werd het effect van de QTLs geverifieerd in bijna-isogene lijnen (NILs). De NILs van *Rphq2*, *Rphq3*, *Rphq4* and *Rnhq* (QTL-NILs) waren beschikbaar in de genetische achtergrond van lijn L94. L94 is extreem vatbaar voor *P. hordei*, en in het zaailingstadium ook enigszins vatbaar voor sommige niet-aangepaste roestschimmels. De experimentele gerstlijn SusPtrit is eveneens vatbaar voor *P. hordei* maar in het zaailingstadium ook zeer vatbaar voor ten minste negen soorten niet-aangepaste roestschimmels. In hoofdstuk 3 ontwikkelden we NILs voor *Rphq2*, *Rphq3, Rphq11*, *Rphq16* en twee allelen van *Rnhq*, namelijk die van L94 en van Vada, in de genetische achtergrond van SusPtrit. Het effect van elk QTL in de genetische achtergronden van L94 en SusPtrit werd niet alleen bepaald met verschillende isolaten van *P. hordei*, maar ook met verschillende soorten en isolaten van niet-aangepaste roestschimmels. De QTL-NILs suggereerden dat de effecten van de genen voor partiële resistentie afhingen van roestschimmelsoort en -isolaat. Sommige introgressies veroorzaakten resistentie tegen een breder spectrum van roestschimmels dan andere. Het breedste spectrum werd gevonden voor de introgressie van QTL *Rphq11*. Mogelijk overschatten de NILs het spectrum van effectiviteit van de genen voor partiële resistentie, want sommige NILs bevatten elders op het genoom ook nog enkele ongewenste fragmenten van het donorgenoom, en de introgressie met het resistentie QTL bevat mogelijk verscheidene gekoppelde genen voor resistentie, elk met een nauwer spectrum van effectiviteit. De introgressie zou in dat geval een resistentie geven tegen een spectrum

van roestschimmels dat de combinatie is van de spectra van verscheidene gekoppelde resistentiegenen. Onder voorbehoud van de mogelijkheid van koppeling tussen diverse nauw-spectrum resistentiegenen wijzen onze resultaten erop dat sommige genen zowel een rol spelen in partiële als in nietwaard resistentie. De gegevens suggereren ook dat genetische achtergrond een rol kan spelen in de resistentie die door de QTL-introgressies wordt veroorzaakt.

De NILs zijn een goede stap naar fijnkartering van de QTLs, zoals in een vorige studie gedaan is voor *Rphq2*. In Hoofdstuk 4 stelden we ons tot doel twee andere interessante QTLs voor partiële resistentie fijn te karteren, namelijk *Rphq11* en *Rphq16*. Daarvoor gebruikten we echter niet de NILs voor deze twee QTLs. Na bevestiging van de effecten van *Rphq11* en *Rphq16* in vroege terugkruisings nakomelingen ontwikkelden we recombinanten die homozygoot waren voor de *Rphq11*/*Rphq16* QTL allelen en homozygoot vatbaar voor het resistentie-QTL elders op het genoom. De genetische achtergrond van deze "gefixeerde QTL-recombinanten" splitste nog uit, maar was naar verwachting niet relevant voor het resistentieniveau. Nog voor de NILs gereed waren konden we op deze manier *Rphq11* fijnkarteren in een interval van 0.2 cM en *Rphq16* in een interval van 1.4 cM. Het meest waarschijnlijke kandidaat gen voor *Rphq11* is een phospholipid hydroperoxide glutathione peroxidase (PHGPx). Dit gen komt overeen met de nieuwe *Rphq11* piekmerker – WBE129, welke ligt binnen het fijnkarterings interval van 0.2 cM en welke ook uit een e-QTL studie in Steptoe/Morex naar voren kwam als een van de kandidaat genen voor *Rphq11*. Er werd geen duidelijk kandidaat gen gevonden voor *Rphq16*.

Een QTL moet fijngekarteerd worden in een voldoende klein genetisch interval om fysieke kartering mogelijk te maken. *Rphq2* ligt in een genetisch interval van 0.1 cM, wat voldoende moet zijn voor fysieke kartering.

In Hoofdstuk 5 beschrijven we de ontwikkeling van twee "non-gridded" Bacterial Artificial Chromosome (BAC) bibliotheken (BAC-libraries) van de gerstaccessies Vada en SusPtrit. Op basis van de groottes van de BAC-klooninserties schatten we dat het genoom van Vada ongeveer 2,6x en dat van SusPtrit ongeveer 3,7x vertegenwoordigd is in de respectievelijke BAC-libraries. De dekking van het Vada genoom is van zelfde orde van grootte als die van de BAC-library van Morex HVVMRXALLhB en die van het SusPtrit genoom als die van HVVMRXALLeA. De dekking van het genoom werd ook geschat door bepaling van het voorkomen van microsatellietmerkers. Die methode suggereerde dat de libraries een 5,0x (Vada) en 6,9x (SusPtrit) dekking hebben. Op basis van de insertiegroottes schatten we dat de BAC-library van Vada een kans geeft van 93% om een bepaalde sequentie vertegenwoordigd te zien, en de BAC-library van SusPtrit een kans

van 98%. In combinatie zouden de BAC-libraries een 99% kans geven om een bepaalde sequentie te vinden.

We vonden drie overlappende BAC-klonen voor Vada die samen het gehele genetische interval voor *Rphq2* dekken. De fysieke grootte van deze Vada BAC-contig is ongeveer 195 Kbp. Voor SusPtrit konden we geen sluitende contig vinden voor *Rphq2*. De fysieke lengte die door de drie BAC-klonen daar wordt overbrugd schatten we op ongeveer 226 Kbp. Met de TriAnnot procedure annoteerden we 12 genen in zowel de Vada- als de SusPtrit-contig, maar slechts vier van deze genen kwamen zowel in de Vada- als in de SusPtrit-contig voor. De kandidaat genen voor *Rphq2* zouden een resistentiefactor in Vada kunnen zijn of een vatbaarheidsfactor in SusPtrit. De peroxidasen en kinasen zijn goede kandidaten voor *Rphq2*. Het is mogelijk dat een van de peroxidase- of kinasegenen in het fysieke interval van *Rphq2* het effect op het resistentie fenotype verklaart. Een andere mogelijkheid is dat peroxidase- of kinasegenen functioneren als complex QTL. Een aantal genen voor Seven in absentia eiwitten (SINA) komen ook in aanmerking als kandidaat gen. De genfamilies die in eerdere studies door klonering geïdentificeerd werden als verantwoordelijk voor partiële resistentie waren niet vertegenwoordigd in de *Rphq2* regio.

Om de kandidaat genen functioneel te testen, stellen we voor stabiele transformatie van een vatbare accessie uit te voeren door middel van *Agrobacterium*, bij voorkeur de lijn SusPtrit. Deze lijn is echter, zoals zovele gerstgenotypen, niet efficiënt te transformeren via deze methode. In hoofdstuk twee beschrijven we de ontwikkeling van een verdubbelde haploiden (DH) populatie (n=122) uit een kruising van SusPtrit met Golden Promise met als doel een "Golden SusPtrit" lijn te ontwikkelen die de hoge vatbaarheid van SusPtrit tegen zekere niet-aangepaste roestschimmels zou combineren met de efficiëntie van Golden Promise voor genetische transformatie. De DH populatie werd getest op resistentieniveau tegen vier niet-aangepaste roestschimmels en een isolaat (*Ph*.1.2.1) van de dwergroestschimmel *P. hordei*. Uit twaalf DH lijnen met de hoogste vatbaarheid voor de geteste niet-aangepaste roestschimmels selecteerden we er vier om hun transformeerbaarheid te bepalen. We verkregen een DH lijn (SG062N) die een transformeerbaarheidsefficiëntie had van 11 tot 17 transformanten per 100 onrijpe embryo's. Het niveau van vatbaarheid van SG062N tegen niet-aangepaste roestschimmels was vergelijkbaar met of zelfs hoger dan die van SusPtrit. De vatbaarheid voor *P. hordei*, gemeten als latentie periode in het zaailingstadium, is even hoog als die van SusPtrit. SG062N werd omgedoopt tot 'Golden SusPtrit', en zal fungeren als waardevolle experimentele lijn die SusPtrit kan vervangen in toekomstige nietwaard en partiële resistentie studies, met name waar gebruik gemaakt gaat worden van stabiele transformatie met kandidaat genen die verantwoordelijk kunnen zijn voor verschillen in resistentieniveaus tegen aangepaste en niet-aangepast roestschimmels.

Acknowledgements

Studying in Wageningen University (WUR) is tough and challenging. I am very grateful to everyone who has accompanied and helped me to achieve this milestone of my life journey.

My golden opportunity to study abroad does not come easy. I must thank University Malaysia Sarawak for the scholarship to study abroad. I wish to thank Professor Dr Cheksum Tawan and Associate Professor Dr Sim Soon Liang for their guidance.

My six years in Wageningen started with my MSc study. In an MSc thesis market, I met my supervisor, Dr Thierry C. Marcel. I would like to express my greatest appreciation to Thierry who gave me the opportunity and patiently supervised my MSc thesis project. You were a great motivator. Later, you became my PhD daily supervisor and helped me to kick start my PhD study. After about one year, you were offered a position in INRA and became my external supervisor. I really admire your supervising skills and hopefully I can be as good one day.

To pursue a PhD study has always been my aim ever since I stepped into WUR. I would like to express my deep gratitude to Dr Rients Niks for offering the opportunity. Dear Rients, the first thing you taught me was, the "student-teacher interaction" should be nonhierarchical. I really appreciate this type of relationship which I never had before back in my home country. I admire your attentiveness to detail and scientific enthusiasm which are crucial to be successful as a scientist. Thank you for giving tons of support, idea, and encouragement, without which, I may not have come this far. I would like to offer my special thanks to Professor Dr Richard Visser for hosting me at the Laboratory of Plant Breeding. You have kindly helped me in various ways to make sure my PhD study run smoothly.

I wish to thank my students for their inputs to this thesis. Allo Dido, Rene Kuijken, Romain Bouchon, Wang Chun and Zhang Yun Meng, I wish you all a great future ahead. My thanks also go to the secretaries, Annie, Letty, Mariame, Nicole and Janneke, also to the technicians Anton, Brian, Wendy, Koen, Fien, Irma, Doret and Danny for all the supports and kindness.

All work and no play make Jack a dull boy. I am glad to have a bunch of friends who constantly keeps my life interesting in the lab as well as in Wageningen. Thank you to Dr. Antoine Gady, Dr. Bjorn Kloosterman and Dr. Efstathios Roumeliotis for keeping the atmosphere in the office lively. Dr. Gao Wei and Dr. Nasim Mansoori, you two were wonderful "new" office mates. I cherish the moment we had together in the small office. We have supported and encouraged each other to achieve our goals, but the best part is the never ending laughter in our office and the constantly available sweeties, crisps and fruits. The laughters and all the sweeties/crisps are the major sources of energy to keep us going. Also, I wish to thank Dr. Nicholas Champouret, Krissana Kowitwawnich, and Dr. Zhang Ning Wen for the constructive advices that help me to move one step after another to achieve my goals. I would like to express my appreciation to Dr Hossein Jafary and Dr Reza Aghnoum who taught me a lot in the lab as well as sharing their research experience with me. My gratitude also goes to Dr Arwa Shahin, Dr Anitha Kumari, Mirjana Vukosavljev, Natalia Carreno, Thomas Liebrand and many more in the Laboratory of Plant Breeding for the friendship we established.

A lab can be a dull place if you were only accompanied by bacteria colonies, solution bottles, various tubes and a radio which plays the same playlist over and over again. Fortunately, there were amazing and interesting people in the lab; Krissana Kowitwawnich, Huang Ping Ping, Dr. Gao Wei, Shairul Izan Ramlee and Tereza Vozábová. The time we spent together in the lab with brilliant jokes is one of the most unforgettable memories.

Living abroad with minimum cooking skill is a bad idea, especially for a food loving person like me. I am thankful to "chef" Chen Xu who taught me his legendary cooking skills, without whom I might still shoving sandwiches, salads and frozen pizzas down my throat until I graduate. Then came Fan Ling Ling, Huang Ping Ping, Zhang Yun Meng, Song Yan Ru, Qi Wei Cong, Dr. Chen Xi, Cheng Ji Hua, Wang Yan and many more Chinese friends who introduced me to more different Chinese cuisines. Having the chance to eat and learn how to cook different Chinese cuisines of my ancestors were great experiences.

When I arrived in Wageningen, I was the only Malaysian student. Few days later, Nurulhuda Ramli came and then there were two of us. Nurulhuda, you are a great friend. Both of us were struggling to get used to the new study culture and environment. We managed to overcome our fears and performed well. Now, you also started your PhD in WUR, and I wish you all the best. A year later, many more Malaysian came to study in WUT. I would like to thank Shairul Izan Ramlee, Nurulhuda Khairudin, Hisfazilah Saari and many more Malay friends for the friendship we established. My special thanks are

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One cannot claim to have studied or lived abroad without integrating with the locals to learn and experience their culture. I wish to thank Arjan Wieringa, Bart Slager, Susan Laurier, Andries Middag, Maartje Middag, Bart van Tuijl, Marian Oortwijn and Gert Aarkel. You all gave me the chance to experience and understand the Dutch culture first hand. I really appreciate the efforts to bring me around the Netherlands and Europe. Travelling by car and bicycle are definitely far more breathtaking and in style than flying over layers of dull clouds. You all are welcome to Sarawak, Malaysia and having me as your personal tour guide.

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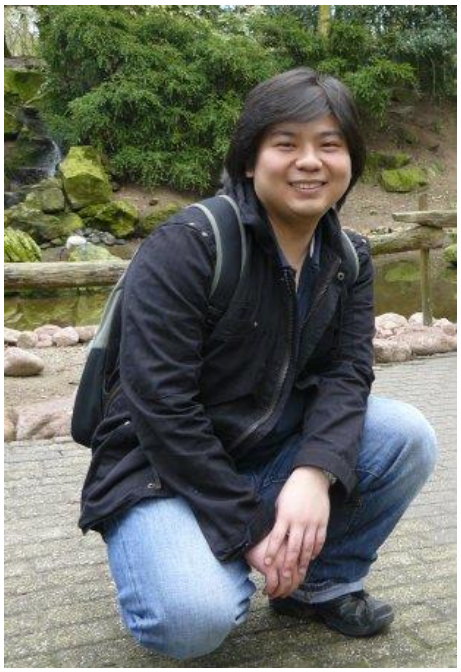
Thank You

“Let us be grateful to the people who make us happy; they are the charming gardeners who make our souls blossom” – Marcel Proust

About The Author



Freddy Yeo Kuok San was born on 2nd April, 1981 in Kuching, Sarawak, Malaysia and grew up in a small town called Serian. He obtained his Diploma in Agriculture from University Putra Malaysia in 2002.

Subsequently, Freddy continued his study in University Malaysia Sarawak (UNIMAS) and graduated in 2005 with a BSc. (Hons) in Plant Resource Science and Management. He was presented the Royal Education Award of UNIMAS. After graduated in 2005, he worked as a research assistant position in Institute of Health and Community Medicine. End of 2005, UNIMAS offered Freddy a Tutor position in the Faculty of Resource Science and Technology.

In August 2006, he was offered a scholarship by UNIMAS to further his study abroad. In September 2006, he started his MSc degree in Wageningen University and graduated in 2008 with an MSc, in Plant Sciences specialized in Plant Breeding and Genetic Resources. Then, in September 2008, he started his PhD research at the Department of Plant Breeding, Wageningen University. This thesis is the outcome of his PhD research. Freddy will join Faculty of Resource Science and Technology, UNIMAS after finishing his studies in Wageningen University.

List of Pulications

Yeo FKS, Wang C, Marcel TC, Lorriaux A & Niks RE Barley partial (host) and nonhost resistance QTL-near-isogenic lines against barley homologous and heterologous rusts. Proceedings of the 13th International Cereal Rusts and Powdery Mildews Conference 2012. Beijing, China

Yeo FKS, Hensel G, Vozábová T, Martin-Sanz A, Marcel TC, Kumlehn J, Niks RE (2014) Golden SusPtrit: a genetically well transformable barley line for studies on the resistance to rust fungi. Theoretical Applied Genetics 127:325-337

Yeo FKS, Bouchon R, Kuiken R, Loriaux A, Boyd C, Niks RE and Marcel TC. High resolution mapping of genes involved in plant stage specific, partial resistance of barley to leaf rust. To be submited

Yeo FKS, Martin-Sanz A, Wang C, Loriaux A, Marcel TC, and Niks RE. Specificity of barley near-nonhost and partial resistance QTLs towards adapted and non-adapted rust fungi. To be submited

Yeo FKS, Kohutova Z, Huneau C, Niks RE, Chalhoub B, Leroy P, Marcel TC. Non-Gridded BAC libraries of the barley cultivars Vada and line SusPtrit for physical mapping of *Rphq2*, a partial resistance QTL to *Puccinia hordei*. To be submited

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Freddy Yeo Kuok San
Date: 18 September 2014
Group: Plant Breeding, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project (higly recommended)**	
"Cloning and confirmation of barley sequences that determine basal resistance of barley to specialized *Puccinia* rust fungi"	Sep 16, 2008
► **Writing or rewriting a project proposal**	
"Cloning and confirmation of barley sequences that determine basal resistance of barley to specialized *Puccinia* rust fungi"	March 2009
► **Writing a review or book chapter**	
MSc courses	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD students day, Leiden University, Leiden, NL	Feb 26, 2009
2nd European Retreat for PhD Students in 'Experimental Plant Sciences', Cologne, Germany	Apr 15-17, 2010
EPS PhD students day, Utrecht University, Utrecht, NL	Jun 01, 2010
► **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 22, 2009
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15,2010
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 03, 2011
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 06-07, 2009
NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 19-20, 2010
NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 04-05, 2011
NWO - ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 02-03, 2012
Bioexploit meeting	Mar 31-Apr 01, 2009
Bioexploit final meeting	Feb 15-16, 2011
► **Seminars (series), workshops and symposia**	
Invited seminars (T. Nurnberger, 'Patterns and recetors in plant immunity'; Cyril Zipfel, 'Receptor kinase signalling in plant innate immunity')	2009
13th QTL-MAS Workshop	Apr 20-21, 2009
International Triticeae Mapping Initiative (ITMI) Summer Workshop, Clermont-Ferrand, FR	Aug 31-Sep 04, 2009
Invited seminar Kirsten Bomblies, 'Genetic incompatibility and the plant immune system'.	2010
International Triticeae Mapping Initiative (ITMI) Summer Workshop, Mexico City	Sep 05-09, 2011
SPICY symposium and workshops	Mar 07-09, 2012
► **Seminar plus**	
► **International symposia and congresses**	
12th International Cereal Rust and Powdery Mildew Conference (ICRPMC), Antalya, Turke)	Oct 13-16, 2009
13th International Cereal Rust and Powdery Mildew Conference (ICRPMC), Beijing, China)	Aug 28-Sep 01, 2012
► **Presentations**	
Oral: International Cereal Rust and Powdery Mildew Conference (ICRPMC)	Oct 13-16, 2009
Oral: EPS theme 2 symposium	Jan 15,2010
Poster: 2nd European Retreat for PhD Students	Apr 15-17, 2010
Poster: NWO - ALW meeting. Lunteren	Apr 19-20, 2010
Oral: 12th International Triticeae Mapping Initiative (ITMI) Summer Workshop.	Sep 05-09, 2011
Poster: NWO - ALW meeting. Lunteren	Apr 02-03, 2012
Oral: 13th International Cereal Rust and Powdery Mildew Conference (ICRPMC)	Aug 28-Sep 01, 2012
► **IAB interview**	
Meeting with a member of the International Advisory Board	Jan 14, 2011
► **Excursions**	
Subtotal Scientific Exposure	*20.0 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
PhD course 'Gateway to Gateway technology', Wageningen University, NL)	Nov 17-21, 2008
Postgraduate course 'Bioinformatics - A User's Approach'	Aug 30-Sep 03 , 2010
► **Journal club**	
Member of literature discussion group "Plant Breeding"	2008-2012
► **Individual research training**	
Subtotal In-Depth Studies	*5.7 credits**

4) Personal development	date
► **Skill training courses**	
Mobilising your Sceintific Network	Jun 03 & 10, 2010
Interpersonal communication for PhD Students	Oct 26-27, 2010
Techniques for writing and Presenting a Scientific Paper	Dec 06-09, 2011
Caeer Assessment	May 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**36.3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the

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

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