Effectiveness of QTLs for Resistance of Barley to Homologous and Heterologous Grass Rust Species

By

Allo Aman Dido

A Thesis Report Submitted to the Laboratory of Plant Breeding in Partial Fulfillment of the Requirement for the Degree of Master of Science in Plant Biotechnology

(Specialization Molecular Breeding and Pathology)

February 2010

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Supervision:

Dr. Ir. Rients E. Niks

Freddy Yeo Kuok San

Examiner: Prof. Dr. R. G. F. Visser

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I dedicate this work to my family, who dreamt of me getting a postgraduate profession and to instill into me, the value of hard work.

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IN THE NAME OF ALLAH (GOD), MOST GRACIOUS, MOST MERCIFUL

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Abbreviations

AFLPs	= Amplified Fragment Length Polymorphisms
AUPDC	= Area under the Disease Progress Curve
CAPS	= Cleaved amplified polymorphic sequence
DH	= Doubled Haploid
EA	= Early abortion
ff. spp	= Formae speciales
HR	= Hypersensitive response
IM	= Interval mapping
LOD	= Logarithm of Odds
MAS	= Marker Assisted Selection
MQM	= Multiple QTL mapping
NIL	= Near isogenic line
NP	= Non-penetrating
OARI	= Oromia Agricultural Research Institute
OWB	= Oregon Wolf Barley
PR	= Partial Resistance
PRI	= Plant Research International
QTL	= Quantitative trait loci
RFLPs	= Restriction Fragment Length Polymorphisms
RIF	= Relative Infection Frequency
RIL	= Recombinant inbred line
RLP50S	= Relative Latent Period in Seedlings
rMQM	= restricted Multiple QTL mapping
SARC	= Sinana Agricultural Research Center
SNP	= Single/Simple Nucleotide Polymorphisms
SSR	= Simple Sequence Repeats
WUR	= Wageningen University and Research

Summary

Many quantitative trait loci (QTLs) in different barley populations were discovered since 1998 for resistance to *P. hordei* and heterologous rust species. Work on PR and non-host resistance was continued and most effective QTLs were introgressed to SusPtrit genetic background. The objectives of current study were: first, to evaluate whether these larger-effect QTLs show specificity in their reaction to homologous and heterologous rust isolates; and second, to see whether these QTLs show plant growth stage dependency or not. In addition there were parallel works to develop more markers which will be used in fine mapping of *Rnhq* by using polymorphism between the QTL donors (Vada and L94) and SusPtrit and also to confirm the fine-mapping of *Rphq11* and *Rphq16* using homozygous recombinant lines.

Evaluation of specificity of larger-effect QTLs in their reaction to homologous rust (*P. hordei* isolate 1.2.1) showed that *Rphq2* on Su-*Rphq2* and L94-*Rphq2* had larger effect than *Rphq3* on Su-*Rphq3* and L94-*Rphq3*. In Vada-*rphq3*, the result was not as expected. *Rphq11* on Su-*Rphq11* also had significant effect than *Rphq16* on Su-*Rphq16* in seedling disease test. The effect of *Rphq3* is small on Su-*Rphq3* and L94-*Rphq3*. Nonhost QTL (*Rnhq*) also had effect on homologous rust. Su-*Rnhq-V* had larger effect than Su-*Rnhq-L* as compared to SusPtrit.

The histology of resistance study on fungal growth revealed that the QTL-NILs varied for infection units scored: non-stoma penetration (NP), early abortion (EA), established colonies and colony sizes ($P < 0.05$). Significant variations were observed between SusPtrit NILs and SusPtrit in EA and established infection units. The result illustrated that PR QTLs, *Rphq2, Rphq3, Rphq11* and *Rphq16* and the non-host resistance QTL, *Rnhq* (*Rnhq-V*), had an effects on both partial resistance towards homologous leaf rust, as well as non-host resistance towards heterologous leaf rusts, *P. triticina, P. hordei-murini* and *P. hordei-secalini*. Also, it was observed that positive association was found between parameter of RLP in seedling stage and proportion of EA abortion at infection sites, which indicated that there is a possible association between partial resistance QTLs and non-host resistance QTLs. Furthermore, high negative correlation between RLP and RIF was observed indicating that some of the genes for resistance to these rusts are either linked or have pleiotropic effects.

A disease test at different leaf layers (plant stages) of homologous rust showed that Those QTLs (*Rphq2* and *Rphq11*) which were effective at seedling stage were also effective across all plant stages with gradually decreasing effect with plant development as co as plants grew older. *Rphq3* which had consistent effect in all leaf layers confirmed the same result that it is a plant stage independent QTL.

For heterologous rusts, the effect of *Rnhq-V* was studied on three rust species; *P. hordei-murini (Phm), P. hordei-secalini (Phs)* and *P. triticina* isolate 'Flamingo' at three stages (leaf layers). Infection frequencies are higher at seedling stage and dramatically decrease as plants grow older in all three rust species tested on both SusPtrit and Su-*Rnhq-V*. The difference between lines tends to be reduced with higher leaf layer in all three tested inappropriate rust species. However, this would be not because of less effectiveness of the *Rnhq*.

As to the development of markers for *Rnhq,* three co-dominant CAPS markers were developed. According to the estimation of linkage map position by RECORD, only one marker (ABC01200-1) was mapped inside the *Rnhq* region while, the other two markers (ABC02539-4 and ABC03559-1) mapped outside the *Rnhq* region.

In the study of fine mapping of partial resistance QTLs, *Rphq11*and *Rphq16, Rphq11* was fine mapped into two genetic windows: between Uni19962 and WBE144 (0.1cM) and WBE130 and GBMS244 (0.4cM) interval between GBS0512 and GBMS244 flanking markers. On the other hand, *Rphq16* located between ABC11948_3 and TC181991_2 markers at 3.1cM size interval.

Chapter 1. General Introduction

1.0 Barley

Cultivated barley (*Hordeum vulgare* L.) belongs to the family Poaceae, in the *Triticeae* tribe (Von Bothmer *et al.* 1995). Barley is considered to be one of the founding species of modern agriculture. Cultivars are mainly classified on the basis of vernalization requirement (spring or winter type), spike morphology (six row, two row and some intermediate forms), end use (malting or feed) and presence of hull on seed (hulless or hulled) (Bard *et. al.* 2000). This hardy crop can grow in a wide variety of environments that include extremes of latitude and altitude where other crops are not adapted to (Harlan, 1976).

Barley provides an excellent system for genome mapping and genetic studies because of its diploid nature, low chromosome number ($2n = 2x = 14$) and availability of information on the genetics of host resistance, including partial and hypersensitive resistance, to *Puccinia hordei.* It can also be used as a model crop for the investigation of the genetics and mechanisms of resistance to inappropriate rust fungi (Zhang *et al.* 1994).

1.1 Barley leaf rust life cycle and infection process

1.1.1 Life cycle

Barley leaf rust, caused by the fungal pathogen *Puccinia hordei*, represents an important foliar disease occurring throughout the world. Yield losses up to 32% have been reported in susceptible cultivars. It is a macrocyclic, heteroecious rust pathogen with the dikaryotic stage limited in nature to *H. vulgare* and the sexual stage to *Ornithogalum* species (Anikster, 1982). The life cycle of *P. hordei* has been classified into five stages on the basis of production of different spore types. Among the five spore stages, the urediniospore stage is economically the most damaging. Each urediniospore has the potential to infect the same host plant or another host plant. Symptoms of infection are pale spots on the leaves, followed by the emergence of orange brown uredosori that contain fungal spores (Golegaonkar, 2007).

After completion of the host life cycle, alternate host and wild *Hordeum* spp. act as a "green bridge" for survival of urediniospores. The green bridge is important in the continuation of the vegetative life cycle of the pathogen where the sexual cycle does not exist (Anikster *et al.* 1976).

1.1.2 Infection process

Rust occurs on many species of plant, but in most cases any one species of rust can only infect one species of plant.

When a rust conidium (sometimes termed conidiospore, asexual, non-motile spore of a fungus) lands on a plant surface, a weak, hydrophobic interaction is formed between the spore and the cutin of the plant cell surface (Osherov and May, 2001). This is the process of spore attachment which prevents the spore from being washed off.

After attachment, the spore will germinate. The germination tube will elongate and try to locate the stoma through thigmotropism (is a movement in which an organism moves or grows in response to touch or contact stimuli). The germ tube grows in a random manner until it reaches a ridge between epidermal cells. At this point, it will start to grow perpendicular to the ridge, greatly increasing its chances of locating a stoma (Osherov and May, 2001).

Once the germ tube detects ridges that match the dimensions of the stomatal lips of its host species, appressorium will be formed. It is a structure that functions to both firmly anchor the fungus and aid in penetration. From the appressorium an infection peg grows down into the plant and between the mesophyll cells (Deising *et al.* 2000). Subsequently, haustorial mother cells (HMC) are formed near the mesophyll cell wall. Later on, a feeding structure called haustorium is formed. The plant cell membrane invaginates around the main haustorial body and the space between the two membranes is extra-haustorial matrix. Besides acting as a feeding structure, haustoria are also important for host-pathogen communication. The rust fungus will then continue to grow and invade the plant until it is ready for sporulation (Voegele and Mendgen, 2003).

1.2 Types of resistance

Resistance is defined as the ability of a host plant to reduce the growth and or development of a pathogen. In this report, attempts are made to describe some of the most commonly used classifications of host and nonhost resistances of cereal crops to rust diseases. In broad terms, resistance to rust pathogens can be classified based on growth stage (adult plant resistance versus seedling resistance), genetics (major genes versus minor genes) and durability (durable versus non durable).

Qualitative and quantitative (or partial) resistances are the two kinds of host resistance that exist against this pathogen. Qualitative resistance is expressed as a hypersensitive host response (HR) (chlorotic or necrotic spots). It is also called vertical resistance due to its race specificity. As reported by Robinson (1976), in vertical resistance there are single genes for resistance in the host plant (R-genes), and there are also single genes for parasitic ability in the parasite (Avr-genes). Hypersensitive resistance is an active defense mechanism which involves cell death surrounding the infection area. As a result, the development of pathogens will be arrested, and the infection will be controlled. It is governed by major genes designated as *Rph* (*Pa*) genes (Kicherer *et al.* 2000) which are race specific and function on a gene-for-gene basis (Parlevliet, 1976b; Parlevliet, 1983). The major findings of the genetic inheritance of traits (characters) by Mendel (1865), the genetic basis of resistance by Biffen (1905), physiological specialization in a rust pathogen by Stakman and Levine (1962), and the concept of gene-for-gene interaction by Flor (1956), have all enabled plant breeders to develop plant genotypes with major gene resistance. The ease with which major genes can be incorporated and the high level of protection conferred by them are the major reasons for the wide use of this approach. However, often cultivars with single resistance genes give temporary protection, lasting only until the occurrence of a new mutant pathotype, the increase of virulent pathotypes already present in the pathogen population, or the introduction of virulent pathotypes (Niks and Lindhout, 2006).

Quantitative resistance or partial resistance (PR) is expressed as a reduced epidemic built-up of the leaf rust pathogen and is controlled by few to many genes (Parlevliet and Van Ommeren, 1975). It is also known as non-hypersensitivity, horizontal, or host basal resistance. PR is race non-specific (Parlevliet, 1978). However, some researches (Qi *et al.* 1999; Marcel *et al.* 2007) reported that individual QTLs for PR are race specific and

may function on a minor-gene-for-minor gene model (Parlevliet and Zadoks, 1977). PR is durable compared to hypersensitivity resistance. Johnson (1984) stated the term "durable resistance" to refer to resistance that remained effective in a cultivar grown for many years in wide array of environments in the presence of the pathogen. This is due to the difficulty of the pathogen to adapt to multiple loci. It can be used in a breeding program to improve the level and durability of resistance of barley. Furthermore, QTLs for PR to leaf rust can easily be accumulated (Niks *et al.* 2000a).

The nonhost resistance of barley towards leaf rust is also known as nonhost basal resistance. It is the most durable, complete and common type of immunity of plants to potential pathogens which by definition occurs in all genotypes of a plant species to all genotypes of a pathogen species (Niks, 1987; Heath, 2000). None of the individuals of nonhost plant species allow any member of a potential pathogen species to successfully reproduce (Heath, 2000). Pathogens to which nonhost resistance is effective are called heterologous, inappropriate or nonhost pathogens (they are called "heterologous" in this report). This definition of nonhost resistance is not necessarily applicable in some occasions because some genotypes of nonhost species can be infected by some heterologous pathogens to a limited extent and under special circumstances (Niks, 1987). A few barley accessions, for example, are in the seedling stage somewhat susceptible to rust species like the wheat leaf rust fungus (*P. triticina*) and the wall barley leaf rust (*P. hordei-murini* (*Phm*)) (Niks, 1987; Zhang *et al.* 1994; Niks et al. 1996; Hoogkamp *et al.* 1998). For such phenomenon, near nonhost (intermediate or marginal host) status has been proposed when only few accessions are moderately susceptible to a normally heterologous pathogen (Niks, 1987). Because of its near-nonhost status for some heterologous rust species, *H. vulgare* L. is a useful model crop to study the genetics and the mechanisms of resistance against heterologous rust fungi such as *P. triticina* and *Phm* (Zhang *et al.* 1994; Jafary *et al.* 2006).

The mechanism of non-host resistance can be a combination of pre-haustorial (non-hypersensitive) and post-haustorial (hypersensitive) resistances (Hoogkamp *et al.* 1998). More is known about the mechanism of nonhost resistance than about its genetics. The genetics of near-nonhost resistance can be studied by crossing some rare genotypes showing moderate susceptibility to some resistant genotypes (Niks *et al.* 1996).

At the Department of Plant Breeding of Wageningen University, research on PR dates back to the early 1970s, when Parlevliet started his work on barley - barley leaf rust (Parlevliet, 1975). Since then, many aspects of PR have been determined by using field studies, molecular markers, QTL mapping, microscopy (histology) and comprehensive information has been collected about the genetics and mechanisms of PR in barley to leaf rust (*P. hordei*) (Parlevliet, 1975; Parlevliet and van Ommeren, 1975; Parlevliet, 1976a; Parlevliet, 1976b; Parlevliet and Kuiper, 1977; Niks, 1982; Niks and Kuiper, 1983; Qi *et al.* 1998; Qi *et al.* 1999; Qi et al. 2000; Niks *et al.* 2000b; Marcel et al. 2007). The histological observations on infected barley lines suggested that PR and nonhost resistance might be due to the same mechanism (Niks, 1983). A number of QTLs in different barley populations were discovered since 1998 for resistance to *P. hordei* (homologous rust) and heterologous rust species (Table 1.1 and Appendix 1.1) and were named *Rphq* (after Resistance to *Puccinia hordei* QTL) and *Rnhq* (after Resistance to non-host QTL), respectively.

Table 1.1. Summary of QTLs conferring partial resistance against *P. hordei* isolate 1.2.1 and heterologous rust species.

QTL	Population			Reference
	Name	Type	Line	
Rphq1 – Rphq6	L x V	RIL	103	Qi et al., 1998
Rphq2, Rphq3, Rphq4, Rphq7, Rphq8, Rphq9, Rphq10, Rphq20 and *Rphq21*	L x V	RIL	103	Qi et al., 1999
Rphq3, Rphq10,Rphq11, Rphq12 and *Rphq13*	L94 x 116-5	RIL	117	Qi et al., 2000
Rphq2, Rphq3, Rphq4 and *Rnhq*[a]	L x V	RIL	103	Niks et al. 2000a
Eight QTLs resistance to heterologous rusts[b]	Su × V	RIL	152	Jafary et al. 2006
Ten QTLs resistance to heterologous rusts[b]	Su × CC	RIL	113	Jafary et al. 2006
7 QTLs resistance to heterologous rusts[b]	OWB	DH	94	Jafary et al. 2006
Rphq12, Rphq16, Rphq17, Rphq18, and *Rphq19*	OWB	DH	94	Marcel et al., 2007
Rphq8, Rphq11, Rphq14 and *Rphq15*	S x M	DH	150	Marcel et al., 2007
Rphq1, Rphq2, Rphq3, Rphq4 Rphq7, Rphq17 Rphq20 and *Rphq21*	L x V	RIL	103	Marcel et al., 2008

[a] First QTL contributed resistance to heterologous rusts (*P. triticina* and *Phm*)
[b] QTLs contributed resistance to four heterologous rusts (*P. triticina, Phm, Phs* and *p. persistens)*

The mapping populations used in those studies showed that every mapping population had different sets of QTLs, showing hardly any overlap with other mapping populations. This indicated that QTLs for basal resistance are numerous and show a high level of specificity.

1.3 Description of QTL Parental lines

The QTLs that were studied in this thesis were mapped in different mapping populations (Table 1.1). L94 x Vada is a recombinant inbred lines (RIL) mapping population consisting of 103 lines. L94 is a line from an Ethiopian landrace, with black and naked seeds and is extremely susceptible to barley leaf rust (*P. hordei*). 'Vada' is an obsolete Dutch cultivar developed from the cross '*Hordeum laevigatum*' × 'Gold', with white, covered seeds, and has a high level of PR to *P. hordei* (Neervoort and Parlevliet, 1978).

The Steptoe x Morex mapping population is a double haploids (DH) population (Kleinhofs et al. 1993). Steptoe is six rowed feed-type barley (Muir and Nilan, 1973). Morex is a six-rowed cultivar used as standard in the American malting industry (Rasmusson and Wilcoxson, 1979).

The Oregon Wolfe Barley (OWB) population is a double-haploid spring barley mapping population derived from F_1 (Costa *et al.* 2001). The two parents are Dom (containing dominant morphological marker stock) and Rec (containing recessive morphological marker stock) (Wolfe, 1972).

1.4 Development of a research line susceptible to heterologous rusts

Earlier screens of barley accessions for susceptibility to *P. triticina* and *Phm* (Niks *et al.* 1996; Hoogkamp *et al.* 1998) allowed identification of several accessions that showed some degree of susceptibility to these rust fungi. Atienza *et al.* (2004) made crosses between barley accessions which exhibited relatively high number of pustules and/or high infection types when infected with *P.triticina and Phm*. Then they tested the F_2 lines for susceptibility to *P. triticina* and *Phm* at the seedling stage. They selected the most susceptible F_2 plants and grown to adult plant stage and crossed between the two crossing combinations to obtain double cross (DC) plants. Each DC plant was grown to develop DC-S_1 lines by selfing. The most susceptible plants within the most susceptible

DC-S_1 lines were selected and selfed for several cycles without selection. Later, they challenged susceptible DC-S_5 lines with *P. triticina* and *Phm*. The DC-S_5 line with the highest number of pustules per leaf and the highest infection type (IT) was selected and named SusPtrit (Sus = Susceptible, P = *Puccinia*, trit = *triticina*) and SusPmur (Sus = Susceptible, P = *Puccinia*, mur = hordei-*murini*), respectively (Atienza *et al.* 2004). SusPtrit is not only exceptionally susceptible to *P. triticina* and *Phm*, but is also susceptible to several other heterologous (inappropriate) rust fungi; however, it is fully resistant to several other grass rust fungi, such as the leaf rust fungus *P. recondita* of rye, suggesting that the nonhost defence mechanisms in SusPtrit are not totally weakened (Atienza *et al.* 2004). This line was as susceptible to *P.hordei* as line L94. SusPtrit was used as a recurrent parent in NILs development program for the QTLs of our interest. (i.e. PR QTLs - *Rphq2*, *Rphq3*, *Rphq11*and *Rphq16* and Nonhost resistance QTL- *Rnhq*).

1.5 Development of QTL-Near isogenic lines (NILs)

After the mapping of the QTLs by different group of researchers, the study was continued with the incorporation of the most consistent QTLs into SusPtrit (Atienza et al. 2004) to obtain Near Isogenic Lines (NILs) by marker assisted selection (MAS). These NILs allow a detailed study on the effect of each QTL of our interest under uniform genetic background.

The most promising and widely cited benefit of molecular markers in plant improvement is MAS (Masojc, 2002). Given the lengthy breeding cycle and considerable resources needed to incorporate disease resistance, molecular markers for specific traits can significantly improve the efficiency of resistance breeding.

In the backcross program, positive selection was performed to select plant(s) for further backcross to ensure the QTL of interest is introgressed into the receiver plant, SusPtrit. If QTL donor plants have additional QTLs other than the QTL of our interest, negative selection was performed to eliminate the additional QTLs from being introduced into the recipient genome. Selection for target QTLs and against additional QTLs was performed using two or more flanking markers as recommended by Visscher *et al.* (1996). The flanking markers used in our QTL-NILs development program are listed in Table 1.2.

After 5-6 backcross and successful introgression of QTL of interest and getting rid of the undesirable QTLs, the genetic background outside the introgressed region will not interfere or the interference will be small enough to not hamper the detection of the QTL by phenotyping.

Table 1.2. Flanking markers used for the marker assisted selection of NILs for five target QTLs (Yeo, 2008)

			Flanking markers					
QTL	**Chrom.**	**Position* (cM)**	**Proximal markers**			**Distal markers**		
			Name	**Type**	**Position (cM)**	**Name**	**Type**	**Position (cM)**
Rphq2	2H	152.0	P15M51	SCAR	152.0	GBMS216	SSR	153.5
Rphq3	6H	62.2	GBM1212	SSR	55.1	ABG388	CAPS	72.4
			WBE103	CAPS	61.3			
Rphq11	2H	95.1	Bmag0125	SSR	89.8	GBM1062	SSR	95.7
						GBMS244	SSR	102.7
Rphq16	5H	160.0	ABG391	CAPS	156.3	ABG390	CAPS	161.0
						GMS002	SSR	183.0
Rnhq	7H	84.1-85.8	SKT1	CAPS	85.8	GBM1303	SSR	86.6

* Peak marker position,

1.6 Organization of the report

This thesis presents three different preliminary studies on the association between host and nonhost resistance of barley.

Chapter 2: Unraveling possible association between QTL for partial resistance and nonhost resistance.

The first focus is on the effect of QTLs resistance to homologous and heterologous rust species. Seedlings of QTL-NILs were challenged with three heterologous and one homologous (host) rust species. We tried to evaluate whether QTLs of our interest, *Rphq2, Rphq3, Rphq11, Rphq16* and *Rnhq* may show specificity in their reaction to homologous and heterologous rust species. Also we evaluated the mechanism of resistance, especially whether the resistance is based on hypersensitivity or non-hypersensitivity. We studied the histology of infection of *Phm, P.hordei-secalini (Phs),* and *P. triticina* in each QTL-NIL. Various components of infection were evaluated, including non-penetrating, early abortion, the percentage of established colonies (with sporogenic and without sporogenic tissues) and colony size of infection units.

Chapter 3: Evaluation of plant stage dependency of QTLs to homologous and heterologous rust pathogen isolates

QTL-NILs were grown to have different developmental stages, from first leaf to flag leaf. This helped to determine the plant stage dependency of the QTLs of our interest.

Chapter 4: Marker development for *Rnhq*, a non-host resistance QTL

The main goal of this experiment is to develop new PCR based molecular markers using information from Potokina *et al.* (2008). The newly developed SCAR and CAPS markers mapped around *Rnhq* can be used in fine mapping of *Rnhq*.

Chapter 5: Fine mapping of the partial resistance QTLs, *Rphq11* and *Rphq16*

The objective of this study is to fine-map *Rphq11* and *Rphq16* using homozygous recombinant lines. The homozygous recombinant lines were challenged with *P. hordei* isolate 1.2.1. The phenotypic data together with genotypic data generated previously were used to pin point *Rphq11* and *Rphq16*.

Chapter 6: General discussion and summary

Findings about specificity and mechanisms of host and non-host immunity are discussed in this chapter. The possible role and nature of QTL for resistance to homologous and heterologous rust fungi as well as the future use of the QTL-NILs are explained.

Chapter 2. Unraveling possible association between QTL for partial resistance and nonhost resistance.

2.0 Introduction

Partial resistance is usually considered as a durable type of disease resistance, to which pathogens not easily adapt. Plants that are partially resistant can still be infected by the pathogen, but they show a much reduced rate of infection compared to susceptible plants, caused by a lower rate of colonization by the fungus (Parlevliet, 1975; Niks and Rubiales, 2002; Niks and Marcel, 2009). As QTLs can confer certain level of resistance by their accumulated effects, the adaptation of a pathogen to make each QTL resistance-allele ineffective is more difficult than to make just one gene ineffective in case of monogenic resistance (Lindhout, 2002), particularly when each QTL-gene encodes a different gene product.

Near-isogenic lines (NILs) differing with regard to disease QTLs provide valuable material for a more detailed study into the genetic basis of quantitative resistance. Development of such NILs allows the evaluation of a QTL in a nearly uniform genetic background, overcoming the difficulties of identifying QTL phenotypes (Marcel *et al.* 2007). QTL-NILs do not only provide a better estimate for the effect of single QTL alleles, but also provide a better insight into QTL x pathogen and QTL x environment interactions. Furthermore, QTL-NILs may provide a starting point for the unraveling of functional genes underlying these loci and possibly be useful for positional cloning (van Berloo *et al.* 2001).

This study uses the NILs developed for partial resistance QTLs *Rphq2, Rphq3, Rphq11,* and *Rphq16,* and a nonhost resistance QTL, *Rnhq,* to evaluate whether these relatively large-effect QTLs may show specificity in their reaction to homologous and heterologous rust isolates. Also, to evaluate the mechanism of resistance at tissue and cell level, especially whether the resistance is based on hypersensitivity or non-hypersensitivity (for those rusts of course to which the QTL is effective.

2.1 Materials and methods

2.1.1 Plant materials

NILs with SusPtrit genetic background (Table 2.1) and having resistance QTLs, *Rphq2*, *Rphq3*, *Rphq11*, *Rphq16* and *Rnhq* (*Rnhq-V* and *Rnhq-L*), were used for this study.

Table 2.1. QTL-NILs used in this study

QTL-NILs	Donor line
Rphq2-BC_5S_1	Vada
Rphq3-BC_6S_1	Vada
Rphq11-s.F_2*.*BC_5S_1	Steptoe
Rphq16-BC_6S_1	Dom
Qnh.L-F_2*.*BC_5S_1	L94
Qnh.V.F_2*.*BC_5S_1	Vada

The parental lines for each respective NILs were used as a reference. For *Rphq2-*BC_5S_1 and *Rphq3-*BC_6S_1 besides SusPtrit and Vada were used as reference, L94, L94-NILs (L94-*Rphq2* and -*Rphq3*) and Vada-NILs (Vada-*rphq2*, and -*rphq3*) were included as well. For the histology assays, host plants corresponding to the rust species under observation were added as a reference.

2.1.2 Inoculum

Four isolates of rust fungi were used (Table 2.2) in infection studies. These pathogens were multiplied on their respective host species as listed in Table 2. Urediniospores were collected and dried in desiccators for 5-7 days before used for inoculation.

Table 2.2. Rust isolates used in this study

Pathogens	Host plant	Common name
P. hordei isolate 1.2.1	*Hordeum vulgare*	Barley leaf rust
P. hordei-murini	*H. murinum*	Wall barley leaf rust
P. hordei-secalini	*H.secalinum*	Meadow barley leaf rust
P. triticina isolate “Flamingo”	*T.aestivum*	Wheat leaf rust

2.1.3 Genotyping QTL-NILs

Genotyping of each quantitative trait loci-near isogenic lines (QTL-NILs) was performed before sowing to confirm the introgression of donor resistant allele by using molecular markers flanking the QTL region (Table 2.3). For this purpose DNA of each QTL-NILs was isolated following the CTAB isolation method according to Wang *et al.* (1993).

Table 2.3. List of markers used in genotyping of QTL-NILs.

Name(s)	Chrom.	Type	°Tm	RE(s)	Linked QTL
besV76P5D5AR	2H	ASPCR	56	-	*Rphq2*
k00345	2H	CAPS	56	*Sdu I*	*Rphq2*
scP15M51-204	2H	SCAR	56		*Rphq2*
ABG388	6H	CAPS	58	*Nla III*	*Rphq3*
WBE201	6H	CAPS	58	*Mnl I*	*Rphq3*
GBM1212	6H	SSR			*Rphq3*
HVM14	6H	SSR			*Rphq3*
GBS0512	2H	CAPS	58	*Aci I - (Hpy99 I)*	*Rphq11*
TC134748	2H	CAPS	47	*ApoI = XapI*	*Rphq11*
GBM1062	2H	SSR			*Rphq11*
GBMS244	2H	SSR			*Rphq11*
DsT-33	5H	SCAR	45	-	*Rphq16*
Scsnp03275_2	5H	CAPS	65	*BglII*	*Rphq16*
GMS002	5H	SSR			*Rphq16*
MWG2031	1H	CAPS	55	*Mwo I*	*Rnhq*
SKT1	1H	CAPS	60	*Alu I*	*Rnhq*
WBE101	1H	CAPS	52	*HpyCH4 IV*	*Rnhq*
GBM1303	1H	SSR			*Rnhq*

2.1.4 Phenotyping QTL-NILs with homologous rust isolate

Seeds of QTLs-NILs were sown in 37 x 39cm boxes in two rows along with reference lines. Depending on the availability of seeds, 1-2 seeds were sown for each NIL. Once the primary leaves are fully grown (7-8 days) they were fixed horizontally with adaxial side up to obtain an equal spore distribution over the leaves. The secondary leaves (if any) were clipped. The inoculations were carried out with freshly collected spores of *P. hordei* isolate 1.2.1. A total of 3.5 mg of spores was used per box. The inoculum was diluted 10 times with lycopodium spores before dusted over the tray using the inoculation tower to obtain uniform spore distribution. On each box one object slide was placed for spore germination check. The inoculated boxes were placed in a humidity chamber to incubate the spores overnight (8 hours) at 100% relative humidity

in the dark at 18°C. After incubation, the inoculated boxes were transferred to a greenhouse compartment where the temperature is set at 14 ± 3°C with 30-70% relative humidity. The experiment was carried out in two replications. The latent period (LP) was measured one week after inoculation. The LP50S was then calculated with the following formula:

$$\mathbf{LP50 = T1 + (T2- T1) \times \frac{(N100-2-N1)}{(N2-N1)}}$$

T1 = the time just before 50% of the pustules are mature

T2 = the time just after 50% of the pustules are mature

N1 = number of mature pustules at T_1

N2 = number of mature pustules at T_2

N100/2 = half of the total mature pustules number

To normalize the results, the relative latency period (RLP50S) was calculated relative to the LP50S of SusPtrit which set at 100 (Parlevliet, 1975).

2.1.5 Phenotyping QTL-NILs with heterologous rust isolates

The QTL-NILs were grown as described above (Section 2.1.4) except that in this case susceptible host plants were included. Ten to twelve days after sowing, completely unfolded primary leaves were fixed horizontally with the adaxial side up, and inoculated with approximately 7-10mg of spores per box using a settling tower (Atienza *et al.* 2004). For each QTL-NIL and reference lines, two seedlings were inoculated of which one seedling was sampled for histological studies as described below. The second seedling was used for macroscopic phenotyping. After inoculation, the boxes were incubated as described in section 2.1.4. To avoid cross contamination of rusts, the settling tower and other tools were cleaned with 70% ethanol before and after use. LP was measured six days (*Phs*) and eight days (*Phm* and *P. triticina*) after inoculation. Additionally, the level of infection was quantified by estimating the following traits: infection frequency (IF, pustules/cm^2), flecks (F, non- sporulating infection sites/cm^2), frequency of visible infection sites (VIF, IF/total amount of visible infection sites/cm^2), and TotF (total amount of visible infection sites/cm^2) by using a metal frame with 1 cm^2 window to elucidate the existing difference among the lines. RLP50S and Relative Infection Frequency (RIF) were calculated by setting the RLP50S and RIF of SusPtrit to 100 as stated above. Analysis of variance for both RLP50S and RIF was carried out

using GenStat statistical software (11.1th edition). All genotypes were grown in one box and the experiment was carried out in two replications.

2.1.6 Histological evaluation of pathogenesis

Preparation of leaf samples for fluorescence Microscopy

Seven days after inoculation, the flecks start to be visible. Leaf segments of approximately 2-3cm long from the middle of primary leaves were collected from each plant-rust combination and put in separate tubes. Collected leaves were prepared as whole mount for fluorescence microscopy according to Rohringer *et al.* (1977), except that Uvitex 2B (Ciba-Geigy) was used instead of Calcofluor. Labels for each line were written on strip of paper using pencil to avoid washing away by water and alcohol during staining. The leaf segments were immediately fixed and bleached by boiling for 1.5 minutes in a water bath in lactophenol-ethanol (1:2 v/v). Some boiling stones were added to the water bath and one stone to each of the tube to prevent sudden eruptions of the contents of the tubes,. After the leaves were bleached, the lactophenol-ethanol was poured off and they were washed 1x 30 minutes in ethanol (50%) and in 0.05N NaOH (2g/l), respectively one after the other. The washed leaf segments were rinsed 3x in water and soaked for 30 minutes in 0.1 M Tris/HCl buffer (pH 8.5). After 5 minutes of staining in a solution of 0.1% Uvitex in the same buffer, they were rinsed thoroughly 4x in water and then washed for 30 minutes in a solution of 25% glycerol. Finally, to prepare the slides, small drops of glycerol were added on the slide before the leaf samples were put along the longitudinal axis of the slide and the leaves samples were embedded on slide with the adaxial side facing up. Then the slide cover was carefully placed on the samples (taking care that no air bubbles between cover glass and leaf sample).

Observation of infection units under UV-Microscope

For ease of inspection, different classes of infection units were set based on status of infection unit where an infection unit is described as non-penetrating (NP), early aborted (EA) and established (Niks, 1981, 1982, 1983). The established class was further classified into two classes based on the phase of colonies development that is, those with sporogenic tissues and without sporogenic tissues. The preparations (slides) were adjusted (screened) under UV-microscope with 10x10. The detailed observation

and scoring were done with a 10x10 and 40x10 magnification. The preparations were screened starting from one of the corners and moving horizontally along longitudinal axis of the leaves. The outmost stomatal rows were excluded from observation to avoid possible border effects. Also overlapping and infection points close to air bubble were ignored. The infection hyphae were scored as “established” type (more than six haustorial mother cells) or “early aborted” type (having six or less haustorial mother cells). Established infection sites were measured for their longest diameter using the eye-piece micrometer. A total of 50 or more infection sites per plant-rust combination were scored. Pictures were taken using a canon power shot A620 digital camera and Axiovision LE 4.6 software. The data collected were analyzed using GenStat statistical software (11.1th edition).

2.2 Results

The host plants were not included in the statistical analysis as their averages were much higher and adding them would make it impossible to distinguish differences between the parental lines.

2.2.1 Effect of PR and nonhost resistance QTLs towards homologous rust *P.hordei* isolate 1.2.1.

QTL-NILs with partial resistance QTLs and nonhost resistance QTLs had longer LP than on susceptible reference line, SusPtrit. As shown in Fig. 2.1, all SusPtrit NILs had higher RLP50S compared to reference line, SusPtrit.

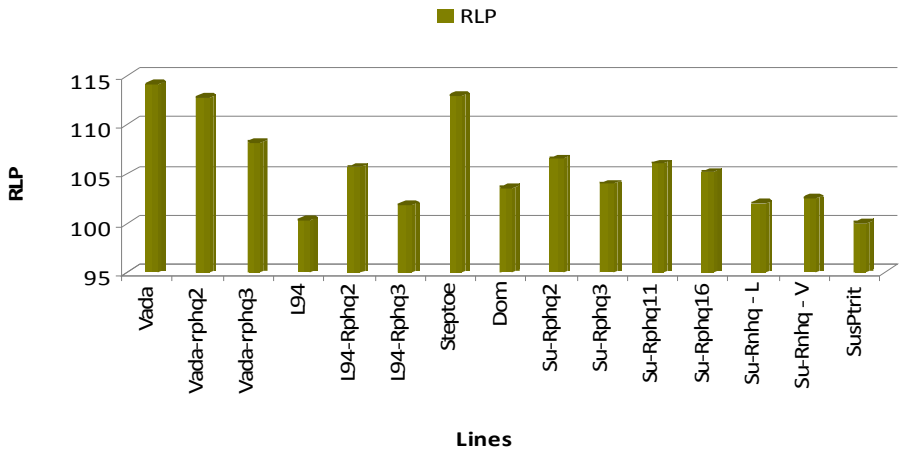

Fig. 2.1 RLP50S of *P. hordei* isolated 1.2.1 on SusPtrit NILs and the reference lines at seedling stage.

Rphq2 had an effect on Su-*Rphq2* and L94-*Rphq2*, which increased the RLP50S with 6.5% and 5.7%, respectively. However, the effect of *Rphq2* is not as expected in Vada-*rphq3*. This could be due to error in LP measurement.

Based on RLP50S, SusPtrit NIL with *Rphq3* also had longer LP than SusPtrit, indicating that, this QTL contributed to the effect on PR of Su-*Rphq3*. Furthermore, it has effect in resistance in Vada-*rphq2* and L94-*Rphq3*.

Other partial resistance QTLs, *Rphq11* and *Rphq16*, showed significantly ($P<0.05$) higher RLP50S on Su-Rphq11 and Su-Rphq16, respectively, as compared to SusPtrit.

On the other hand, *Rnhq* QTL on Su-*Rnhq-V* had relatively positive effect on resistance than that of Su-*Rnhq*-L. However, both Su-*Rnhq-V* and Su-*Rnhq*-L had 3% and 2% higher RLP50S than SusPtrit, indicating that *Rnhq* also had effect towards homologous rust.

2.2.2 Effect of partial resistance and nonhost resistance QTLs towards heterologous rust isolates

The parameters measured in macroscopic observation were RLP50S and RIF, while in microscopic observation; four infection statuses (non-penetrating, early aborted, established infection units and colony size) were measured. Measuring the LP of the heterologous rusts was difficult because on resistant lines not all flecks give rise to uredia and the presence of chlorosis covering infection sites. However, attempts were made to score carefully as not to increase experimental errors.

There were no sufficient infection (flacks) observed on Vada and Steptoe with all three tested rust fungi. However, very few numbers with long sporulation period was observed on Vada with *Phs*, while on Steptoe with all three rust species. There were typically large pustules which could be of *P. hordei* isolate 1.2.1 and they were excluded from counting.

The RIF of SusPtrit NILs with PR QTLs ranges from 38 % (Su-*Rphq2*) to 63% (Su-*Rphq16*) on seedlings infected by *P. triticina* (Fig. 2.2). On SusPtrit, the number of pustules is higher as compared to QTL-NILs, that means, on NILs small number of flecks were observed. SusPtrit -NILs (both from host and nonhost QTLs) had higher infection frequency for *P. triticina* compared to *Phs* and *Phm*. Su-*Rphq3* had lower RIF

for *Phs* and higher for *Phm* as compared to Su-*Rphq2* and Su-*Rphq11*. Su-*Rphq16* had about 40% RIF by both *Phs* and *Phm*.

The NILs with Su-*Rnhq-V* had lower infection frequencies than those with Su-*Rnhq*-L in case of infection with *Phm* and *Phs*, while they do have similar effect on *P. triticina* (Fig. 2.2).

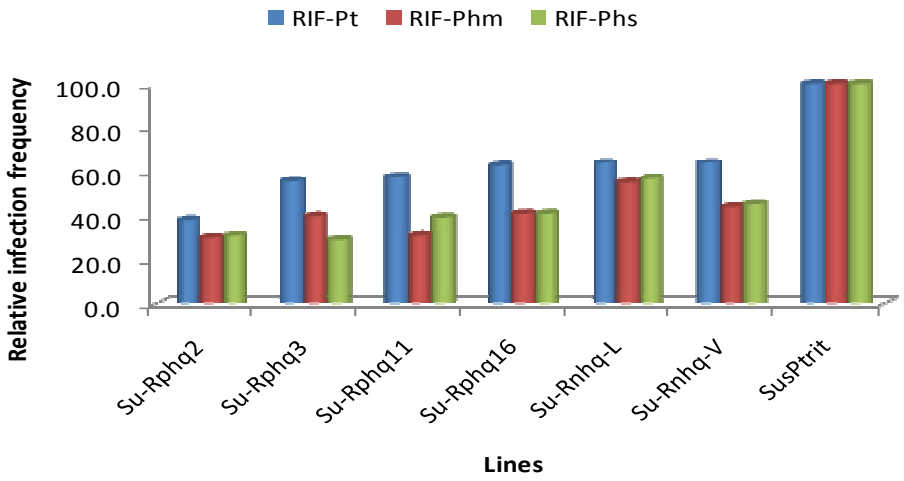

Fig.2.2 RIF of *P. triticina*, *Phm* and *Phs* on QTL-NILs and reference line at seedling stage.

As shown in Fig. 2.3, Su-*Rphq2*, *Su-Rphq3*, *Su-Rphq11* and *Su-Rphq16* had longer RLP50S on *P. triticina* relative to SusPtrit.

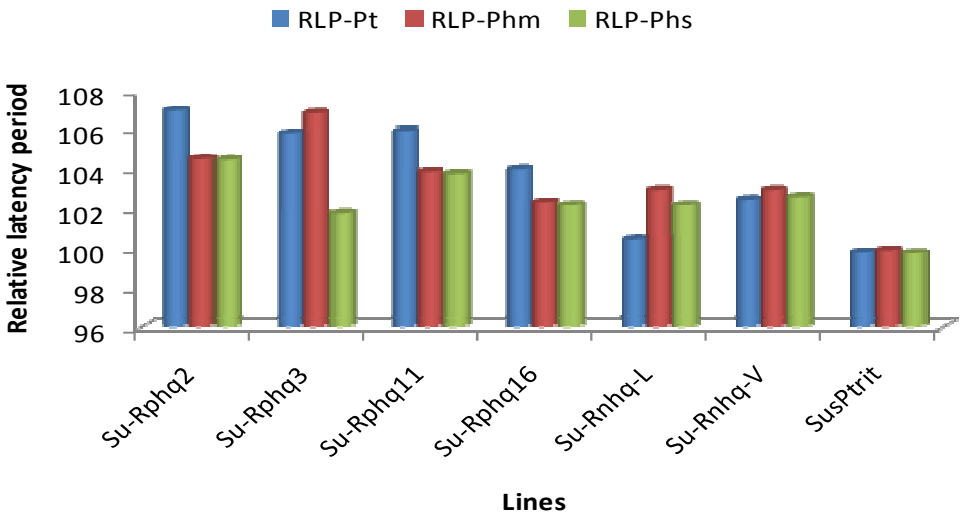

Fig. 2.3 RLP of *P. triticina*, *Phm* and *Phs* on SusPtrit NILs and reference line at seedling stage.

Rphq3 on Su-*Rphq3* had longer RLP on *Phm* as compared to compared to other PR QTLs. For *Phs*, *Rphq2* had higher RLP50S followed by *Rphq3* and *Rphq11* on Su-*Rphq3* and Su-*Rphq11*, respectively (Fig. 2.3). However, *Rphq3* on Su-*Rphq3* had lower RLP compared to other PR QTLs on *Phs*.

Nonhost resistance QTL, *Rnhq*, on Su-*Rnhq-V* and Su-*Rnhq-L*, showed an effect on RLP for infection three tested inappropriate rust species, with more effect from Su-*Rnhq-V*.

On the SusPtrit NILs it was observed that the percentage of non-penetrating (NP) infection units (this non-penetration of infection units was recognized by the absence of substomatal vesicle (SSV)) of *P. triticina* and *Phm* on average, were almost twice as high as in the susceptible line, SusPtrit (Fig. 2.4; Appendix 2.4 and Appendix 2.6). For *Phs* on the other hand, the percentage of non-penetrating infection units showed no significant differences. Higher percentage of NP was observed in Su-*Rphq3* for both *P. triticina*, for *Phm* and *Phs* higher values were in Su-*Rphq2*. In case of non-host SusPtrit NILs, Su-*Rnhq-V* had higher percentage of NP than that of Su-*Rnhq-L* for the three rusts species tested (Fig. 2.4; Appendix 2.4-2.6).

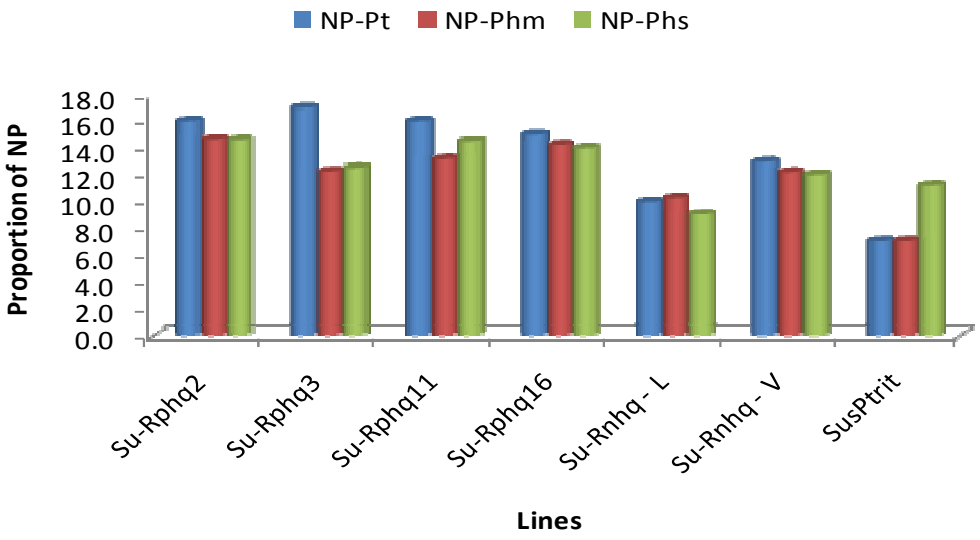

Fig. 2.4 Proportion of NP infection units (percentage of infection units which fail to enter stoma and make branch) of *P. triticina*, *Phm* and *Phs* on SusPtrit NILs and reference line at seedling stage.

In near isogenic lines with PR QTLs, the percentage of EA infection units ranged from 28% in Su-*Rphq16* to 35% in Su-*Rphq2* for *P. triticina*. However, the percentage of EA was higher in case of other two inappropriate rusts species (for *Phs* it ranges from 56% to 63%) and (for *Phm* ranges from 45% in Su-*Rphq16* to 56% in Su-*Rphq2* (Fig. 2.5; Appendix 2.4-2.6).

The EA infection units in NILs with non-host QTLs (*Rnhq*) were below 40%, except that Su-*Rnhq-V* on *Phs* had about 43%. Furthermore, Su-*Rnhq-V* had relatively higher EA than that Su-*Rnhq-L* in all three rusts species tested (Fig.2.5).

Large proportions of infection units of *Phs* were arrested before forming branches, though data not shown the same was observed for infection with *P. triticina* and *Phm*. In general, QTL-NILs had higher proportions of EA as compared to SusPtrit when observed through microscopy.

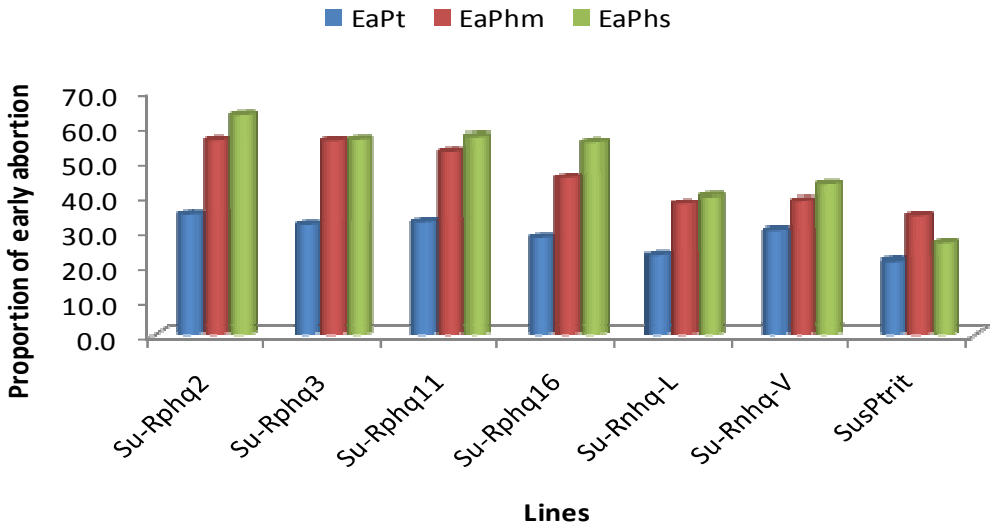

Fig. 2.5 Proportion of EA infection units of *P. triticina*, *Phm* and *Phs* on SusPtrit NILs and reference line at seedling stage.

The established colonies were classified according to their phase of development in to two categories, namely, those with sporogenic tissues (WST) and without sporogenic tissues (WOST). There is a significant variation between QTL-NILs and susceptible line, SusPtrit, in proportion of established infection units with and without sporogenic tissues at seven days after inoculation.

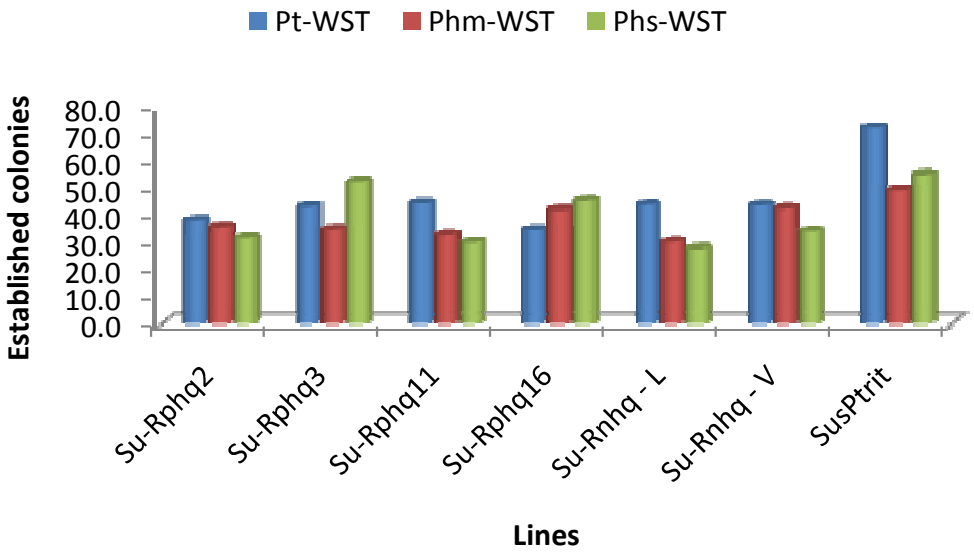

Fig. 2.6a Proportion of established infection units with sporogenic tissues (WST) of *P. triticina*, *Phm* and *Phs* on SusPtrit NILs and reference line at seedling stage.

In NILs with partial resistant QTLs, less than half of established colonies in SusPtrit were with sporogenic tissues in all tested rust species (Fig. 2.6a), while in case of that of established infection units without sporogenic tissues (Fig. 2.6b), the percentage is higher for *P. triticina* as compared to SusPtrit (Appendix 2.4; 2.5 and 2.6).

As shown in Fig. 2.6a and 2.6b, SusPtrit NILs had lower percentage of established infection units for all tested inappropriate rust species as compared to reference line, SusPtrit. From all tested NILs, lower percentages of established infection units (*P. triticina* 49.0%, *Phm* 29.5% and *Phs* 23.6%) were observed in NIL with *Rphq2*.

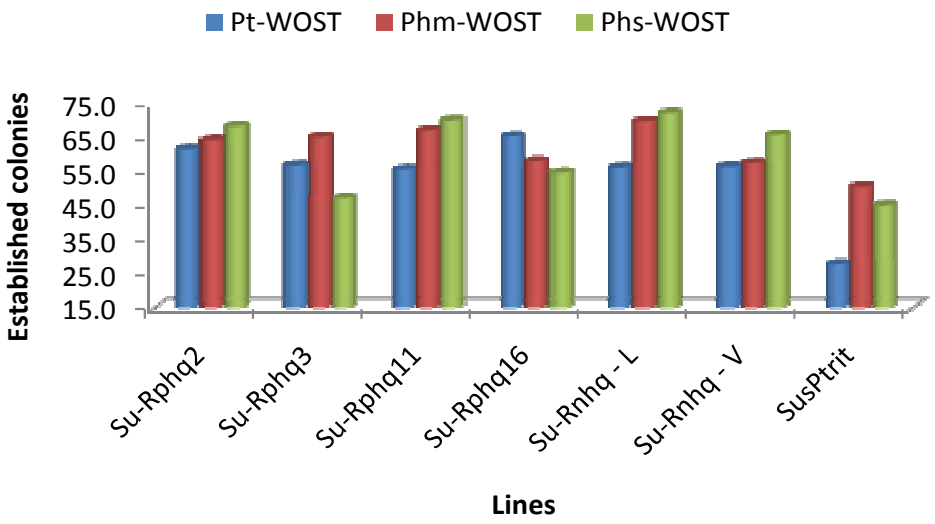

Fig. 2.6b Proportion of established infection units without sporogenic tissues of *P. triticina*, *Phm* and *Phs* on SusPtrit NILs and reference line at seedling stage.

In NILs with *Rnhq* (Su-*Rnhq-V* and Su-*Rnhq-L*), the proportions of established colonies with sporogenic tissues were lower than in SusPtrit infected with all three inappropriate rust species, while the percentage is higher than that of SusPtrit for established colonies without sporogenic tissues (Appendix 2.4; 2.5 and 2.6).

There was significant difference in colony size, both with and without sporogenic tissues between NILs containing PR QTLs and SusPtrit infected with *P. triticina*. However, the colony size difference between NILs and SusPtrit infected with *Phm* and *Phs*, was only for infection units with sporogenic tissue, while for infection units without sporogenic tissue the sizes were almost comparable with SusPtrit, but showed some more chlorosis (Fig. 2.7 and 2.8; Appendix 2.4; 2.5 and 2.6).

The colony size of infection units with sporogenic tissue on Su-*Rnhq-L* and Su-*Rnhq-V* infected with all tested rust species are relatively lower than SusPtrit, but higher than PR QTL containing NILs (Fig. 2.7). For infection units without sporogenic tissues, there is significant difference for *P. triticina*, while the difference is negligible for *Phm* and *Phs* (Fig. 2.8). This difference indicates variation in the resistance of nonhost reaction to inappropriate rusts among them.

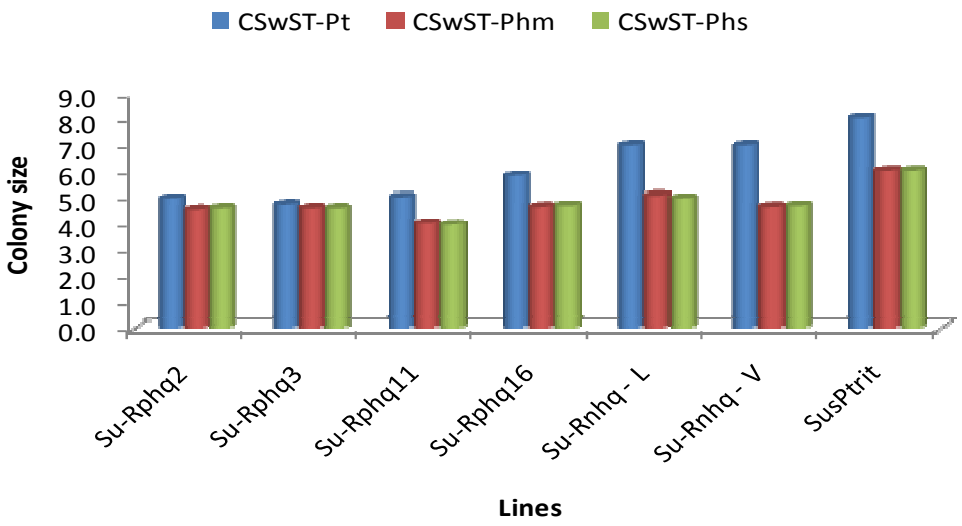

Fig. 2.7 Colony size of infection units with sporogenic tissues of *P. triticina*, *Phm* and *Phs* on QTL-NILs and reference line at seedling stage.

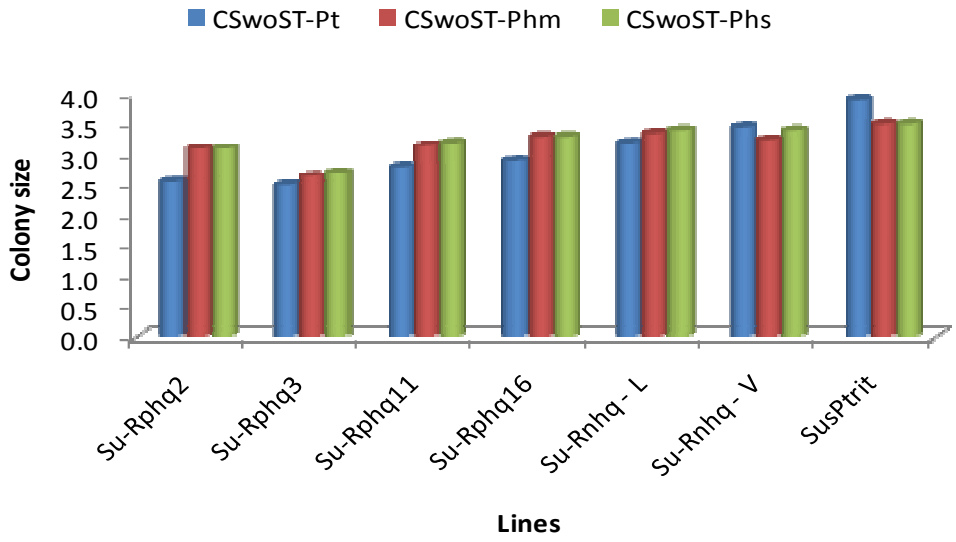

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Fig. 2.8 Colony size of infection units without sporogenic tissues of *P. triticina*, *Phm* and *Phs* on QTL-NILs and reference lines at seedling stage.

2.2.3 Correlations between measured components of resistances for tested rusts

The association in prehaustorial resistance was quantified by calculating correlation coefficients (*r*) among the infection parameters measured for partial resistance and nonhost resistance to tested rust species. In the case of association, a negative correlation would be expected between RLP to *P. hordei* and FVIS of other rust fungi.

As a result, higher negative correlation was observed between relative latency period (RLP) of *P. hordei* isolate 1.2.1 and relative infection frequency (RIF) of *P. triticina, Phm* and *Phs,* indicating that some of the genes for resistance to these rusts are either linked or have pleiotropic (the genetic effect of a single gene on multiple phenotypic traits) effects. The higher negative correlation was also observed between parameters measured from those tested inappropriate rust fungi (Table 2.4).

Table 2.4 Correlation coefficients (*r*) among infection parameters measured from four rust fungi.

Parameters	RLP *Ph*	RIF*Pt*	EA*Pt*	RIF*Phs*	EA*Phs*	RIF*Phm*	EA*Phm*
RLP *P. hordei* isolate 1.2.1	1.00						
RIF *P. triticina* 'Flamingo'	-0.81	1.00					
EA *P. triticina* 'Flamingo'	0.85	-0.84	1.00				
RIF *P. hordei-secalini*	-0.87	0.93	-0.86	1.00			
EA *P. hordei-secalini*	0.94	-0.91	0.90	-0.93	1.00		
RIF *P. hordei-murini*	-0.92	0.94	-0.86	0.98	-0.92	1.00	
EA *P. hordei-murini*	0.81	-0.79	0.87	-0.80	0.91	-0.76	1.00

Note: RLP = relative latency period, RIF = relative infection frequency, EA = percentage of early abortion

Furthermore, the higher positive correlation (r = 0.81 to 0.94) was also observed between RLP of *P. hordei* isolate 1.2.1 and EA of *P. triticina* isolate 'Flamingo', *Phm* and *Phs* (Table 2.4). Correlation between RIF of *P. triticina* isolate "Flamingo" and RIF of *Phm* and *Phs* was also higher. In general, higher positive association was observed between different parameters measured (RLP, RIF, and EA), indicating that there is a possible association between partial resistance quantitative trait loci and nonhost resistance quantitative trait loci.

2.3 Discussion

The present work was aimed to investigate the specificity of the three effective PR QTLs and a nonhost resistance QTL toward homologous and heterologous rust isolates. For this purpose, QTL-NILs seedlings with SusPtrit genetic background was challenged with *P. hordei* isolate 1.2.1, *P.hordei-secalini*, *P. hordei-murini* and *P. triticina* isolate 'Flamingo'.

Against *P. hordei* isolate 1.2.1, *Rphq2*, *Rphq3, Rphq11* and *Rphq16* significantly increase the LP observed in Su-*Rphq2*, Su-*Rphq3,* Su-*Rphq11* and Su-*Rphq16* as compared to SusPtrit. The effect of *Rphq3* in the seedling stage was smaller than that of the other three PR QTLs (Fig. 2.1). This is in agreement with the previous studies (Qi *et al.* 1998; Qi *et al.* 1999; Marcel *et al.* 2007). On the other hand, the effects of non-host QTLs (both on Su-*Rnhq-L* and Su-*Rnhq-V*) were not statistically significant. However, they have positive effect on resistance towards *P. hordei*.

The macroscopic observation revealed that all SusPtrit NILs had lower RIF and longer LP as compared to SusPtrit. Of these NILs, lower RIF were observed in lines with *Rphq2* (for *P. triticina, Phm* and *Phm*), *Rphq3* (for *Phs*) and *Rphq11* (for *Phm*). Nonhost resistance QTLs, showed nonhost resistance on Su-*Rnhq-V, having* 45% RLF for both *Phm* and *Phs,* while the percentage was the same with Su-*Rnhq-L* for *P. triticina*.

Concerning, microscopic observations, the distribution of infection units type based on counting of $\geq$50 infection units per plant revealed differences between QTL-NILs and SusPtrit for *P. triticina, Phs,* and *Phm*. These differences could be in proportion of non-penetration, EA, formation of sporogenic tissue and colony sizes. The proportion of NP for both PR QTL NILs and nonhost QTL NILs was significantly ($P < 0.05$) higher than SusPtrit's for *P. triticina* and *Phm.* For *Phs,* the effect did not show significantly higher values. The highest average value of NP was observed in SusPtrit-NILs containing *Rphq2* (Su-*Rphq2*) for both *Phm* and *Phs,* however, for *P. triticina* it was observed in NILs containing *Rphq3* (Su-*Rphq3*).

Of the four infection unit classes studied in this experiment, it seems that EA and established infection units appeared to be the parameters with the largest contrasts

between lines to see the existing variation among the NILs containing PR QTLs (*Rphq2*, *Rphq3*, *Rphq11*, *Rphq16*), non-host QTL, (*Rnhq*-V and *Rnhq*-L) and susceptible line, SusPtrit. Thus, higher proportions of EA were observed on NILs containing *Rphq2* QTL except in Vada-*rphq3*, for all inappropriate rust species. The occurrence of large proportions of EA combined with host cell necrosis has been reported as a typical feature of nonhost reaction (Niks, 1982). On non-host plant, infection units are arrested between formation of haustorial mother cell (HMC) and first haustoria often accompanied with limited cell collapse (Niks, 1982). In his early abortion study, Niks (1982) reported that the EA in partially resistant barley seedling resembles the nonhost reaction. In the present result, particularly on Sus-*Rphq2* and Sus-*Rphq11*, EA infection units agree with this fact (Appendix 2.4; 2.5 and 2.6).

Significantly larger proportions of established colonies with sporogenic tissues were observed in SusPtrit as compared to SusPtrit NILs with PR and non-host QTLs (Fig. 2.6a). Microscopical observations made in this study on the mechanism of non-host resistance confirm that for a large part of the resistance is pre-haustorial. Large proportions of colonies were arrested before HMC formation. Even, when the colonies were established, they were smaller in size in partial resistance QTL-NILs as compared to SusPtrit (Fig. 2.7 and 2.8).

The possible association between partial resistance and non-host resistance, the correlation of the effects between partial resistance QTLs and non-host resistance QTL towards homologous and heterologous rusts was analyzed. There was high negative correlation between RLP of *p. hordei* and RIF of *P. triticina*, *Phm* and *Phs*. Also, high positive correlation between RLP of *P. hordei* and EA of *P. triticina*, *Phm* and *Phs* (Table 2.4) was observed. This suggested that genes have dual effectiveness to both rusts, or close linkage of genes showing that there is an association in genetics of resistance to *Phm* and *Phs*. As stated in Hoogkamp *et al.* (1998), Niks *et al.* (2000) and Jafary *et al.* (2006), this significant association between parameters for PR to *P. hordei* and the loci for resistance to inappropriate heterologous rusts revealed that these two traits are associated with each other.

In general, the result of current study illustrated that partial resistance QTLs, *Rphq2*, *Rphq3, Rphq11 and Rphq16* and the non-host resistance QTL, *Rnhq,* in barley, have

an effects on both partial resistance towards homologous leaf rust, as well as non-host resistance towards heterologous leaf rusts, *P. hordei-murini* and *P.hordei-secalini*. Furthermore, higher positive correlation was observed between parameter of relative latent period in seedling stage and proportion of early abortion at infection sites, which indicated that there is a possible association between partial resistance QTLs and non-host resistance QTLs. Higher negative correlation was also observed between RLP of *P. hordei* isolate 1.2.1 and RIF of *P. triticina, Phm* and *Phs*, indicating that some of the genes for resistance to these rusts are either linked or have pleiotropic effects.

Chapter 3. Evaluation of plant stage dependency of QTLs to homologous and heterologous rust pathogen isolates

3.0 Introduction

The responses of different barley genotypes to *P. hordei* were assessed in greenhouse tests at seedling growth stages and in the field at adult plant growth stages (Parlevliet, 1975; Parlevliet and Kulievit, 1986; Marcel *et al.* 2008; Yeo, 2008). For these the long-time standard barley leaf rust *P. hordei* isolate 1.2.1 was used to evaluate the level of partial resistance. Partial resistance is believed to be one of the components of basal resistance, which provides the first line of defence against adapted and unadapted microbial intruders.

The effect of *Rphq2*, *Rphq11* and *Rphq16* are plant stage dependent (Marcel *et al.* 2007), that they are effective only at seedling stage. *Rphq3* on the other hand has a strong and consistent effect at both seedling and adult plant stages. *Rnhq* is a QTL for nonhost resistance, and was effective to *Phm* and *Phs* at seedling stage (Jafary *et al.* 2006). The objective of this research is to see whether these QTLs show plant growth stage dependency by using their NILs.

3.1 Materials and methods

3.1.1 Plant materials

We used the same QTL-NILs with SusPtrit genetic background for resistance QTLs were used in Chapter 2 for this study, except for Su-*Rphq16*. The QTLs-NILs seeds were sown together with their respective reference lines. The sowing was done two times a week to ensure sufficient plants of each QTLs-NIL and reference lines at the required stage and was extended for eight weeks to have different plant stages. For each QTL-NIL and reference lines, 2 and 3 seeds, respectively were sown in a pot of 14cm diameter. At the 8th week, the seeds were sown in boxes (39cm x 37cm) as they were used at seedling stage (Chapter 2) for this experiment. The plants were raised in the greenhouse compartments in three replications.

3.1.2 Inoculum

Eight different stages of plants for each partial resistance QTL-NIL were inoculated with barley leaf rust *P. hordei,* isolate 1.2.1. For Su-*Rnhq,* only three different growth stages were tested *P. triticina, Phm* and *Phs*; plants with first leaves, second leaves and third leaves because the adult plants are resistant to these inappropriate rusts. For inoculation of those plants at each stage, 1mg (*P. hordei* 1.2.1) and 2mg (heterologous rusts) of spores diluted 10 times with lycopodium spores was used as inoculum for each pot. Then, the inoculum was sprayed over the plants as uniformly as possible. These inoculations were not performed in the settling tower. For those plant materials at seedling stage, they were inoculated as described in Chapter 2. The plants were then placed in a humidity chamber overnight (8 hours) at 100% relative humidity in the dark at 18ºC to allow the spores to germinate. After incubation, the plants were transferred to a greenhouse compartment where the temperature was set at 14 ± 3C^{o} with 30-70% relative humidity.

3.1.3 Data collection and analysis

Five to eight days after inoculation, when the infection flecks appear, observation zones containing proper density of flecks were delimited by marker. The observations started when the susceptible line showed the first mature pustules. The latency period (LP50) of three to five plants per QTL-NILs and two to three plants per parental line per stage in three replications and averages were considered to reflect the level of partial resistance for each QTL-NILs and donor lines. For all lines two leaves per pot per plant stages were scored.

For heterologous rusts, the frequency of visible infection sites (VIS; the number of both flecks and pustules per cm^2) and infection frequency (IF; the number of pustules per cm^2) following Jafary *et al.* (2006) were evaluated as described for seedling disease test in Chapter 2. Also, the latency period (LP) of the fungi on each plant was evaluated.

3.2 Result

3.2.1 Plant stage dependency of partial resistant QTLs

All lines tested show an increase in LP from the primary leaf up to the flag leaf. However, the LP of susceptible check, SusPtrit, was lower than the QTL-NILs and other parental lines which carry resistance genes (Appendix 3.1 and 3.2).

LPs for SusPtrit were the shortest, averaging from 192 to 200 hours from first leaf to forth leaf (Appendix 3.1). Compared with SusPtrit, LP differences were slightly larger for QTL-NILs (201 to 240hrs), Vada NILs (218 to 258 hrs) and L94-NILs (205 to 248 hrs) across all leaf layers (growth stages) (Appendix 3.1).

Rphq2: From first leaf to third leaf layers, *Rphq2* has longer RLP than *Rphq3* and up to second leaf layers compared to *Rphq11* on NILs with SusPtrit genetic background (Fig.3.3). Its effect starts to gradually decrease from second and fourth leaf layer onwards on L94-*Rphq2* and Sus-*Rphq2*. In general, the effect of *Rphq2* in Sus-*Rphq2* and L94-*Rphq2* is not significant above forth leaf layers (Fig 3.1- 3.3; Appendix 3.1). On the other hand, in Vada background NIL with *rphq3,* longer LP was observed after six leaf layer due to the presence of *Rphq2*. Its effect was lower than *Rphq3* from first, second and forth leaf layers (Fig. 3.1).

Rphq3: The effect of this QTL is gradually increased after third leaf stage showing consistent effect in all developmental stages (Fig. 3.1-3.3), except in Vada-*rphq2*. The effect of *Rphq3* was lower than *Rphq2* on L94-*Rphq2* from first to second leaf layers (Fig. 3.2). It is also, lower than *Rphq2* and *Rphq11* from first leaf to third leaf layers on Su-*Rphq2* and Su-*Rphq11*, respectively (Fig. 3.3). On the sixth leaf layer, its effect becomes equal to *Rphq2* and *Rphq11* on Su-*Rphq2*, and Su-*Rphq11*, respectively. However, its effect increases afterwards (Fig 3.3). In Vada-NILs with *rphq2*, the effect of *Rphq3* was higher than that with *rphq3* up to forth leaf layers though it has lower effect at third and sixth leaf layer afterwards (Fig. 3.1).

Rphq11: The effect of *Rphq11* on Su-*Rphq11* was higher than Su-*Rphq3* from first leaf to third leaf layers (Fig. 3.3). As plants grow higher, the RLP of Su-*Rphq11* increased as in other QTLs-NILs.

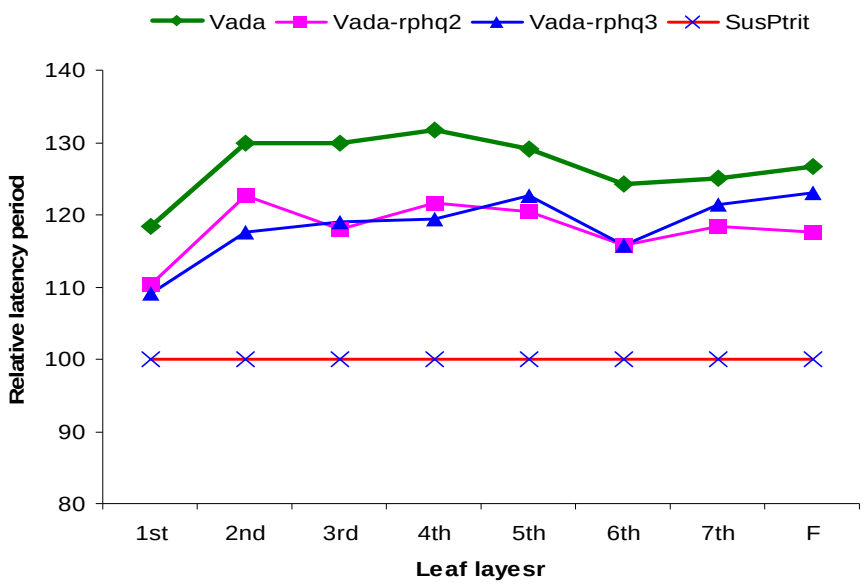


Fig. 3.1 RLP50 of Vada and Vada-NILs relative to SusPtrit infected with *P. hordei*.

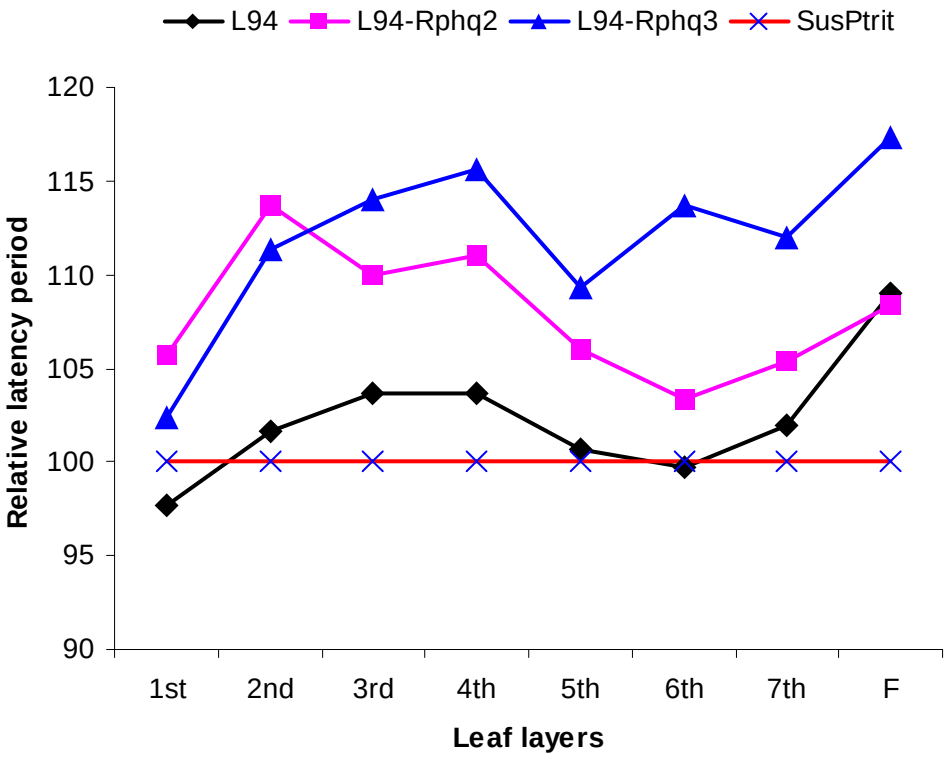


Fig. 3.2 RLP50 of L94 and L94-NILs relative to SusPtrit infected with *P. hordei*.

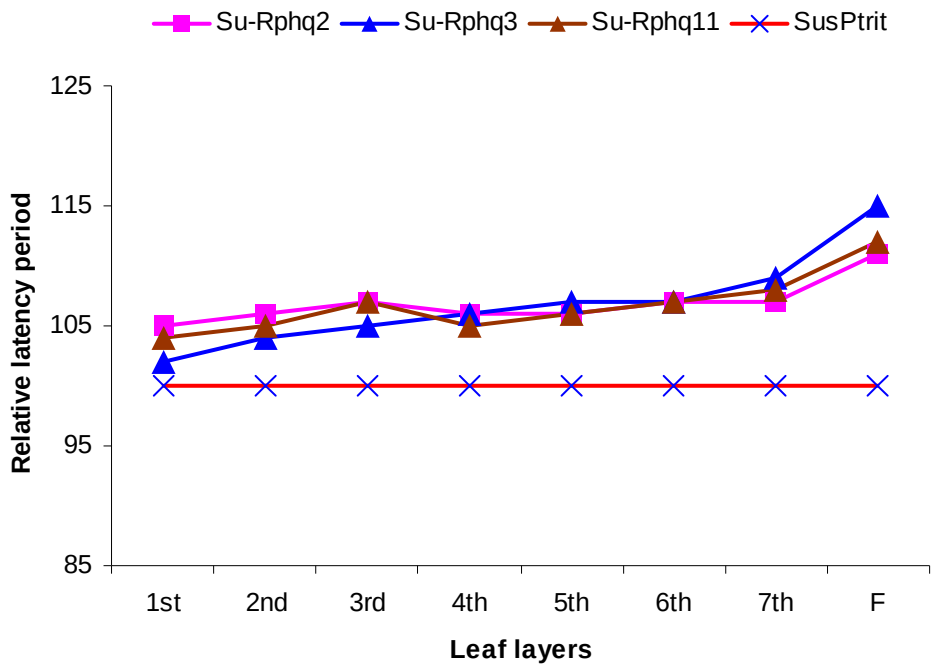


Fig. 3.3 RLP50 of Su-*Rphq2,* Su-*Rphq3* and Su-*Rphq11* relative to SusPtrit infected with *P. hordei*.

3.2.2 Plant stage dependency of nonhost resistance QTLs (*Rnhq-V*)

Effects of Su-*Rnhq-V* to the three inappropriate rust fungi were assessed by determining the relative latency period (RLP) and number of macroscopically visible infection sites.

The level of infection established by inappropriate rusts rage from immune (no pustules and less than three flecks per cm^2) to susceptible. Infection frequencies are higher at seedling stage and dramatically decrease as plants grow higher in all three rust species tested (Table 3.1). The uredia size of infected leaves was small and had chlorosis on Su-*Rnhq-V* NILs at seedling stage as compared to that of SusPtrit.

As shown in Table 3.1, for infection with *P. triticina,* effect of *Rnhq-V* showed significant effect on Su-Rnhq-V only at second leaf stage. In case of *Phm* at first leaf stage, the effect is not significant; however there was positive effect at second leaf stage. It had positive effect on RLP at first and second leaf stage for *Phs*. However, the effect seems to decrease at second and third leaf stages. In general, the effect of *Rnhq-V* on Su-*Rnhq-V* on RLP tends to decrease with increment in leaf layer in all

three tested inappropriate rust species. Parental line 'Vada' showed no sporulating uredia on both *P. triticina* and *Phm* except very few on *Phs*, as a result RLP was not scored for this line on those two rust species (Table 3.2).

Table 3.1. RIF of Su-*Rnhq*-V, SusPtrit, and L94 infected with *Pt, Phm* and *Phs*.

Lines	***P. Triticina* (Flamingo)**			***P. hordei-murini***			***P. hordei-secalini***		
	1	2	3	1	2	3	1	2	3
Su-Rnhq-V	66.3	38.0	33.0	37.5	22.0	18.0	39.7	33.7	25.0
Vada	2.1	-	-	-	-	-	6.0	2.0	-
L-94	73.1	47.0	39.5	63.0	57.0	53.0	77.3	69.0	63.0
SusPtrit	100.	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 3.2. RLP50 of Su-*Rnhq*-V, SusPtrit, and L94 infected with *Pt, Phm* and *Phs*.

Lines	***P. Triticina* (Flamingo)**			***P. hordei-murini***			***P. hordei-secalini***		
	1	2	3	1	2	3	1	2	3
Su-Rnhq-V	101	105	103	102	104	103	104	103	103
Vada	-	-	-	-	-	-	117	122	-
L-94	103	105	108	103	105	109	101	103	107
SusPtrit	100	100	100	100	100	100	100	100	100

3.3 Discussion

Adequate standardization of plant age, inoculum density and quality, and environmental conditions is required to recognize true differences in susceptibility. In this research, the environmental conditions during plant growth prior to inoculation, during exposure of inoculated plants to dew, and during post-dew development were sufficiently defined and controlled to provide an acceptable level of variation in disease development attributable solely to environmental factors.

The influence of plant development on disease resistance is a crucial break in our understanding of plant–pathogen interactions. Plants are generally more susceptible to disease in early than in late phases (Develey-Rivière and Galiana, 2007). This may reflect an increase in resistance over time, with plants already resistant to a pathogen

increasing their ability to control infection and colonization at a precise growth phase. Alternatively, a host plant susceptible to a virulent pathogen at early stages of growth may acquire disease resistance during its development.

Near-isogenic lines (NILs) differing with regard to disease resistance QTLs provide valuable material for a more detailed study into the genetic and molecular dissection of the mechanisms underlying the emergence of disease resistance during host development. Such NILs allows the evaluation of QTL in a nearly uniform genetic background, overcoming the difficulties of identifying QTL phenotypes (Marcel *et al.* 2007). QTL-NILs do not only provide a better estimate for the effect of single QTL alleles, but also provide a better insight into QTL x pathogen and QTL x environment interactions.

In this study, three most effective QTL-NILs with SusPtrit background, *Rphq2*, *Rphq3,* and *Rphq11* contributed in resistance to homologous rust were used to evaluate the effect of each QTL at different plant development stages.

As shown in Fig. 3.3, *Rphq2* on Su-*Rphq2* had higher effect from first to third leaf layers as compared to *Rphq3* on Su-*Rphq3*. Its effect starts to relatively decrease after fourth leaf layers in SusPtrit background NILs. *Rphq2* is effective at seedling stage and gradually lose its effect as plant grows higher. The effect of *Rphq3* on Su-*Rphq3* consistently increases from first leaf layer to adult plant stage. This consistency in effect indicates that this QTL is stage independent. On the other hand, *Rphq11* on Su-*Rphq11* had no as such statistically significant difference from *Rphq2* and *Rphq3 o*n Su-*Rphq2* and *Su-Rphq3,* respectively. It tended to have an effect intermediate between *Rphq2* and *Rphq3* (Fig. 3.3).

In previous studies (Niks *et al.* 2000a; Marcel *et al.* 2008) it was reported that *Rphq2* had a strong effect in the seedling stage but almost no effect in adult plant stage, while *Rphq3* was effective in seedling and adult plant stages indicating that *Rphq3* is plant stage independent. Also it was reported (Yeo, 2008) that, *Rphq11* was effective in seedling stage. However, in present study, it was observed that those QTLs which were effective at seedling stage were also effective across all plant stages with gradually decreasing effects as plants grew older. This could be because, previously, these QTLs were reported to be plant stage dependent because they were mapped at

seedlings but not at adult stage. Here we used NILs where only the QTL of interest is in the plant material. So, this may indicates that the QTL which was reported to be plant stage dependent may not be as reported when they are evaluated individually in a NIL due to the fact that QTLs do function throughout the plant stage but its effect is smaller than other detected QTLs in a mapping population which may suppressed its detection using MapQTL. However, the effect of *Rphq3* is consistent in all leaf layers observed, indicating that this QTL is plant stage independent as reported in previous studies. As far as *Rnhq* is concerned, Su-*Rnhq*-*V* had positive effect in resistance at first leaf layer; however, its effect seems to decrease as plants grew older.

Chapter 4. Marker development for *Rnhq*, a non-host resistance QTL

4.0 Introduction

Molecular markers are applied extensively in fundamental research like QTL mapping and map-based cloning. They are useful in developing resistant cultivars, especially in developing cultivars with pyramided resistance genes (Castro *et al.* 2003). Although many markers linked to resistance genes are known, often the closest markers are not sufficiently tightly linked, therefore, identification of additional markers is required before marker-aided selection or initiation of map-based cloning can be considered. With the development of molecular marker techniques like amplified fragment length polymorphisms (AFLP) saturated genetic maps became more commonly available for a great number of plant species (Vos *et al.* 1995).

Rnhq is a QTL for nonhost resistance, which was mapped by Niks *et al.* (2000a) in L94 x Vada RIL populations. This population was initially constructed to study partial resistance against barley leaf rust (*P. hordei*) but it also segregates for the level of resistance to inappropriate rust fungi *Phm* and *P. triticina* both at macroscopic level (Zhang *et al.* 1994) and at microscopic level (Hoogkamp *et al.* 1998). *Rnhq,* was mapped on the long arm of chromosome 1 (7H), is effective to *Phm* and to a lesser extent to *P. triticina* at seedling stage (Niks et al. 2000a).

Work on *Rnhq* was continued and a near isogenic line with L94 background was created. Fine mapping using homozygous recombinants screened from crosses between resistant NIL L94-*Rnhq* and susceptible L94 was done by van Dijk (2007). A NIL with Vada allele in SusPtrit background is now available, but there is also one NIL with the L94 allele in SusPtrit background.

Rnhq was found to be effective towards *P.triticina*, and *P. persistens* by Jafary *et al* (2006) and effective to *Phm* and *Phs* by van Dijk (2007). The later author did not reconfirm the effect of *Rnhq* towards *P.triticina*. The author reason that the resistance to *P. triticina* was found using a L94xVada derived RIL population instead of the L94xL94-*Rnhq* population which he used for characterization of *Rnhq*.

van Dijk (2007) used molecular markers like SCAR and CAPS marker converted from AFLP markers mapped in L94 x V population (Qi *et al.* 1998) and SSR markers to fine-map *Rnhq*. Currently, *Rnhq* was fine mapped into a genetic window of 0.78 cM between the GBM1359 and SKT1 markers (Fig. 4.1). More markers are needed to give a higher resolution on *Rnhq* position.

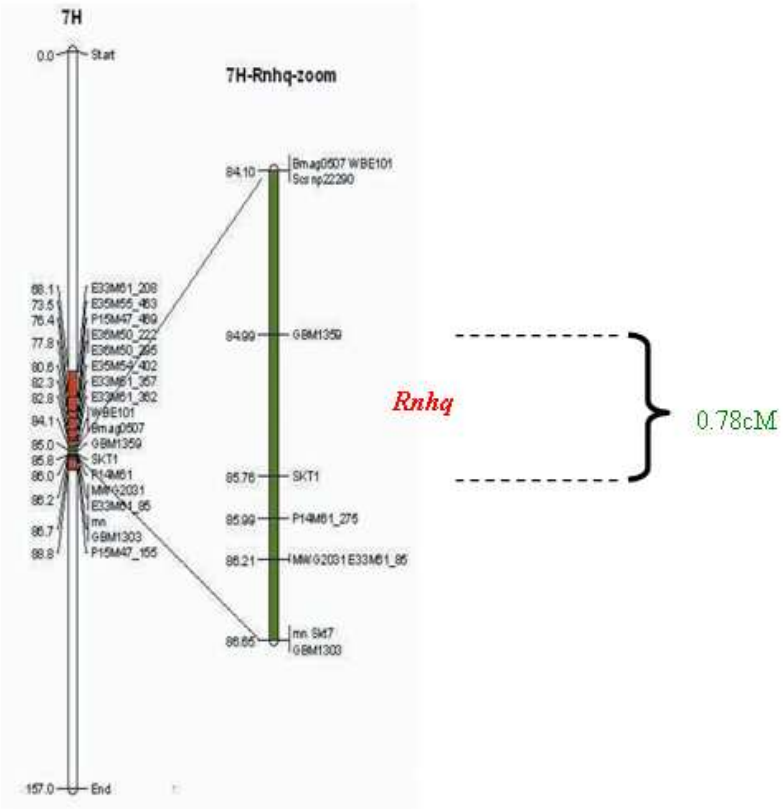


Fig 4.1 The 0.78 cM genetic window of Rnhq flank by GBM1359 and SKT1 (van Dijk, 2007).

Therefore, the objective of the present study is to develop more markers which will be used in fine mapping of *Rnhq*.

4.1 Materials and methods

4.1.1 Primer design and marker generation

According to Potokina *et al* (2008), Affymetrix microarray can be used to profile individuals from segregating populations and accurately identify polymorphic genetic markers called transcript derived markers (TDM). The TDM were integrated into a barley consensus map (Aghnoum *et al.* 2010). A total of 22 TDM were selected to be used for marker development (SCAR and CAPs). They were mapped within the

region of *Rnhq* flanked by WBE101 and GBM1303. The size of the region is 2.6 cM in the Barley Integrated Map 2007 and 5.3 cM in the Barley Integrated Map 2008. A total of 61 primer combinations we designed using the DNAstar software. A gradient-PCR was performed for each pair of primers in order to determine the optimal annealing temperature, following the PCR mix shown in Appendix 4.1.2 (a). Using the optimal annealing temperature the primer pairs were used to prime Vada, L94 and SusPtrit parental DNA. A SCAR is observed when polymorphism is observed between the parental lines PCR products. If not, the PCR products with a clear single band were sent for Quickshot^{TM} sequencing. The sequence information then was used to search for presence of SNP, which was exploited for the development of CAPS or derived-CAPS (dCAPS) markers. Suitable restriction enzymes were identified with the dCAPS finder program (http://helix.wustl.edu/dcaps/dcaps.html). Once the appropriate restriction enzyme had been identified, the polymorphism was verified between the parental lines 'Vada', L94 and SusPtrit. The markers were then mapped using RECORD (van Os *et al.* 2005) on L94 x Vada or Vada x SusPtrit mapping population.

4.2 Result

Forty one out of 61 primer combinations showed amplification after gradient PCR. However, only 31 primer combinations were repeatable to obtain PCR product of Vada, L94 and SusPtrit DNA (Appendix 4.1.1) by using the optimum annealing temperature. There were no SCAR markers obtained. The PCR products of the 31 primer pairs were sent for sequencing.

Out of the 31 primer pairs, only 3 primer pairs, ABC01200-1, ABC02539-4 and ABC03559-1, can be developed into CAPS markers (Table 4.1; Fig. 4.2). No polymorphism was observed on the other 28 primer pairs.. ABC01200-1 (83.9cM), was mapped near the peak marker SKT1 of *Rnhq*, while ABC02539-4 and ABC03559-1 were mapped proximal (79.3 cM) to and distal (87 cM) from the peak marker (Fig. 4.3).

Table 4.1. New markers developed in this study for *Rnhq*

Primers	Annealing (^{0}C)	RE	Marker type	Position (cM)	Recognition site
ABC02539-4	60	DdeI	CAPS (co-dominant)	79.3	GGCC
ABC01200-1	60	SpeI	CAPS (co-dominant)	83.9	ACTAGT
ABC03559-1	60	BfaI	CAPS (co-dominant)	87.0	CTAG

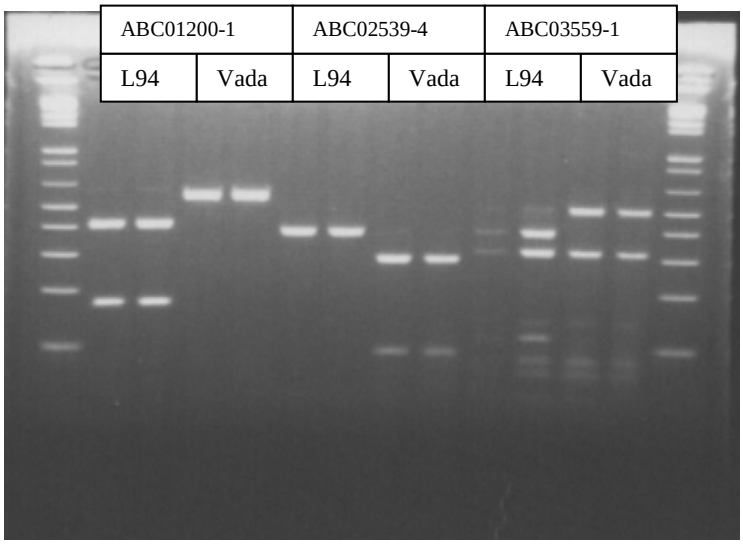


Fig. 4.2 Profile of markers developed for *Rnhq*

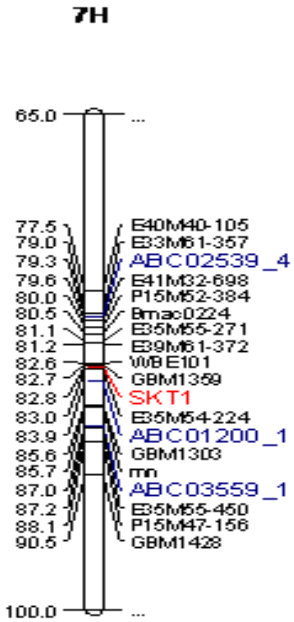


Fig. 4.3 Map order of newly developed molecular markers (Blue color), Red = the peak marker of the QTL.

4.3 Discussion

After the invention of polymerase chain reaction (PCR) technology, a large number of approaches for generation of molecular markers based on PCR were detailed, primarily due to its apparent simplicity and high probability of success. The development of PCR-based markers has been widely used for the construction of genetic maps and has greatly changed the prospects for application of molecular markers to study populations and to accelerate breeding (Rafalski *et al.* 1991; Rafalski and Tingey 1993).

Advantages of CAPS markers include the involvement of PCR requiring only low quantities of template DNA (50–100ng per reaction), the co-dominance of alleles and robust.

Accordingly, the development of new molecular markers around *Rnhq* permits the construction of high-resolution genetic maps to position the QTLs more accurately. Based on the estimation of linkage map position by RECORD, the two newly developed co-dominant CAPS markers (ABC02539-4 and ABC03559-1) mapped outside the *Rnhq* region. These three markers (ABC02539-4, ABC01200-1 and ABC03559-1) were mapped at distance of 3.5cM (proximal), 1.1cM and 4.2cM (distal), respectively from peak marker, SKT1. Of these three markers, only ABC01200-1 is within the boundary of the flanking markers (WBE101 and GBM1303) which van Dijk (2007) used to search for recombinants. Their accurate position need to be confirmed using substitution mapping.

To conclude, a total of 3 new CAPS markers were developed for *Rnhq*. They may increase the resolution of the current genetic map and maybe useful of fine mapping of *Rnhq*. More new markers are desirable. The development of new molecular markers around *Rnhq* region provided good flanking markers to be used later-on in fine mapping *Rnhq*.

Chapter 5. Fine mapping of partial resistance QTLs,*Rphq11* and *Rphq16*

5.0 Introduction

Quantitative trait locus (QTL) mapping is a highly effective approach for studying genetically complex forms of plant disease resistance. With QTL mapping, the roles of specific resistance loci can be described, race-specificity of partial resistance genes can be assessed, and interactions between resistance genes, plant development, and the environment can be analyzed (Young, 1996). The accuracy of QTL mapping is dependent on the density of markers, the accuracy of the trait analysis, the size of the population used and the size of the explained variation of the QTL (Van Ooijen, 1999 cited in Lindhout, 2002).

The mapping of leaf rust resistance genes and identification of molecular markers closely linked to them can facilitate the transfer and pyramiding of resistance genes in barley breeding programs through marker-assisted selection. It also may provide a foundation for map-based cloning strategy.

Rphq11 and *Rphq16* were assigned to barley chromosomes 2H in Steptoe x Morex population and 5H in OWB mapping population, respectively (Marcel *et al.* 2007). Substitution mapping done by Kuijken (2009) using seven strategic recombinant families for *Rphq11* region helped to narrow down the genetic interval into 0.8 cM between molecular markers Uni19962 and GBM1062 (Fig. 5.1A). Regarding *Rphq16,* Bouchon (2009) used 12 strategic recombinants families for fine mapping and found that *Rphq16* should be between MWG2249 and GBS0408, a genetic window of 3.6 cM (Fig. 5.1B). The genetic window of *Rphq11* and *Rphq16* obtained was determined by using progenies from a limited number of heterozygous recombinants. The respective QTL is still segregating in those materials. Heterozygous recombinants can be used for fine mapping purpose. However, there is a limitation in providing good statistical support.

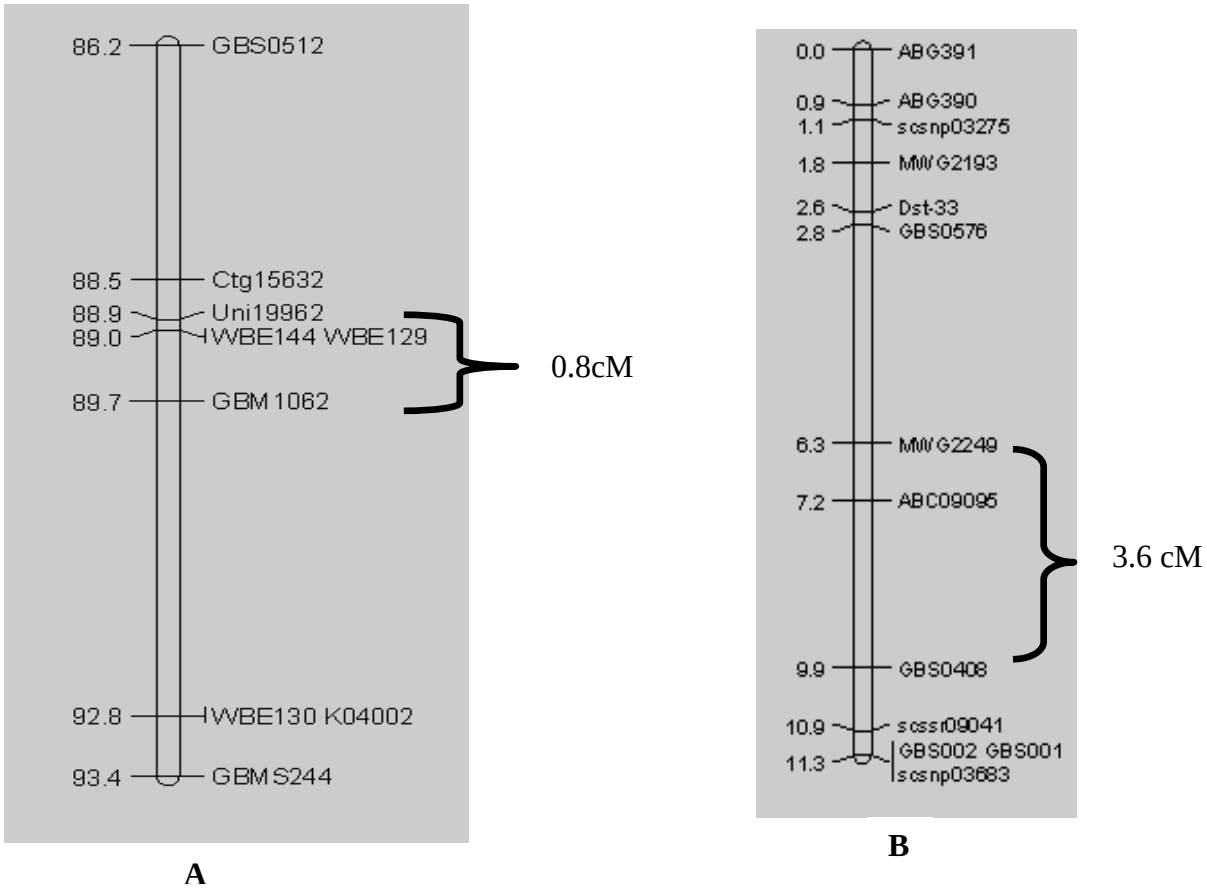


Figure 5.1. Substitution mapping of *Rphq11* (Kuijken, 2009) (A) and of *Rphq16* (Bouchon, 2009) (B).

Therefore, the main goal of this experiment is to confirm the fine-map of *Rphq11* and *Rphq16* using homozygous recombinant lines generated from the previous materials. Homozygous recombinant lines are more suitable to give clear and statistically significant result on fine mapping.

5.1 Materials and methods

5.1.1 Plant materials

Thirty-four and 32 F_6 homozygous recombinant lines derived from a cross between Steptoe x SusPtrit and Dom x SusPtrit, respectively, were used for this study. Parental lines such as Vada, L94, Steptoe, Morex, Dom, Rec, and QTL-NILs of *Rphq11* and *Rphq16* (QTL11-s-F_2-BC_5S_1 and Su-QTL16-BC_6S_1, respectively) were also included as a reference in each fine mapping population.

5.1.2 Pathogen material and phenotyping of the St x Sus and Dom x Sus

The spores of *P. hordei* isolate 1.2.1 were multiplied on susceptible barley line L94. Spores were collected and dried for short period (5-7 days) until used for inoculation. Seeds of the homozygous recombinant lines were sown in boxes (37 x 39 cm) along with the reference lines. Ten to twelve days after sowing, the first seedling leaves were fixed in horizontal position, the adaxial side facing up. The inoculations were carried out by using 3.5 mg spores per box resulting in a deposition of about 200 urediniospores per cm^2 as described by Qi *et al.* (1998). The inoculum was diluted 10 times with lycopodium spores before to be dusted over the tray. The dusted tray was placed in humidity chamber to incubate the spores overnight (8 hours) at 100% relative humidity in the dark at 18^oC. After incubation, the seedlings were transferred to a greenhouse compartment where the temperature was set at 14 ± 3^oC with 30-70% relative humidity.

The relative latency period at seedling stage (RLP50S) was then calculated relative to the latency period (LP) of SusPtrit, where SusPtrit was set as 100, as described by Parlevliet (1975). The disease test was in 3 replicates for *Rphq11*, and 2 replicates for *Rphq16*. The genotype and phenotype data were used to determine the new genetic windows of *Rphq11* and *Rphq16*, respectively.

To determine whether there was a genotypic effect on RLP50S within homozygous recombinants, an unbalanced ANOVA design was performed. Lines within one family were grouped according to their genotype (AA or BB) and this was used as a blocking factor. A genetic effect was considered to be present in a family at a significance level of P = 0.05.

5.1.3 QTL mapping

As a complement, the set of homozygous recombinant lines were treated as a mapping population. QTL analysis in both populations were performed by using MapQTL® 6 (van Ooijen, 2009) software programme. In order to map QTLs for resistance to rust species, interval mapping (IM) which combined the collected phenotypic data and the genotypic data, was performed in all replications and the average separately. The IM allowed us to see which markers had a high (i.e. LOD>1) and/or significant (i.e. LOD>3) LOD score. Such markers were then selected as cofactors and an Automatic

Co-factor Selection (ACS) was performed to retain only the most significant co-factor. With the set of co-factors retained, multiple QTL mapping (MQM) was run. If new peak markers were detected, new cofactors were selected and a new ACS was performed before repeating the MQM analysis. When no additional peak markers were noticeable (i.e. stable LOD profile), a restricted MQM was run to characterize the detected QTLs: LOD value, percentage of the variation explained. Significant LOD thresholds at level of 5% were obtained by running permutation test (PT) on data sets. The position of the QTLs was determined by calculating the 2-LOD intervals:

- *[LOD of the peak marker] - 2:* it gives an interval of positions in which the QTL is located with an error rate of 0.05.

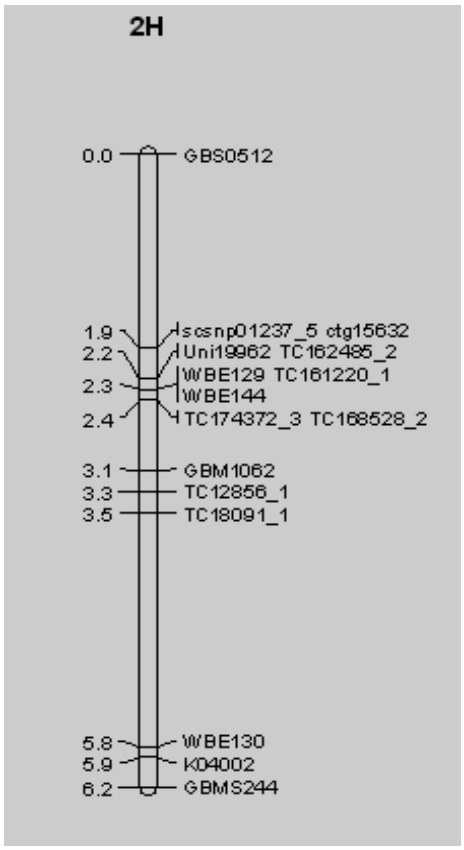
The genetic distance between the flanking markers of respective QTL (i.e., *Rphq11* and *Rphq16*) was calculated by dividing the total number of recombinants with the total number of gametes used in the recombinant selection.

5.2 Results

5.2.1 Fine mapping of *Rphq11*

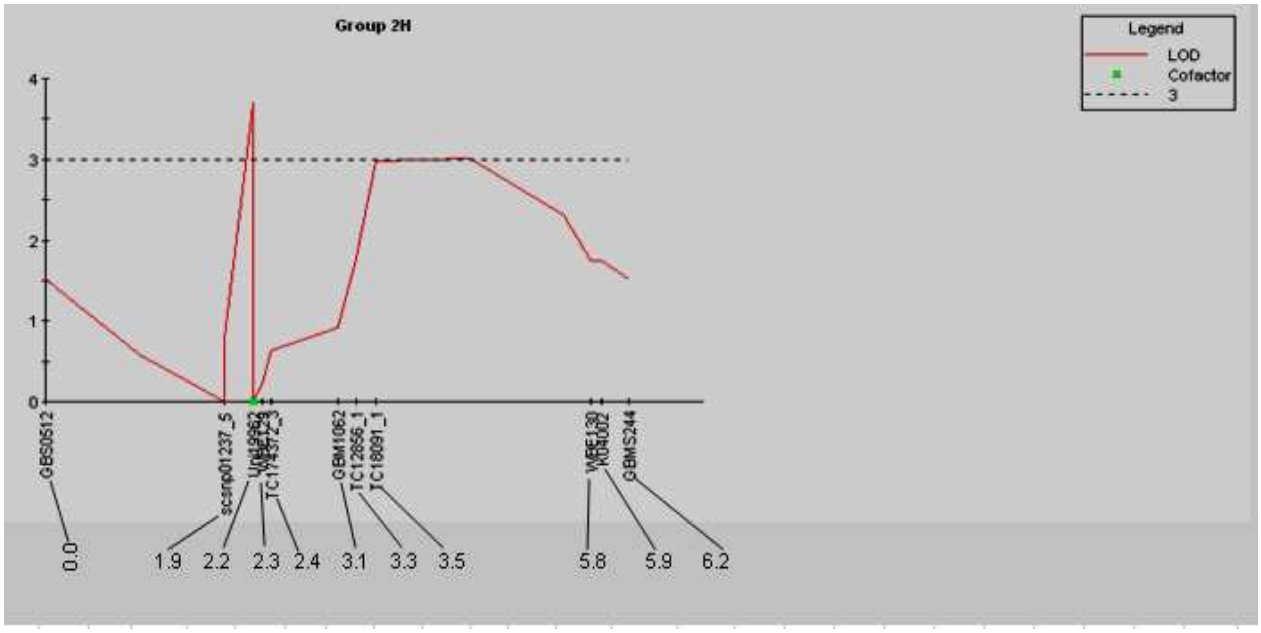
The identification of homozygous recombinants was performed from a F6 population derived from a Steptoe x SusPtrit cross in which *Rphq11* is segregating. 730 heterozygous plants were screened for recombination event between the CAPS marker GBS0512 and SSR marker GBMS244 flanking the QTL region. There were a total of 91 recombinants obtained and revealed a genetic distance of 6.2cM between flanking markers (Fig. 5.2A).

The previous studies (Yeo, 2008; Kuijken, 2009) in the Steptoe x SusPtrit population give important clues that *Rphq11* is positioned in the region between GBM1062 and Uni19962 (Fig. 5.1A). The molecular markers of special interest, which were reported by Kuijken (2009) as an important candidate to explain the partial resistance conferred by *Rphq11* was also situated in between this region.



(A)

Fig 5.2 New linkage map of the *Rphq11* (A) and LOD profile of peak marker (B). Numbers on the left side of the bar represent the genetic distance between molecular markers, based on the new genetic positions after mapping with 34 F_6 homozygous recombinants. The distance between markers is in cM.



(B)

The lines ST112.2.1, ST42.2, ST95.1 and ST64.1 were excluded from analysis (both ANOVA and MapQTL® 6), because they only have one replication phenotypic data. Also, lines ST66.1 (with genotype BB) ST107.1, ST44.8, ST17.1, ST14.1, ST45.1 and ST6.2 (with genotype AA) showed RLP50S which contradict with their genotype and excluded from analysis.

According to analysis of an unbalanced design using GenStat regression, plants with genotype AA for marker (s) in between estimated regions of QTL, showed a lower RLP50S compared with lines having the resistant genotype BB of Steptoe at the same marker (Table 5.1). Significant differences in RLP50S were observed between genotypic groups with AA and BB.

Table 5.1 Mean RLP50S of homozygous recombinant lines carrying the SusPtrit AA allele or Steptoe BB allele

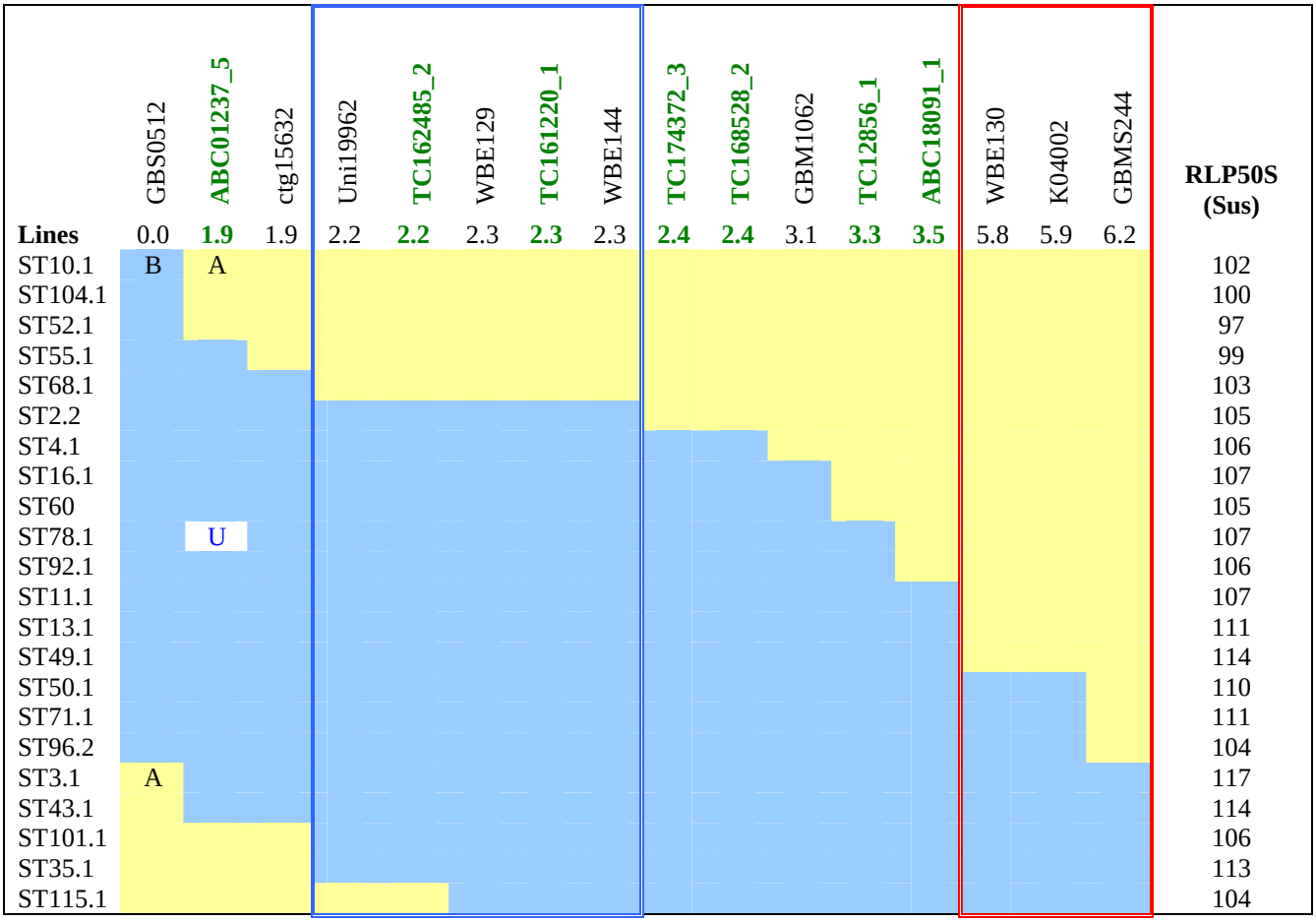
Marker (s)	Genotypes	RLP50S	LSD (5%)	P-value
WBE129/TC161220_1/WBE144	AA	100.8[a]	2.95	<0.001
	BB	109.2[b]		
ABC18091_1	AA	103.3[a]	2.71	<0.001
	BB	110.7[b]		

With the help of genotypic and phenotypic data obtained from these homozygous recombinants, it was observed that *Rphq11* had two genetic windows flanked by markers Uni19962 and WBE129/TC161220_1/WBE144 (0.1cM) and WBE130 and GBMS244 (0.4cM), respectively (Table 5.2).

The attempt to fine map *Rphq11* was also carried through QTL mapping approach. The homozygous recombinant lines were treated as a mapping population. MapQTL analysis showed that the LOD score values of each replicates were lower than 3, while that of average was greater than 3. The peak markers observed after we run ACS and rMQM were Uni19962 and ABC18091_1 (Fig. 5.2B).

According to synteny with rice, it was observed that the physical sizes of the two genetic windows of *Rphq11* are approximately 22kb and 43kb, respectively. We also tried to find the candidate genes found in the genetic window flanked by Uni19962 and WBE129/TC161220_1/WBE144. They are glutathione peroxidase and serine racemase. On the other genetic window which is flanked by GBMS244 and WBE130, protein kinase and SHR5-receptor-like kinase were found.

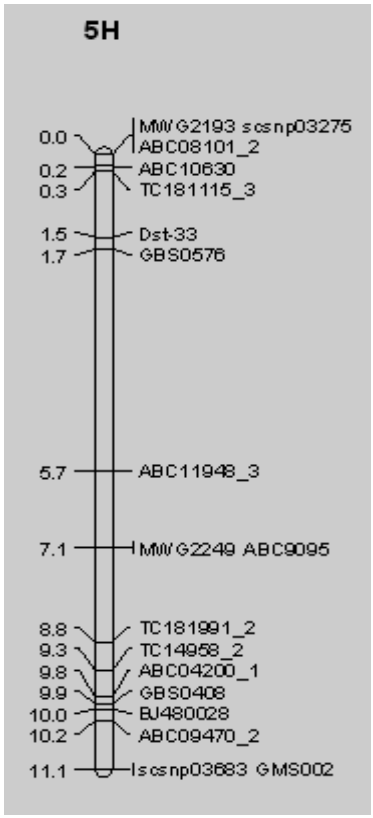
Table 5.2 Results of the genotyping and phenotyping (RLP50S) of the homozygous recombinants in which *Rphq11* is segregating selected for this study. The order of the markers* and the genetic distances are based on the new genetic map obtained in this study.



* Yellow area = SusPtrit introgression, Pale blue = Steptoe introgression, Blue line = location of the QTL *Rphq11*, A= represent homozygous SusPtrit allele, B= represent homozygous Steptoe allele, U = Unknown, Red colored = lines with data from one replication, Blue colored = lines with phenotypic data conflicting with genotypic data, Markers in green are newly included markers.

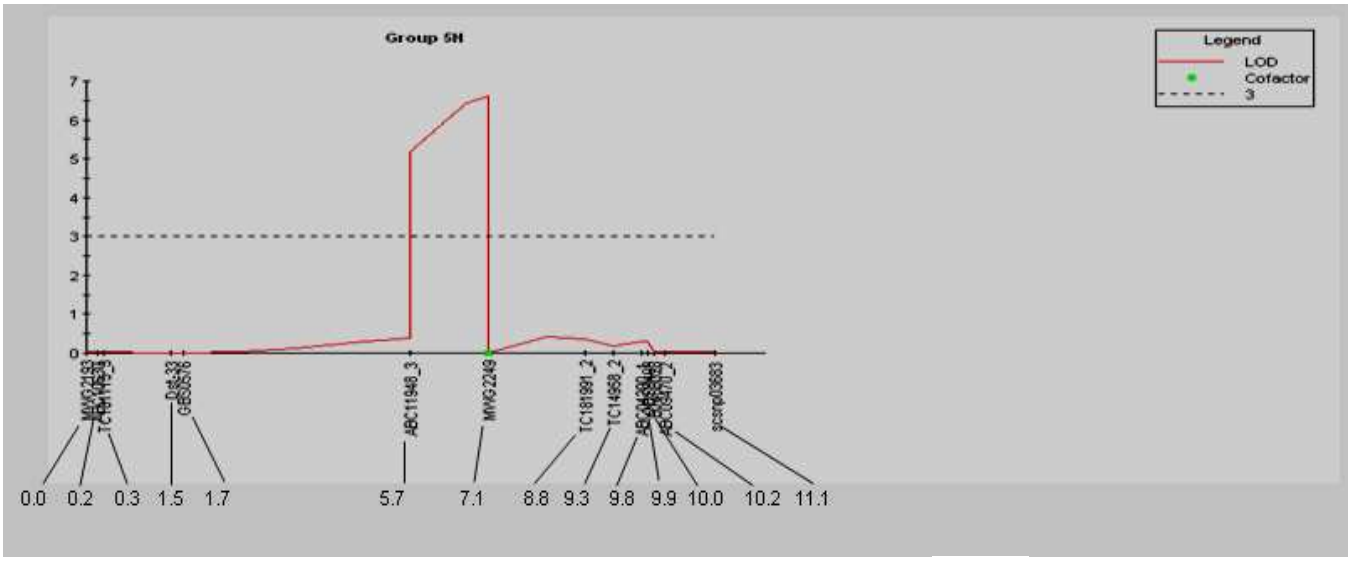
5.2.2 Fine mapping of *Rphq16*

Thirty one recombinants in the region of *Rphq16* have been identified so far and progeny lines of those recombinants were selected for homozygosity. These identified homozygous recombinants were used for fine mapping.



(A)

Fig 5.3 New linkage map of the *Rphq16* (A) and LOD profile of peak marker (B). Numbers on the left side of the bar represent the genetic distance between molecular markers, based on the new genetic positions after mapping with 31 F_6 homozygous recombinants. The distance between markers is in cM.



(B)

Bouchon, 2009) reported that *Rphq16* is found in the region between MWG2249 and GBS0408 (Fig. 5.1B).

Before the start of analysis, lines Dom196.3, Dom1.3, Dom111.3, Dom 113.2, Dom201.7, and Dom98.4 were excluded because they have only one replication data. According to the analysis of an unbalanced design using GenStat regression, plants with resistant genotype AA for marker (s) in between estimated region of QTL, showed a higher RLP50S (107.1) compared with lines having the susceptible genotype BB of SusPtrit (100.8) at the same marker (Table 5.3).

Table 5.3 Mean RLP50S of homozygous recombinant lines carrying the Dom AA allele or SusPtrit BB allele

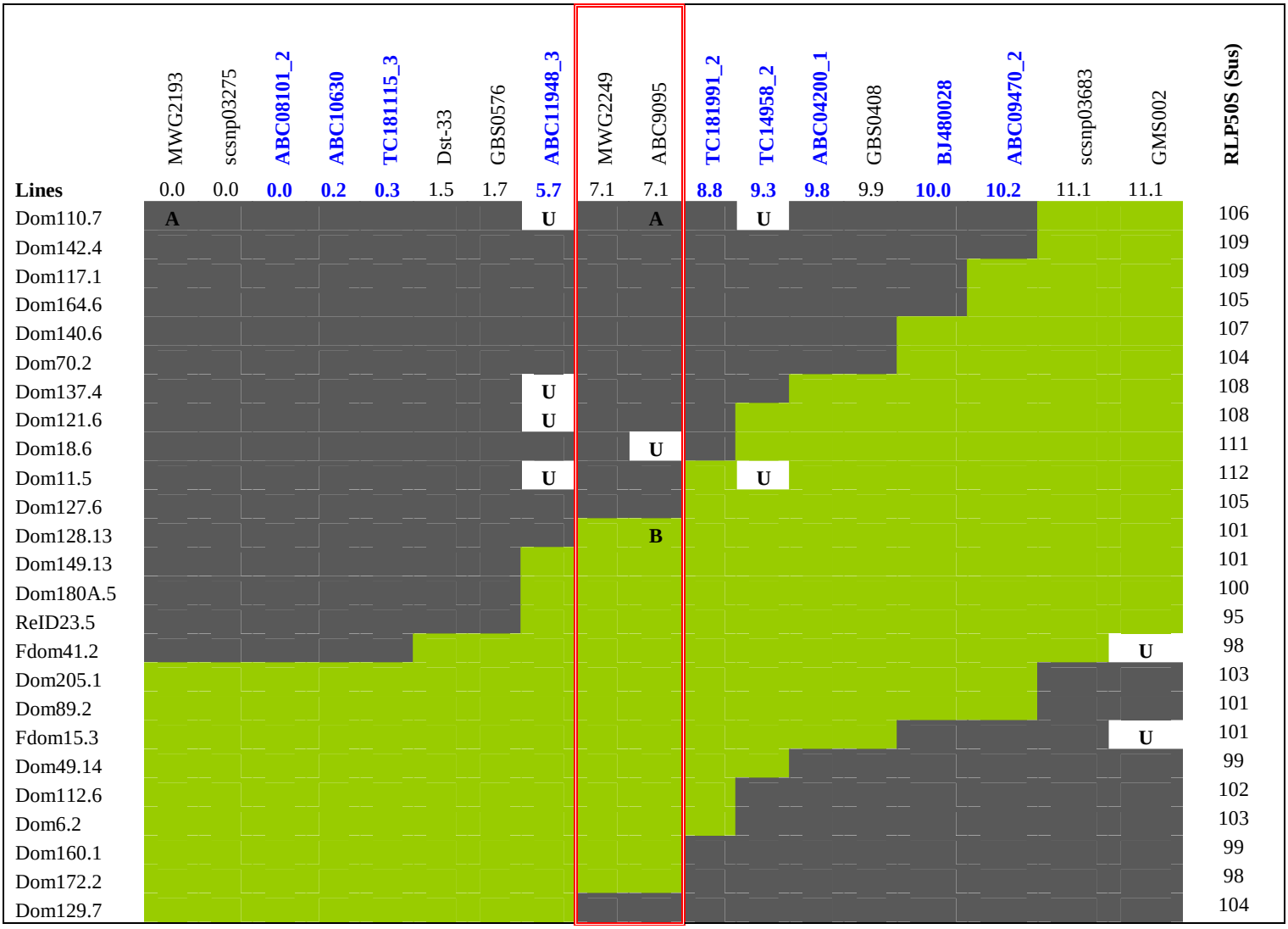
Genotypes	Marker	RLP50S
AA	ABC9095/MWG2249	107.1[a]
BB	ABC9095/MWG2249	100.8[b]
LSD (5%)		1.90
P-value		<0.001

As shown in the Table 5.4, new molecular markers were included between flanking markers ABG391 and GBS002 to increase the resolution of the genetic map. We found that the *Rphq16* was estimated to be between ABC11948_3 and TC181991_2 (3.1cM).

Again the homozygous recombinant lines were treated as a mapping population and subjected to QTL mapping analysis. Unlike to Rphq11, the analysis showed that the LOD score values of each replicate and the average were higher than 3 (Figure 5.3B). The peak marker was MWG2249. However, ABC9095 was also collocated with MWG2249. Also, after we run rMQM we found that there was other marker (ABC11948_3) with LOD score value more than 3.

Based on synteny with rice, it was observed that the physical distance of *Rphq16* genetic window is 175kb. The candidate genes found in the genetic window are oxidoreductase and glutathione S-transferase.

Table 5.4 Results of the genotyping and phenotyping (RLP50S) of the homozygous recombinants in which *Rphq16* is segregating selected for this study. The order of the markers and the genetic distances are based on the new genetic map obtained in this study.



Lime area = SusPtrit introgression, Gary area = Dom introgression, A= represents homozygous Dom allele, B= represents homozygous SusPtrit allele U = Unknown, Red box = putative location of *Rphq16*, Red color = lines with data from one replication, Blue color = newly included molecular markers.

5.3 Discussion

For both *Rphq11* and *Rphq16* the information obtained from previous studies (Marcel *et al.* 2007; Yeo, 2008; Kuijken, 2009 and Bouchon, 2009) gave an important indication on which genetic region to focus using respective homozygous recombinants. The fine map obtained in this study showed that, in both cases, the QTLs were mapped in new positions which is different from Kuijken (2009) and Bouchon (2009), respectively.

Rphq11 is fine mapped into two genetic windows flanked by Uni19962 and WBE129/TC161220_1/WBE144 and WBE130 and GBMS244 (Table 5.2). The genetic windows are now 0.1cM and 0.4cM, respectively. According to synteny with rice, the physical sizes of the two genetic windows of *Rphq11* are approximately 22kb and 43kb, respectively. The candidate genes found in the genetic window flanked by WBE144 and K14 are glutathione peroxidase and serine racemase. Glutathione peroxidase (GPX) is a family of multiple isozymes which catalyzes the reduction of H_2O_2, organic hydroperoxides and lipid hydroperoxides using glutathione as a reducing agent, and thus helps to protect the cells against oxidative damage (Flohé and Günzler, 1984), while serine racemase is an enzyme that degrades D-serine in plants, suggesting that the main physiological function of plant serine racemase might be to degrade serine to reduce D- serine (Sugimoto *et al* 2009). In the other genetic window flanked by GBMS244 and WBE130, protein kinase and SHR5-receptor-like kinase were found. Protein kinase induced by cold and salt stresses while receptor-like kinase (RLK), named SHR5 which may participate in signal transduction involved in the establishment of plant–endophytic bacteria interaction, respectively.

For *Rphq16*, the QTL was estimated to be in a genetic window of 3.1cM and found in between the newly included molecular markers with ABC11948_3 and TC181991_2 (Fig. 5.3A and Table 5.4).This attempt of fine mapping manage to reduce a total of 0.5 cM from the previous genetic window (3.6 cM, Bouchon, 2009). However, it seems like the present result did not further fine map the QTL. This could be due to the position of MWG2249 and ABC09095. In previous genetic map these two markers were resolved, however, in present new genetic map, they did not resolve. This is because there was no recombination was found between these two markers. In the future study, in orders to deeply study *Rphq16*, more molecular markers are

needed in the region of 3.1 cM between MWG2249 and ABC09095. Based on synteny with rice, it was observed that the physical distance of *Rphq16* genetic window is 175kb. The candidate genes found in the genetic window are oxidoreductase and glutathione S-transferase. Oxidoreductase involved in signal transduction, and growth regulation, while glutathione S-transferase used as chemical defense in plants.

Chapter 6. General discussion and summary

6.1 General discussion

Partial resistance to leaf rust (*P. hordei*) in barley is a quantitative resistance that is not based on hypersensitivity. This resistance hampers haustorium formation resulting in a long latency period in greenhouse tests. Four and one most consistent QTLs contributed resistance to homologous and heterologous rusts, respectively, were introgressed into the susceptible barley research line SusPtrit by marker-assisted backcrossing, to obtain quantitative trait loci-near isogenic lines (QTL-NILs). In a QTL-NIL, the target QTL becomes the major genetic source of variation because of the absence of other segregating QTLs (Marcel *et al.* 2008).

In previous studies, Qi *et al.* (1998, 1999), Niks *et al.* (2000a) identified *Rphq2* as being one of the QTLs with the greatest effect in the seedling stage and *Rphq3*, as plant stage independent QTL, while Marcel *et al.* (2007) and Yeo (2008) identified *Rphq11* and *Rphq16* as effective QTLs at seedling stage, in SxM and OWB RIL population, respectively.

In present study, evaluation of QTL-NILs at seedling stage confirmed that *Rphq2* had a significant effect on RLP in seedlings stage on *P. hordei* isolate 1.2.1. It prolonged the LP by 12, 15 and 11 hours on, Su-*Rphq2*, Vada-*rphq3* and L94-*Rphq2*, respectively as compared to SusPtrit (Appendix 2.3). *Rphq3* on Su-*Rphq3* had higher effect in seedlings compared to L94-*Rphq3*. On the other hand, *Rphq11* and *Rphq16* prolonged the LP by 11 and 10 hours on Su-*Rphq11* and Su-*Rphq16*, respectively (Appendix 2.3). For QTL-NILs with *Rnhq* (Su-*Rnhq*-L) which was from parental line L94 did not have significant effect, indicating that it has lower effect on *P. hordei*. However, Su-*Rnhq*-*V* seems to have significant effect due to the presence of 'Vada' allele as compared to SusPtrit (Appendix 2.3).

As to the study of heterologous rust fungi, *P. triticina* isolate 'Flamingo', *Phs* and *Phm*, *Rphq2* had significant effect than *Rphq3*, *Rphq11*and *Rphq16* on RIF of *P. triticina* and *Phm (*Appendix 2.4; 2.5 and 2.6). Regarding microscopic observations, pre-haustorial types of resistances were conferred by *Rphq2, Rphq3, Rphq11* and

Rphq16 on Su-*Rphq2,* Su-*Rphq3,* Su-*Rphq11,* and Su-*Rphq16,* respectively, due to high percentage of early abortion as compared to SusPtrit (Fig. 2.5). This high percentage of EA is an indicative of the failure of haustorium formation by the infection units in all rust species. Relative to susceptible check line, SusPtrit, the percentage of EA is higher for Su-*Rnhq-V* by *P. triticina, Phm* and *Phs*, indicating that there is influence of partially resistant donor line genes on these rusts. However, for Su-*Rnhq-L* the differences were not statistically significant, except for *Phs*. In L94 and in 'Vada' background, both corresponding NILs had larger percentage of EA (Appendix 2.4; 2.5 and 2.6). In general, the observed results revealed that the underlying resistance mechanism cloud be mainly based on EA of infection units and to some extent associated with low host cell necrosis around established sites, which was also observed as chlorosis macroscopically.

In previous studies it was reported that, *Rphq2* and *Rphq11* had significant effect at seedling stages, while *Rphq3* had consistent effect in all growth stages. In this study, it was also observed that, all QTLs hade effect across all plant stages with gradual decrease in effects as plants grew older (Fig. 3.2 and 3.3). Previously, these QTLs were reported to be plant stage dependent because they were mapped at seedlings but not at adult stage. As a result it becomes difficult to conclude that their effect is plant stage dependent. This could be due to the fact that, the QTL do function throughout the plant stage but it's effect is smaller than other detected QTLs in a mapping population which may suppresses its detection using MapQTL and/or the QTL may have a negative interaction with other QTLs. For, *Rphq3* on the other hand, the effect is consistent in all leaf layers observed, indicating that this QTL is plant stage independent as reported in previous studies. The effect of *Rphq3* in Vada-*rphq2* was also seem to perform as expected (Fig. 3.1).

In disease test of inappropriate rust species at three leaf layers with nonhost QTL, *Rnhq,* on Su-*Rnhq-V,* it was observed that the infection frequency was higher at first leaf layer and decrease as plants grew older.

With the attempt to saturate the *Rnhq* regions with more markers through development of more CAPS and SCAR markers, only few primer pairs showed amplification after gradient PCR. Of those amplified primer pairs we could managed

to develop only three (ABC02539-4, ABC01200-1 and ABC03559-1) co-dominant CAPS molecular markers.

The fine-mapping of partial resistance QTLs, *Rphq11* and *Rph16*, using homozygous recombinants, the genetic distance between their respective flanking markers was narrowed to 6.2cM and 11.1cM, respectively. Based on phenotypic and genotype data, it was observed that, *Rphq11* fine mapped into two genetic windows between Uni19962 and WBE129/TC161220_1/WBE144 (0.1cM) and WBE130 and GBMS244 (0.4cM), while *Rphq16* located between ABC11948_3 and TC181991_2 (3.1cM).

6.2 Conclusion/Future research

In the experiment to see specificity of QTLs, it was observed that partial resistance QTLs, *Rphq2*, *Rphq3*, *Rphq11* and *Rphq16* and the non-host resistance QTL, *Rnhq*, had an effects on both partial resistance towards homologous leaf rust, *P. hordei* isolate 1.2.1, as well as non-host resistance towards heterologous leaf rusts, *P. triticina* isolate "Flamingo", *P. hordei-murini* and *P. hordei-secalini*. Furthermore, high positive association was observed between RLP in seedling stage and proportion of EA at infection sites, which indicated that there is a possible association between partial resistance QTLs and non-host resistance QTLs. In plant stage dependency of QTLs study it was observed that none of the QTLs are plant stage dependent though they do have variation in effects. For this it still needs to be confirmed. Marker development for fine mapping of *Rnhq* was ended with only three CAPS markers and should be repeated to get more markers. The fine map done in this experiment showed that, *Rphq11* splited into two genetic windows of 0.1cM and 0.4cM. In this both regions, significant differences in RLP50S were observed between genotypes with AA allele and BB allele, indicating that this QTL in these regions is effective. The genetic window of *Rphq16* is 3.1cM which is still relatively large to proceed with physical mapping. It would be interesting to perform a similar study on this QTL by using homozygous recombinants already identified in this study.

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Appendices

Appendix 1.1 Summary of QTLs conferring partial resistance against leaf rust isolate 1.2.1 of different parley populations.

A. Seedling

QTL	Peak marker	LOD	Chr.	Intg. Map08	% Expl	Add. Effect	Donor	Source
Rphq1	E39M61-372	3.2	7H	82.9	3.1	-1.5	Vada	Qi et al., 1998
Rphq2	E38M55-251	14.2	2H	147.9	27.5	-3.7	Vada	Jafary et al., 2008
Rphq3	E37M33-574	16.0	6H	60.7	21.0	-3.9	Vada	Qi et al., 1998
Rphq4	E38M54-247	14.7	5H	15.6	25.3	-14.4	Vada	Qi et al., 1998
Rphq5	E35M61-368	3.0	4H	83.3	4.0	-5.6	Vada	Qi et al., 1998
Rphq6	E41M32-83	4.6	2H	32.3	7.9	-8.0	Vada	Qi et al., 1998
Rphq7	E35M48-160	3.0	5H	112.6	2.8	-1.1	Vada	Marcel et al., 2008
Rphq8	Amy2	3.4	7H	88.1	4.1	-1.2	Morex	Marcel et al., 2007
Rphq9	E40M32-123	4.4	7H	105.9	10.4	-6.4	Vada	Qi et al., 1999
Rphq10	E35M54-548	2.7	4H	19.3	5.1	-4.3	Vada	Qi et al., 1999
Rphq11	MWG503	21.0	2H	89.6	34.1	3.3	Steptoe	Marcel et al., 2007
Rphq14	E42M51-113	3.2	1H	9.1	5.4	-1.7	Vada	Jafary et al., 2008
Rphq15	ABC152A	5.3	6H	22.2	5.5	1.3	Steptoe	Marcel et al., 2007
Rphq16	Bmag0113i	11.4	5H	169.9	27.0	2.6	Dom	Jafary et al., 2008
Rphq17	MWG844B	5.1	3H	59.6	11.9	1.7	Dom	Jafary et al., 2008
Rphq18	GBM1251	3.1	2H	50.4	8.1	-1.4	Rec	Jafary et al., 2008
Rphq19	Bmac0303c	3.7	4H	65.5	7.3	-1.3	Rec	Jafary et al., 2008
Rphq20	E33M58-504	2.8	4H	110.9	2.1	-1.3	Vada	Marcel et al., 2008
Rphq21	P15M53-163	3.9	1H	58.1	3.4	-1.3	Vada	Marcel et al., 2008
Rnhq		9.5	7H		30.0			Niks et al, 2000

B. Adult stages

QTLs	Chr.	Population	Qi et al. 1998		Qi et al. 1999		Qi et al. 2000		Niks et al, 2000	
			LOD	Exp%	LOD	Exp%	LOD	Exp%	LOD	Exp%
Rphq2	2H	L94 x V	3.0	4.1	5.3	10.9			3.0	4.0
Rphq3	6H	L94 x V, L94 x 116.5	10.7	17.4	6.5	13.7	10.1	20.2	10.7	17.0
Rphq4	5H	L94 x V	14.3	25.4	4.5	9.1			14.3	25.0
Rphq5	4H	L94 x V	3.1	4.3					3.1	4.0
Rphq6	2H	L94 x V	5.3	7.7					5.3	8.0
Rphq8	7H	St x M			4.7	9.4				
Rphq9	7H	L94 x V			4.2	7.1				
Rphq10	4H	L94 x V, L94 x 116.5			3.1	6.1	3.1	5.5		
Rphq13		L94 x 116.5					3.7	9.2		
Rphq15	6H	St x M							1.8	3.2
Rphq17	3H	OWB							3.4	5.0
Rphq18	2H	OWB							3.0	11.0

Appendix 2.1 Genotype of QTL-NILs

2.1.1 Genotype of *Rphq2*-NILs

Rphq2	Sdu I k00345	besV76P5D5AR	scP15M51-204	HVM54 SSR-12	HVM40 SSR-21	Bmag0223 SSR-31	GBMS035 SSR-117	GMS006 SSR-135
L94	AA	AA	AA	AA	AA	AA	AA	AA
SusPtrit	CC	CC = AA	CC = AA	CC	CC	CC	CC	CC
Vada	BB = CC	B-	B-	BB	BB	BB	BB	BB
Su-Q2-BC5S1.1	AA	BB	BB	BB	BB	BB	BB	BB
Su-Q2-BC5S1.2	AA	BB	BB	BB	BB	BB	BB	BB
Su-Q2-BC5S1.3	AB	BB	BB	BB	BB	BB	BB	BB
Su-Q2-BC5S1.4	AA	BB	BB	BB	BB	BB	BB	BB
Su-Q2-BC5S1.6	AB	BB	BB	BB	BB	BB	BB	BB
Su-Q2-BC5S1.7	AB	BB	BB	BB	BB	BB	BB	BB
Su-Q2-BC5S1.8	AA	BB	BB	BB	BB	BB	BB	BB
Su-Q2-BC5S1.9	BB	BB	BB	BB	BB	BB	BB	BB

2.1.2 Genotype of *Rphq3*-NILs

Rphq3	*Mnl I* WBE201	*Nla III* ABG388	HVM14 SSR-56	GBM1212 SSR-108
L94	AA	AA	AA	AA
SusPtrit	CC = BB	CC	CC	CC=AA
Vada	BB	B-	BB	BB
Su-Q3-BC6S1.1	BB	BB	BB	BB
Su-Q3-BC6S1.6	BB	BB	BB	BB
Su-Q3-BC6S1.7	BB	BB	BB	BB
Su-Q3-BC6S1.9	BB	BB	BB	BB
Su-Q3-BC6S1.12	BB	BB	BB	BB

2.1.3 Genotype of *Rphq11*-NILs

Rphq11	*Aci I* GBS0512	*Apo I* TC134748	GBMS244 SSR-123	GBM1062 SSR-127
SusPtrit	CC	CC	CC	CC
Steptoe	DD	DD	DD	DD
Su-QTL11.s-F2.BC5S1.6	DD	DD	DD	DD
Su-QTL11.s-F2.BC5S1.8		DD	DD	DD
Su-QTL11.s-F2.BC5S1.12	DD		DD	DD

2.1.4 Genotype of *Rphq16*-NILs

Rphq16	snp03275_2 Bgl II	Dst-33	GMS002 SSR-100
SusPtrit	CC	CC	CC
Dom	EE	EE	EE
Su-Q16-BC6S1.9	EE	EE	EE

2.1.5 Genotype of *Rnhq-L*-NILs

Rnh-L	***HpyCH4IV*** **WBE101**	***Mwo I*** **MWG2031**	**GBM1303 SSR-90**
L94	AA	AA	AA
SusPtrit	CC = BB	CC = BB	CC
Vada	BB	BB	BB
Su-Qnh.L-F2.BC5S.9	AB	AA	BB

2.1.6 Genotype of *Rnhq-V*-NILs

Rnh-V	***HpyCH4IV*** **WBE101**	***Mwo I*** **MWG2031**	***Alu I*** **SKT1**	**GBM1303 SSR-90**
L94	AA	AA	AA	AA
SusPtrit	CC = BB	CC = BB	CC = AA	CC
Vada	BB	BB	BB	BB
Su-Qnh.v-F2.BC5S1.3	BB	X	BB	BB
Su-Qnh.v-F2.BC5S1.6	BB	X	BB	BB
Su-Qnh.v-F2.BC5S1.7		X	BB	BB
Su-Qnh.v-F2.BC5S1.10	BB	X		BB
Su-Qnh.v-F2.BC5S1.12	BB	X		BB
Su-Qnh.v-F2.BC5S1.13	BB	X	BB	BB
Su-Qnh.v-F2.BC5S1.14	BB	X	BB	BB

Appendix 2.2 Histology

2.2.1 Ingredients for preparation of lactophenol solution

250ml lactic acid

500ml glycerin

250ml 20% phenol solution

Then keep in refrigerator

2.2.2 Preparation of 0.1M Tris/HCl buffer (pH 8.5)

Dissolve 12.1gm Tris in 800ml H_2O

Adjust pH to 8.5 with HCl (25%)

Add H_2O till volume is 1 liter

Appendix 2.3 Mean LP and RLP50 of by *P. hordei* isolate 1.2.1 of tested lines at seedling stage.

Lines	LP	RLP
Vada	213.4e	114.1e
Vada-*rphq2*	210.7de	112.7cde
Vada-*rphq3*	202.4cd	108.2bcde
L94	187.6ab	100.3a
L94-*Rphq2*	197.6abc	105.7abc
L94-*Rphq3*	190.3ab	101.8ab
Steptoe	211.1de	112.9de
Dom	193.7abc	103.6ab
Su-*Rphq2*	198.7bc	106.8abcd
Su-*Rphq3*	194.0abc	104.0ab
Su-*Rphq11*	198.0abc	106.0abcd
Su-*Rphq16*	196.4abc	105.2ab
Su-*Rnhq-L*	190.5ab	101.8ab
Su-*Rnhq-V*	191.9abc	102.6ab
SusPtrit	187.0a	100.0a

Appendix 2.4. Mean RLP, RIF and percentage of infection units by *P. triticina* isolate 'Flamingo' of tested lines per classes of infection units: Least significance differences (F-test) among the mean of infection units within each class is presented as a resulted from One-way ANOVA.

Line	RLP	RIF	Status of infection (%)				Colony size (µm)	
			NP	EA	Established		With ST	W/o ST
					With ST[x]	W/o ST		
Vada	**	**	29.0g	71.0g	*	*	*	*
Vada-*rphq2*	**	6.4[y]a	24.2f	69.6fg	*	6.4[a]	*	1.8
Vada-*rphq3*	117.3f	7.2[a]	24.5f	66.2fg	*	9.3a	*	2.1
L94	108.2bcde	69.2e	7.5ab	32.0c	21.5bc	39.0g	5.4de	2.3abcd
L94-*Rphq2*	113.9ef	38.8c	14.0cde	48.0e	18.0b	21.5bc	4.6bc	2.0ab
L94-*Rphq3*	108.0bcde	33.4c	12.0cd	39.0d	20.0	29.0de	4.0ab	2.0ab
Steptoe	118.0[y]f	0.0[y]	32.0g	68.0fg	*	*	*	*
Morex	114.2ef	22.2b	15.5de	64.0f	*	20.5bc	*	2.0ab
Dom	112.3def	13.1ab	14.0cde	63.5f	6.1a	16.0b	4.0ab	3.1efg
Rec	110.1cdef	34.0c	11.5bcd	47.0e	17.5b	24.0cd	3.8a	2.7cdef
Su-*Rphq2*	106.5bcde	37.8c	16.0de	34.5cd	19.0bc	30.0de	5.0cd	2.6bcdef
Su-*Rphq3*	106.0bcde	55.8d	17.0e	31.5c	22.0bc	29.5de	4.8cd	2.5bcde
Su-*Rphq11*	106.4bcde	57.7d	16.0de	32.3c	23.0bc	28.7de	5.1cd	2.8defg
Su-*Rphq16*	104.4bcd	63.0de	15.0de	28.0bc	19.5bc	37.5fg	5.9e	2.9defg
Su-*Rnhq-L*	100.6b	64.2de	10.0abc	22.5ab	29.4d	38.0g	7.0f	3.2fg
Su-*Rnhq-V*	102.6bc	64.2de	13.0cde	30.0c	24.5cd	32.0ef	7.0f	3.5gh
SusPtrit	100.0b	100.0	7.0a	21.0a	52.5	19.5bc	8.1	3.9h
8860 (Wheat)	92.3a	194.7	*	*	*	*	*	*
LSD (5%)	8.1	9.8	4.0	5.8	5.2	5.6	0.7	0.6
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[X] Sporogenic tissue,

[Y] Data only from one replication,

W/o = without

** No sporulation/no flecks,

* Excluded from analysis

Appendix 2.5. Mean RLP, RIF and percentage of infection units by *Phs* of tested lines per classes of infection units: Least significance differences (F-test) among the mean of infection units within each class is presented as a resulted from One-way ANOVA

Line	RLP	RIF	Status of infection (%)				Colony size (µm)	
			NP	EA	Established		With ST	W/o ST
					With ST [X]	W/o ST		
Vada	121.3	2.3 [y]a	19.7fg	80.4h	*	*	*	*
Vada-*rphq2*	111.3 [y]de	4.5 [y]a	17.3def	76.3gh	*	8.0b	*	2.0ab
Vada-*rphq3*	114.1ef	15.2ab	15.5cdef	74.1gh	*	10.4bc	*	2.2ab
L94	101.7b	77.5	10.0ab	22.5a	35.5e	32.0f	5.8ef	3.1de
L94-*Rphq2*	103.7b	42.5def	12.0abc	70.0fg	10.0abc	8.0b	5.1cde	2.4bc
L94-*Rphq3*	102.7b	32.6cd	11.0abc	64.5ef	11.5abcd	13.0bcd	3.9a	2.4bc
Steptoe	116.3f	5.1a	22.8g	75.0gh	*	2.3a	*	1.9a
Morex	112.9def	22.5bc	18.9efg	65.2ef	*	16.0de	*	2.4bc
Dom	109.0cd	54.0efg	15.2bcdef	61.0cde	12.2bcd	12.4bcd	5.2cde	2.4bc
Rec	104.7bc	61.9g	14.0abcde	60.5cde	15.2d	11.0bcd	5.4de	3.1e
Su-*Rphq2*	104.9bc	31.2cd	14.6bcde	63.3def	7.7a	16.4de	4.6	3.1e
Su-*Rphq3*	102.0b	29.4bcd	12.5abcd	56.3cd	15.5d	13.9cde	4.6bc	2.7cd
Su-*Rphq11*	104.0bc	39.1de	14.5bcde	57.0cd	9.0ab	18.8e	4.0ab	3.2e
Su-*Rphq16*	102.2b	40.8de	14.0abcde	55.5c	13.7cd	16.5de	4.7cd	3.3e
Su-*Rnhq-L*	102.2b	57.1fg	9.0a	39.8b	14.1cd	37.1	5.0cd	3.4e
Su-*Rnhq-V*	102.9b	45.7def	12.0abc	43.2b	15.5d	29.3f	4.7cd	3.4e
SusPtrit	100.0b	100.0	11.2abc	26.4a	34.0e	27.7f	6.1f	3.5e
H. secalinum	90.6a	299.2	*	*	*	*	*	*
LSD (5%)	4.7	14.7	4.5	6.5	3.9	4.9	0.7	0.4
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[X] Sporogenic tissue,
[Y] Data only from one replication,
W/o = without,
* Excluded from analysis

Appendix 2.6. Mean RLP, RIF and percentage of infection units by *Phm* of tested lines per classes of infection units: Least significance differences (F-test) among the mean of infection units within each class is presented as a resulted from One-way ANOVA

Line	RLP	RIF	Status of infection (%)				Colony size (µm)	
			NP	EA	Established		With ST	W/o ST
					With ST[x]	W/o ST		
Vada	***	3.8[Y]	19.5h	80.5h	*	*	*	*
Vada-*rphq2*	103.4bcde	9.1a	15.4cdefg	76.6gh	*	8.0b	*	1.8a
Vada-*rphq3*	103.5bcde	11.0a	16.0defgh	73.8g	*	10.6bc	*	2.2abc
L94	100.6bc	82.5cd	10.2ab	22.8a	35.0	32.0j	5.8cd	3.1efg
L94-*Rphq2*	104.7de	30.0ab	11.7bc	68.9f	11.6ab	8.5b	5.1bc	2.4cd
L94-*Rphq3*	103.3bcde	55.9bc	11.7bc	59.6d	12.0ab	16.7ef	3.9a	2.4bcd
Steptoe	112.7g	3.8 [Y]	23.6	74.7g	*	2.0a	*	1.9ab
Morex	109.7fg	11.0a	18.7fgh	65.4ef	*	14.4de	*	2.6cde
Dom	102.9bcd	44.8ab	14.5cd	61.3de	10.8[a]	13.5cde	5.2bc	2.2abcd
Rec	100.9bcd	50.6bc	14.7cde	59.9d	15.5bc	11.3bcd	5.4bcd	3.05efg
Su-*Rphq2*	104.6de	30.3ab	14.7cde	56.1cd	10.4[a]	19.2fg	4.6ab	3.1fg
Su-*Rphq3*	106.9ef	39.7ab	12.3bcd	56.0cd	11.2a	20.6gh	4.6ab	2.7def
Su-*Rphq11*	104.2cde	31.2ab	13.2bcd	52.7c	11.4ab	23.0hi	4.0a	3.2g
Su-*Rphq16*	102.4bcd	40.7ab	14.3bcd	45.2	16.7cd	24.0i	4.7ab	3.3g
Su-*Rnhq-L*	103.2bcde	55.3bc	10.3ab	37.8b	15.7bc	36.5	5.1bc	3.4g
Su-*Rnhq-V*	103.0bcd	44.5ab	12.2bcd	38.5b	20.5d	28.8j	4.7ab	3.3g
SusPtrit	100.0b	100.0d	7.0a	34.2b	29.5	29.9j	6.1d	3.5g
H. murinum	96.0a	184.3	*	*	*	*	*	*
LSD (5%)	3.4	32.5	3.7	4.8	3.9	3.1	0.7	0.4
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

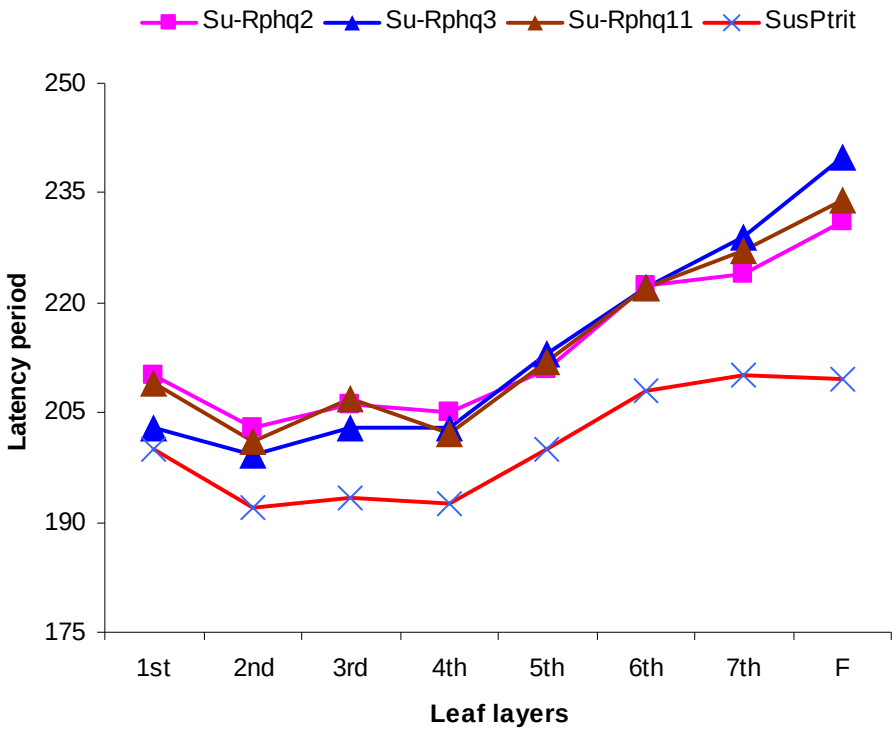
[X] Sporogenic tissue,
[Y] Data only from one replication,
W/o = without,
** No sporulation and flecks,
* Excluded from analysis

Appendix 3.1 Mean RLP* of tested lines at different leaf layers by *P. hordei* isolate 1.2.1: Least significance differences (F-test) presented as a resulted from One-way ANOVA.

Lines	1st	2nd	3rd	4th	5th	6th	7th	F
Vada	118^{g}	130^{g}	130^{f}	132^{e}	129^{e}	124^{f}	125^{f}	127^{d}
Vada-*rphq2*	110^{ef}	123^{fg}	118^{cde}	122^{cde}	120de	116^{e}	119^{def}	118^{bcd}
Vada-*rphq3*	109^{def}	118^{def}	119^{ef}	119^{cde}	123de	116^{e}	121^{ef}	123^{cd}
L94	98^{a}	102^{a}	104^{ab}	104^{ab}	101^{a}	99.7^{a}	102^{ab}	109^{ab}
L94-*Rphq2*	106^{bcde}	114^{cde}	110^{abcde}	111^{abc}	106^{ab}	103^{ab}	105^{abc}	108^{ab}
L94-*Rphq3*	102^{abc}	111^{bcd}	114^{bcde}	116^{bcd}	109^{abc}	114de	112^{cde}	117^{bcd}
Steptoe	113^{f}	121^{ef}	122^{ef}	124de	119^{cde}	115^{e}	115^{cde}	119^{bcd}
Morex	108^{cdef}	118^{def}	119^{def}	120^{cde}	113^{bcd}	110^{cd}	110^{bcd}	113^{bc}
Su-Rphq2	105^{bcde}	106^{abc}	107^{abc}	106^{ab}	106^{ab}	107^{bc}	107^{abc}	111^{ab}
Su-Rphq3	102^{ab}	104^{ab}	105^{ab}	106^{ab}	107^{ab}	107^{bc}	109^{abcd}	115^{bc}
Su-Rphq11	104^{bcd}	105^{ab}	107^{abcd}	105^{ab}	106^{ab}	107^{bc}	108^{abc}	112^{bc}
SusPtrit	100^{ab}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
LSD (5%)	5.3	7.7	10.6	11.2	9.9	3.7	8.8	10.8
F-test	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001

* Means followed by a common letter are not significantly different according to Duncan's LSD test (P<0.05)

Appendix 3.2. Latency period of Su-*Rphq2*, Su-*Rphq3* and Su-*Rphq11* relative to SusPtrit infected with *P. hordei* isolate 1.2.1



F = Flag leaf, Y axis = Latency period (in hr), and X = axis leaf layers.

Appendix 4.1 Summary of primer pairs used for marker development

Nr	Primers	Annealing Tm	Amplification			Remarks
			Vada	SusPtrit	L94	
1	ABC02163	63	Clear	Clear	Weak	
2	ABC03894-1	65	Weak	Clear	Clear	
3	ABC03894-3	63	Clear	Clear	Clear	
4	ABC03894-4	63	Clear	Weak	Weak	
5	ABC05815-1	63	Clear	Weak	Weak	
6	ABC06283-1	63	Clear	Clear	Clear	
7	ABC06793-4	63	Weak	Clear	Clear	
8	ABC06968-1	63	Clear	Weak	Clear	
9	ABC07088-1	65	Clear	Clear	Weak	
10	ABC07088-2	63	Clear	Clear	Weak	
11	ABC10242-2	63	Clear	Clear	Clear	
12	ABC11086-1	63	Clear	Clear	Clear	
13	ABC11086-3	63	Clear	Clear	Clear	
14	ABC11086-4	63	Clear	Clear	Clear	
15	ABC17403-1	65	Weak	Clear	Clear	
16	ABC18212-1	65	Clear	Clear	Clear	
17	ABC18212-2	65	Clear	Clear	Clear	
18	ABC06968-3	58	Clear	Weak	Clear	
19	ABC07088-3	58	Weak	Clear	Clear	
20	ABC10242-1	58	Clear	Clear	Clear	
21	ABC07183	58	Weak	Clear	Clear	
22	ABC07248	58	Clear	Clear	Clear	
23	ABC17403-3	58	Weak	Weak	Weak	
24	ABC01200-1	60	Clear	Weak	Clear	
25	ABC01200-2	60	Clear	Clear	Clear	
26	ABC01200-4	60	Clear	Clear	Clear	
27	ABC02539-1	60	Clear	Clear	Weak	
28	ABC02539-4	60	Clear	Clear	Clear	
29	ABC03559-1	60	Clear	Weak	Clear	
30	ABC02539-3	58	Clear	Clear	Clear	
31	ABC07022-2	58	Weak	Weak	Clear	

Appendix 4.2 PCR conditions used for the development of SCAR and CAPS markers and SSR and PCR profile.

a) PCR mix used for the development of SCAR and CAPS markers and PCR profiles.

Components	Volume (µL)
H_2O (autoclaved)	18.4
PCR buffer S-taq (10x)	2.5
dNTPs (5mM)	1.0
Forward primer (5 pmol/µL)	1.0
Reverse primer	1.0
SuperTaq polymerase (5 units/µL)	0.1
DNA	1.0
Total volume	**25**

b) PCR mix used for SSR PCR profile

Components	Volume (µL)
MilliQ H_2O (autoclaved)	6.6
10 X SB (Super buffer)	1.0
dNTPs [5mM]	0.4
Primer mix F+R [5pmol/µl]	1.0
Taq-polymerase (SuperTaq (5U/µl)	1.0
DNA (50ng/µl)	1.0
Total volume	**10**

Appendix 4.3 An overview of all molecular markers used in mapping Rnhq in this study.

Pop	Chrom.	Type	Marker name	intMap2008	Current Position (cM)	Pattern	Source
LxV	7H	AFLP	E40M40-105	78.819	77.48	L94	Qi et al., 1998
LxV	7H	AFLP	E33M61-357	83.739	78.95	Vada	Qi et al., 1998
LxV	7H		**ABC02539-4**		79.33		
LxV	7H	AFLP	E41M32-698	85.293	79.59	L94	Qi et al., 1998
LxV	7H	AFLP	P15M52-384	81.559	80.01	Vada	Marcel et al., 2007
LxV	7H	SSR	Bmac0224	84.531	80.50	Codom	Marcel et al., 2007
LxV	7H	AFLP	E35M55-271	80.297	81.06	Vada	Qi et al., 1998
LxV	7H	AFLP	E39M61-372	82.891	81.19	L94	Qi et al., 1998
LxV	7H	CAPS	WBE101	83.065	82.58	Codom	Marcel et al., 2007
LxV	7H		GBM1359		82.70		
LxV	7H		SKT1		82.80		
LxV	7H	AFLP	E35M54-224	85.782	82.97	L94	Qi et al., 1998
LxV	7H		**ABC01200-1**		83.90		
LxV	7H	SSR	GBM1303	88.352	85.60	Codom	Marcel et al., 2007
LxV	7H	Gene	m*n*	88.352	85.65	Vada	Qi et al., 1998
LxV	7H		**ABC03559-1**		87.00		
LxV	7H	AFLP	E35M55-450	89.152	87.22	Vada	Qi et al., 1998
LxV	7H	AFLP	P15M47-156	89.958	88.06	Vada	Marcel et al., 2007
LxV	7H	SSR	GBM1428	92.219	90.49	Codom	Marcel et al., 2007