Fine-mapping of nonhost resistance genes in barley

MSc thesis Plant Breeding

Adrichem, Marloes van (931228006020)
WAGENINGEN UR

Supervisors
MSc Mathilde Daniau
Dr. Ir. Rients Niks

June 2018

Abstract

Nonhost resistance is defined as the immunity displayed by all accessions within a plant species against all pathovars of a pathogenic species. This type of resistance would be interesting to use for durable resistance in breeding programmes. However, very little is known about the genetic mechanisms behind nonhost resistance.

During this thesis, nonhost resistance genes against *Puccinia persistens, P. hordei-secalini and P. graminis lolii* were fine-mapped in barley. The used mapping populations are L94 x L94-*Rnhq* and SusPtrit x L94-*Rnhq* BC_1S_1 and BC_1S_2. Previous research identified a QTL, called *Rnhq*, located on chromosome 7H conferred resistance to several heterologous rusts: *P. triticina, P. hordei-murini, P. persistens, P. hordei-secalini and P. graminis lolii*. Later this QTL appeared to consist of three subregions, each conferring resistance to one or several heterologous rusts. Subregion 1 conferred resistance to *P. hordei-murini* and *P.hordei-secalini,* subregion 2 to *P. triticina,* and subregion 3 to *P. graminis lolii.*

The focus of this project was on fine-mapping *P. graminis lolii.* The results of the experiments with the SusPtrit x L94-*Rnhq* population indicate that a resistance gene against *P. persistens* is located in subregion 2 and another resistance gene against *P. graminis lolii* is located in the distal half of subregion 3. For *P. hordei-secalini* contradictory results were obtained regarding its location and possibly even two resistance genes are located in the *Rnhq* region. More fine-mapping is required to confirm the obtained results and to determine the exact location of the resistance genes.

Commented [M1]: Delete

Commented [M2]: … using/in two different barley mapping populations: L94 x L94-Rnhq and SusPtrit x L94-Rnhq (BC1S2 and BC1S2).

Commented [M3]: I would first introduce the Rnhq qtl and then talk about the aim of your thesis and the subsequent results you found.

Commented [M4]: ing

Commented [M5]: Please reorder the second sentence. If you start talking about Pgl, why pursuing directly with Pp and at the end, coming back to Pgl???

Table of Contents

Introduction 1
Materials and Methods 3
Plant material 3
DNA extraction and molecular marker analysis 3
Pathogen material 4
Phenotyping 4
L94-Rnhq x L94 mapping population (P. graminis lolii fine-mapping) 4
SusPtrit-Rnhq x SusPtrit mapping population (P. graminis lolii fine-mapping) 5
SusPtrit-Rnhq x SusPtrit mapping population (P. hordei-secalini) 5
SusPtrit-Rnhq x SusPtrit mapping population (P. persistens) 6
Statistical analysis 7
Results 8
Fine-mapping of P. graminis lolii resistance 8
Region where resistance gene is located narrowed down using L94-Rnhq x L94 population 8
No P. graminis lolii resistance detected when using SusPtrit-Rnhq x SusPtrit mapping population 9
Contradictory indications to where Puccinia hordei-secalini resistance in Rnhq region is located 13
Evidence that Puccinia persistens resistance gene is located in subregion 2 of the Rnhq region 17
Segregation BC_1S_1 mapping population 19
Discussion 20
Future recommendations 22
References 23
Appendix 25
1. Primer sequences 25
2A. Seed bags used for experiments 26
2B. Parental BC_1S_1 plant used to develop BC_1S_2 population 26
3A. Difference between VIS and IF measure 27
3B. Raw data P. graminis lolii experiments using BC_1S_1 population 27
4. Raw data P. hordei-secalini experiment using BC_1S_2 population 36
5. Raw data P. persistens experiments 37
6. LightScanner output 39
7. qPCR material and methods 40

Introduction

Barley is the fourth largest cereal crop in the world, with a production of 148.7 million tons in 2016 (USDA 2017). Rust diseases caused by the pathogenic fungi of the *Pucciniales* order can severely impact yield with losses up to 45% (Lim and Gaunt 1986). This loss is due to abnormal phloem-transport of nutrients and reduced photosynthesis induced by the rust pathogens (Pozsaar and Kiraaly 1966). Rust damage on yield can be reduced by spraying fungicides (Conry and Dunne 1993). On one hand, fungicides result in control of rust diseases. On the other hand, they have a negative impact on the environment and they also cost money resulting in less profit for farmers. Another method to prevent rust infection in cereals is breeding for resistance. Selection of crops can be done by looking for the most resistant phenotypes and / or use genetic selection. The latter, also known as marker assisted breeding, can be used if a resistance genes is known. The selected plants can consequently be used in the next breeding cycle to further improve the crop.

As sessile organisms, plants have evolved a complex immune system to prevent pathogenic attacks. Resistance can be divided into host resistance and nonhost resistance. Pathogens have a very narrow range of host plants for which they can suppress the plant immune system. Host plants and their adapted pathogens co-evolve together. In resistant cultivars, the host recognizes pathogenic effectors via R-genes, whose main class consists of a nucleotide binding (NB) domain and a leucine rich repeat (LRR) domain. This recognition of pathogen effectors by a NB-LRR domain leads to cell death around the initial infection site (hypersensitive response; HR). Due to HR the pathogen is not able to feed and grow and hence the plant will not suffer from disease. This HR resistance mechanism is called host resistance (Flor 1971). However, the disadvantage of host resistance is that a mutation of the pathogen's effector might lead to an infected plant, as the NB-LRR domain cannot recognize the pathogen's effector anymore (Bakkeren et al. 2012; Johnston et al. 2013). To avoid fast breakage of host resistance it is possible to stack multiple resistance genes (R-genes) in one cultivar (gene pyramiding). As more R-genes are present after gene pyramiding the chance that one of the pathogen's effectors will be recognized are increased compared to plants containing only one R-gene. Therefore the cultivar will be resistant for a longer period of time (Singh et al. 2011; Johnston et al. 2013). There is also a non-hypersensitivity resistance called partial resistance. Partial resistance results in a longer latency period or less severe disease symptoms after infection and has a polygenic nature (Qi et al., 1998). Due to the polygenic nature this type of resistance is considered to be more durable compared to single R-genes. The disadvantage, however, is that partial resistance does not confer complete resistance.

In contrast to host resistance, there is also nonhost resistance (NHR). NHR is defined as the immunity displayed by all accessions within a plant species against all pathovars of a pathogenic species (Heath 1981). NHR is achieved through preformed physical barriers like papilla formation, callose formation and subsequent biochemical barriers (Bakkeren et al. 2012). NHR is considered to be a durable form of resistance as multiple genes (quantitative trait loci; QTL) are involved (Jafary et al., 2006).

At first it might not seem useful to study NHR, because nonhost plants do not suffer from pathogens that cannot infect them due to NHR. However, durable disease resistance is a goal for breeders, although at the moment very little is known about how NHR works and how it is inherited. If in the future more is known about NHR it might be possible to integrate it in breeding programmes to achieve durably resistant plants. A major drawback of studying nonhost resistance is that all members of the plant species are resistant to the pathogen you want to study. Furthermore, to study the inheritance of resistance, both resistant and susceptible parents are required. A solution to this problem is to use 'near nonhosts' which are plant-pathogen combinations for which only few accessions within a plant species are moderately susceptible to the pathogenic species (Niks 1988). Barley is a near nonhost to several heterologous rusts such as *P. hordei-murini, P. triticina, P. persistens, P. hordei-secalini and P. graminis lolii* (Atienza et al., 2004)

In 1998, (Qi et al. 1998) started to study partial resistance against barley leaf rust (*Puccinia hordei 1.2.1)* using a recombinant inbred line (RIL) population derived from a cross between Vada and a landrace called L94. This population was also used to create a dense linkage map. For the partial resistance, six QTLs were found (Qi et al. 1998). The same population was also screened with heterologous rusts *Puccinia triticina* and *P. hordei-murini* at seedling stage, to determine if the found QTLs also played a role in the resistance to these heterologous rusts (Niks et al. 2000). During this screening a QTL from Vada was discovered,

which was effective against *P. hordei-murini* and to a lesser extent to *P. triticina.* This nonhost QTL (*Rnhq*) was mapped on the long arm of chromosome 1 (7H). van Dijk et al. (2007) continued the research on the *Rnhq* by using the mapping population L94 x L94-*Rnhq* and narrowed down the region where the resistance to *P. hordei-secalini* and *P. hordei-murini* is possibly located (84.10 - 86.65cM ; 2.55cM). Later research revealed that *Rnhq* consists of three subregions which are all involved in resistance to one or several heterologous rusts. Subregion 1 at 63cM contains a resistance gene for *P. hordei-murini* and *P. hordei-secalini,* subregion 2 at 86cM for *P. triticina* and subregion 3 at 94cM to *P. graminis-lolii* (Salunke et al., 2010). This latest region is of main interest during this thesis.

Additionally, in 2004 a new barley research line, called SusPtrit, was developed by Atienza et al. to study *P. triticina,* as L94 was only very moderately susceptible to this rust. Later, SusPtrit was screened with other heterologous rusts such as *P. hordei-secalini, P. hordei-murini* and *P. graminis-lolii*, which showed an equal or enhanced susceptibility compared to L94. Consequently, SusPtrit is a suitable background for NHR studies.

During this thesis the aim was to further fine-map the gene that confers nonhost resistance to *P. graminis lolii*, which is located in subregion 3 according to Salunke et al. (2010). The L94-*Rnhq* x L94 mapping population was used for this. In addition a new mapping population derived from L94-*Rnhq* x SusPtrit was tested to check whether this population could also be used for fine-mapping of NHR against *P. graminis lolii* as SusPtrit is more susceptible compared to L94 it might facilitate fine-mapping of the NHR gene for *P. graminis lolii* and a BAC library for further map-based cloning is available for SusPtrit.

A side-project of this thesis was fine-mapping of nonhost resistance genes against *P. hordei-secalini* and *P.persistens* in the new L94-*Rnhq* x SusPtrit population. First the *Rnhq* was checked for resistance against *P. hordei-secalini* as it was found by both Jafary et al. (2006) and Van Dijk et al. (2007). Jafary et al. (2006) found QTLs overlapping the *Rnhq* region which conferred resistance to heterologous rusts *P. persistens* and *P. triticina* using a mapping population derived from Vada x SusPtrit. However, Van Dijk, et al. (2007) did not find these resistances in the *Rnhq* region in the L94 x L94-*Rnhq* mapping population as L94 is near-immune to *P. persistens*. Van Dijk et al. (2007) proposed that a more susceptible SusPtrit background would enable a better detection of the QTLs as the differences in susceptibility between the parents would be bigger. Therefore it was expected that fine-mapping the *P. persistens* NHR gene in *Rnhq* would be possible using the new mapping population (L94-*Rnhq* x SusPtrit).

Materials and Methods

Plant material

During this thesis, two mapping populations were used. The first mapping population was derived from the cross L94 x L94-*Rnhq*. L94-*Rnhq* is a near isogenic line (NIL) containing a susceptible L94 background and the *Rnhq* region from Vada and was developed by Niks et al. (unpublished). A new mapping population was developed by Daniau (2015) as infection tests with *P. graminis lolii* resulted in lots of necrotic spots. The new mapping population was made by crossing L94-*Rnhq* x SusPtrit. SusPtrit is a barley research line that is in the seedling stage fully susceptible to *P. triticina* and to some other heterologous rusts among which is *P. graminis lolii* (Atienza et al., 2004). An advantage of SusPtrit over L94 as susceptible parents is that SusPtrit is more susceptible to *P. graminis lolii* than L94, which should facilitate the phenotyping and fine-mapping of the resistance gene. The mapping population derived from L94-*Rnhq* x SusPtrit which was backcrossed to SusPtrit is named SusPtrit-*Rnhq* BC_1S_1. During this project plants from SusPtrit-*Rnhq* BC_1S_1 that were heterozygous for *Rnhq* were selfed, resulting in a BC_1S_2 mapping population. As control Vada, L94-*Rnhq*, L94, SusQnh or Sus*Rnhq,* and SusPtrit were used (Table 1). Only few seeds of SusQnh (with a Vada introgression for subregion 2 and 3) were available, therefore as soon as Sus*Rnhq* (seeds of selfed SusPtrit-*Rnhq* BC_1S_1 plant with Vada introgression for whole *Rnhq* region) was available this one was used as control.

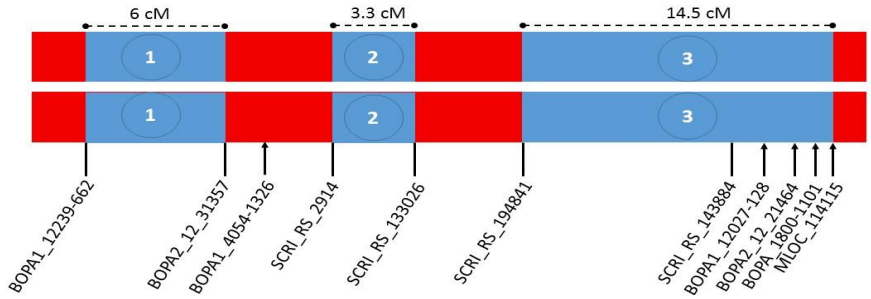
Table 1 Genotype of Rnhq *of the used control lines.*

	Subregion 1		Subregion 2		Subregion 3	
	BOPA1_12239-662	BOPA2_12_31357	SCRI_RS_2914	SCRI_RS_133026	SCRI_RS_194841	SCRI_RS_143884
cM	63.3	66.2	84	85.7	91.6	99.4
Vada	V	V	V	V	V	V
L94-*Rnhq*	V	V	V	V	V	V
L94	L	L	L	L	L	L
Sus*Rnhq*	V	V	V	V	V	V
SusQnh	S	S	V	V	V	V
SusPtrit	S	S	S	S	S	S

DNA extraction and genotyping by LightScanner

Genomic DNA was isolated using the following protocol. 2 cm of fresh plant material was sampled in cluster tubes containing two beads (Ø3.2 mm chrome steel). After addition of 150µl DNA extraction buffer (10% (v/v) Tris_HCl (0.5M, pH=7.5), 1.76% (w/v) NaCl, 10.27% (w/v) sucrose in MiliQ water) the tissue was smashed using a TissueLyser II (Qiagen) for 2 min at 30 Hz/min. The samples were centrifuged for 2 min at 2500 rpm (Heraeus multifuge 3S-R) and then incubated at 70°C for 10 min. The cell debris was pun down by centrifuging for 5 min at 4000 rpm (Heraeus multifuge 3S-R). The supernatant was transferred to a new 96 well plate and diluted to 10ng/µl before being used as a template for further genotyping.

SNP markers in combination with high resolution melting (HRM) analysis were used to determine the genotype of the plants (Wittwer et al., 2003). Regions containing a SNP were amplified using Phire taq enzyme and Veriti 96 wells Thermal Cycler PCR system (Thermofisher Scientific). Used primers and the corresponding location of the SNP are presented in Appendix 1. The LightScanner™ (Idaho Technology Inc.) in combination with LCgreen® Plus (Biofire Diagnostics Inc.) was used for genotyping (Wittwer et al., 2003).



Most SNP markers were designed based on the available consensus map. During this project new SNP *Figure 1 Overview of available SNP markers and newly designed SNP markers (arrows) in* Rnhq *region. SCRI_RS_143884 was first regarded as distal flanking marker of subregion 3.*

markers were designed at the end of subregion 3 in order to locate the gene responsible for resistance against *P. graminis lolii* using the L94-*Rnhq* x L94 population (Table 2; primer sequences in Appendix 1). Figure 1 gives an overview of the location of the flanking markers and newly designed markers.

SNPs BOPA1_12027-128, BOPA2_12_21464 and BOPA1_1800-1101 were mentioned by Salunke et al. (2010) but not available as markers at PBR department of Wageningen UR. These primers were designed based on SNP data present in a consensus map (version 2013) available at the PBR department of Wageningen UR. Additionally a flanking marker for subregion 3 was designed based on genotyping by sequencing (GBS) data from Cynara Romero (unpublished). For the SusPtrit-*Rnhq* x SusPtrit population experiments one marker was designed which is located between subregion 1 and subregion 2 as there was a large gap (Figure 1; Table 2). The marker was designed based on an available consensus map and SNP data at the PBR department of Wageningen UR.

Table 2 Markers used for fine-mapping and their location according to consensus map. Sequences are presented in Appendix 1 (=cM based on the consensus map)*

New marker	Marker	Location (cM)	Subregion
	BOPA1_12239-662	63.3	1
	BOPA2_12_31357	66.2	
X	BOPA1_4054-1326	72.4	
	SCRI_RS_2914	84.0	2
	SCRI_RS_133026	85.7	
	SCRI_RS_194841	91.6	3
	BOPA2_12_21479	94.7	
	BOPA1_2444-437	98.3	
	SCRI_RS_196885	99.1	
	SCRI_RS_143884	99.4	
X	BOPA1_12027-128	99.6	
X	BOPA2_12_21464	128.6 *	
X	BOPA1_1800-1101	128.6 *	
X	MLOC_114115	Flanking	

Pathogen material

Three rusts were used for inoculation: *P. graminis* f. sp. *lolii, P. persistens and P. hordei-secalini*. These rusts were multiplied on their respective host plants: *Lolium perenne, Elytrigia repens and Hordeum secalinum.* Urediniospores were harvested and either, immediately used for inoculation, or stored in a desiccator until used.

Phenotyping

L94-Rnhq x L94 mapping population (P. graminis lolii fine-mapping)

Seeds derived from selfed homozygous recombinants of the L94-*Rnhq* x L94 mapping population were grown in boxes together with Vada, L94 and L94-*Rnhq* as controls. Table 3 shows the genotypes and phenotypes of the homozygous recombinants. Based on this information, progeny seeds were picked for further fine-mapping (data from Mathilde Daniau). The used selection criterium was that the genotypes should be complementary, for example like rec103

and rec1177. Per homozygous recombinant 15±3 seedlings were tested, except for rec103 which had a bad germination level (10%) and yielded only 7 seedlings.

Table 4 Genotypes of BC_1S_1 plants that were selfed to yield BC_1S_2 seeds. BC_1S_2 seedlings were used in a fine-mapping experiment of P. hordei-secalini.

		SCRI_RS_194841 (91.6 cM)	BOPA2_12_21479 (94.7 cM)	BOPA1_2444-437 (98.3 cM)	SCRI_RS_196885 (99.1 cM)	SCRI_RS_143884 (99.4 cM)	Phenotype
Box1	rec242	L	L	L	L	V	R
	rec978	L	L	V	V	V	R
	rec1148	V	V	L	L	L	S
Box2	rec103	V	V	V	L	L	S
	rec1177	L	L	L	V	V	R
Box3	rec1162	L	L	L	V	V	S
	rec2487	L	L	L	V	V	S
	rec2474	L	L	L	V	V	S

Table 3 Genotype and phenotype of eight different homozygous recombinants whose seeds were used for fine-mapping subregion 3.

Twelve days after sowing, completely unfolded primary leaves were placed in a horizontal position with the adaxial side facing up using pins (Π-shaped). Other leaves were removed. Inoculation was performed using a mixture of 1 mg fresh *P. graminis lolii* spores, 10 mg *P. graminis lolii* spores out of liquid nitrogen stock and 80 mg Lycopodium spores per box. A settling tower was used to evenly distribute the spores on the primary leaves. After inoculation the seedlings were incubated overnight in a humidity room. After the pins were removed the boxes were transferred to the greenhouse. At 14 days post infection (DPI) the number of pustules, flecks and necrotic spots was counted and the leaf length and width were measured. These values were used to calculate the visible infection sites (VIS; the number of flecks, pustules and necrotic spots per square centimeter) and the infection frequency (IF; the number of pustules per square centimeter). After phenotyping, plants with a heterozygous *Rnhq* region were transplanted and selfed to get a BC_1S_2 population.

SusPtrit-*Rnhq* x SusPtrit mapping population (*P. graminis lolii* fine-mapping)

A similar approach was used for the SusPtrit-*Rnhq* x SusPtrit experiment. Around 30 seeds, derived from selfing a still segregating BC_1S_1 plant, were sown per box and additionally Vada and SusPtrit were sown as controls. Sus*Rnhq* (having the Vada genotype for the *Rnhq* region) was not yet available to use as control. Per experiment one seed bag (containing seeds derived from selfing one BC_1S_1 plant) was randomly chosen. The used seed bags can be found in Appendix 2A. In total the experiment was replicated eight times. The boxes were inoculated with 6 to 8 mg *P. graminis lolii* spores mixed with 10 times the amount Lycopodium.

SusPtrit-*Rnhq* x SusPtrit mapping population (*P. hordei-secalini*)

For fine-mapping of *P. hordei-secalini* resistance, two experiments were performed using the BC_1S_1 mapping population. Per experiment 30 seeds were sown from a randomly chosen seed bag (Appendix 2A) together with 5 Vada and 5 SusPtrit seeds as control. For one experiment SusQnh was added as additional control, which has the Vada introgression only for subregion 2 and 3, because Sus*Rnhq* was not available yet. The boxes were inoculated with 2 and 3 mg *P. hordei-secalini* spores. An additional experiment was performed once BC_1S_2 seeds were available after selfing BC_1S_1 plants that were heterozygous for the *Rnhq* region. Appendix 2B lists the parents from which the BC_1S_2 were derived.

Large amounts of BC_1S_2 seeds were sown in order to get complementary genotypes in the area of subregion 2 and 3. Table 4 shows part of the genotype on which the selection was based. After genotyping of the seedlings mostly homozygous recombinants genotype were selected and transplanted into a box, which was subsequently inoculated with 2.8 mg of *P. hordei-secalini* spores mix with Lycopodium. The genotype of the selected seedlings used for the experiment are presented in Table 5. As control SusPtrit and Sus*Rnhq* were used.

	Subregion 1		Subregion 2		
	BOPA1_12239-662	BOPA2_12_31357	SCRI_RS_2914	SCRI_RS_133026	
	63.3 cM	66.2 cM	84.0 cM	85.7 cM	# Seeds
rec62	V	V	H	-	32
rec10	H	S	S	-	24
rec44	H	H	V	-	40
rec37	S	S	V	-	8
rec19	S	S	S	-	50

Table 5 Genotypes of BC_1S_2 seedlings that were selected and used in a fine-mapping experiment of P. hordei-secalini.

	Subregion 1		Subregion 2		
	BOPA1_12239-662	BOPA2_12_31357	SCRI_RS_2914	SCRI_RS_133026	
	63.3 cM	66.2 cM	84.0 cM	85.7 cM	# Seeds
Sus*Rnhq*	V	V	V	V	5
SusPtrit	S	S	S	S	5
rec62	V	V	V	-	4
rec62	V	H	S	-	2
rec10	V	S	S	-	5
rec37	S	S	V	-	4
rec19	S	S	S	-	5

SusPtrit-*Rnhq* x SusPtrit mapping population (*P. persistens*)

For fine-mapping of *P. pesistens* resistance, two experiments were performed using the BC_1S_1 SusPtrit-*Rnhq* x SusPtrit mapping population. Per experiment 30 seeds were sown from a randomly chosen seed bag (Appendix 2A). Additionally 5 seeds of Vada and SusPtrit were sown and during one experiment also 5 seeds of SusQnh. The boxes were inoculated with 2 and 4 mg *P. persistens* spores. An additional experiment was performed when BC_1S_2 seeds were available, Appendix 2B lists the parents from which the BC_1S_2 seeds were derived. Similarly to the *P. hordei-secalini* experiment, large amounts of BC_1S_2 seeds were sown and after genotyping seedlings having a wanted homozygous recombinant genotype were selected. Table 6 shows the genotype of the parental BC_1S_1 genotypes. After selection of the seedlings they were transplanted into a box together with SusPtrit and Sus*Rnhq,* which was consequently inoculated with 3.2 mg of *P. persistens* spores mixed with Lycopodium. Unfortunately, only eight seedlings of one homozygous recombinant genotype were available. Table 7 shows the genotype used for inoculation with focus on subregion 2 and 3.

Table 6 Genotypes of BC_1S_1 plants that were selfed to yield BC_1S_2 seeds. BC_1S_2 seedlings were used in a fine-mapping experiment of P. persistens.

	Subregion 2		Subregion 3		
	SCRI_RS_2914	SCRI_RS_133026	SCRI_RS_194841	SCRI_RS_143884	
	84.0 cM	85.7 cM	91.6 cM	99.4 cM	# Seeds
rec58	V	V	V	-	12
rec47	V	V	H	-	52
rec51	H	S	S	-	80
rec3	H	V	V	-	52
rec17	S	S	H	-	52
rec19	S	S	H	-	50

Table 7 Genotype of BC1S2 seedlings used for inoculation with 3.2 mg P. persistens *and number of seedlings used.*

	Subregion 2		Subregion 3		
	SCRI_RS_2914	SCRI_RS_133026	SCRI_RS_194841	SCRI_RS_143884	
	84.0 cM	85.7 cM	91.6 cM	99.4 cM	# Seeds
Sus*Rnhq*	V	V	V	V	5
SusPtrit	S	S	S	S	5
rec3	S	V	V	-	16

Statistical analysis

The statistical program SPSS Statistics 24 was used to perform various statistical analyses. For the *P. graminis lolii* fine-mapping in L94-*Rnhq* x L94 population and the *P. hordei-secalini* and *P. persistens* experiments using the SusPtrit-*Rnhq* x SusPtrit BC_1S_2 population, one-way ANOVA was performed to determine if statistical significant differences between the genotypes were present regarding disease symptoms. Tukey was used as post hoc test if the homogeneity of variances assumption was met, otherwise Games-Howell was used. To test if the average phenotype per marker allele was significantly different, two sample independent t-tests were performed. The resulting p-value was converted to a LOD score (-Log (p-value)).

For the SusPtrit-*Rnhq* x SusPtrit BC_1S_1 experiments a chi-square test was performed to check if the alleles were segregating according to Mendelians law (1:2:1). Next, the phenotypic data were converted to Relative Visible Infection Sites (RVIS), with the average of parent SusPtrit taken as 100%. ANOVA and post hoc test Tukey were performed to determine if there was a statistical significant difference regarding RVIS between the plants homozygous for the Vada allele and those homozygous for the SusPtrit allele.

Results

Fine-mapping of *P. graminis lolii* resistance

Resistance gene is not found to be located in subregion 3 when using L94-Rnhq x L94 population

To fine-map *P. graminis lolii* resistance, a disease test was performed using homozygous recombinants of the L94-*Rnhq* x L94 mapping population and 11 mg *P. graminis lolii* spores (fresh and frozen mixed). The seedlings were genotyped to validate the genotype found in the parental plants (Table 8a). Table 8b shows that the seedlings of recombinant 103 and 242 had a different genotype than expected based on the data from the parental plants. The differences are highlighted in Table 8b. The results of flanking marker MLOC_114115, which was newly designed and tested on the seedlings, are included in Table 8b as well.

Table 8a. Genotypic and phenotypic data of the homozygous recombinant parental plants which were selfed. 8b. Genotypic and phenotypic data of the corresponding offspring. Found genotypic differences are highlighted. The RIF and RVI
Different letters indicate a significant differenc

a.

		SCRI_RS_194841 91.6 cM	BOPA2_12_214 94.7 cM
Box1	rec242	L	L
	rec978	L	L
	rec1148	V	V
Box2	rec103	V	V
	rec1177	L	L
Box3	rec1162	L	L
	rec2487	L	L
	rec2474	L	L

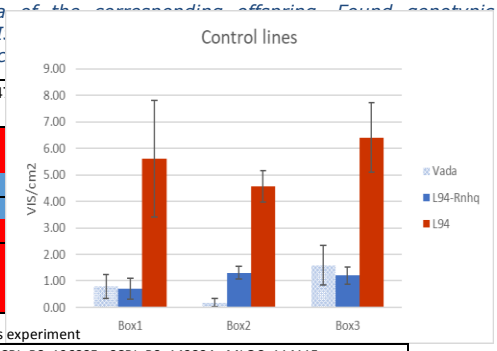


Figure 2 VIS/cm2 of parental lines per box.

b.

		SCRI_RS_194841 91.6 cM	BOPA2_12_21479 94.7 cM	BOPA1_2444-437 98.3 cM	SCRI_RS_196885 99.1 cM	SCRI_RS_143884 99.4 cM	MLOC_114115 Flanking marker	Phenotype	RIF		RVIS	
Box1	rec242	L	L	L	L	L	L	R	0.35	a	0.35	b
	rec978	L	L	V	V	V	V	R/I	0.37	a	0.69	b
	rec1148	V	V	L	L	L	L	S	1.26	b	1.18	a
Box2	rec103	L	L	V	V	V	V	R	0.06	a	0.23	a
	rec1177	L	L	L	V	V	V	R	0.31	a	0.32	a
Box3	rec1162	L	L	L	V	V	V	R	0.24	a	0.27	a
	rec2487	L	L	L	V	V	V	R	0.21	a	0.23	a
	rec2474	L	L	L	V	V	V	R	0.21	a	0.23	a

The phenotype was measured as the visible infection sites (VIS; the number of pustules, flecks and necrotic spots) per cm^2 and the infection frequency (IF; the number of pustules) per cm^2. During this experiment the seedlings were distributed over three boxes (Table 8b). The parental lines had a comparable number of infection sites (Figure 2), but to be able to make fair comparisons between all seedlings the VIS was converted to relative VIS (RVIS; VIS/cm^2 relative to the VIS/cm^2 of the susceptible parent SusPtrit in the same box) and the IF was converted to relative IF (RIF; IF/cm^2 relative to the IF/cm^2 of the susceptible parent SusPtrit in the same box). Jafary et al. (2006) found similar results for RVIS and RIF and used only the RVIS for fine-mapping.

In this experiment both RVIS and RIF are presented in Figure 3, but the difference between RVIS and RIF is small. The ANOVA results regarding the phenotypic data are shown in Table 8b. Regarding the RVIS, recombinants 978 and 1148 are both statistically significantly different from the other recombinants, whereas for the RIF only recombinant 1148 is different. This means that recombinant 1148 is more susceptible than the other recombinants tested and that recombinant 978 is only significantly more susceptible when RVIS is used as phenotypic measure. The difference between RIF and RVIS statistics might be due to the relatively small sample sizes (15±3 seeds; rec103 only 7 seeds). If larger sample sizes are tested it is likely that the results of RIF and RVIS will be more similar.

These phenotypic results are different than was previously found for the parental homozygous recombinants, which is highlighted in Table 8b. In this experiment only one homozygous recombinant was

clearly susceptible (rec1148), whereas for recombinants rec103, rec1162, rec2487 and rec2474 also susceptible phenotype was expected based on the parental data.

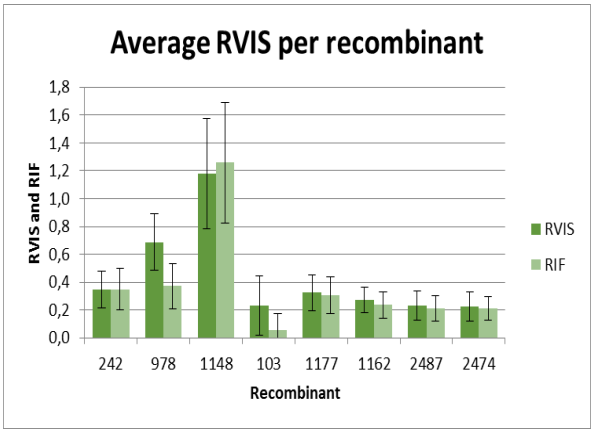


Figure 3 Average RVIS and RIF of homozygous recombinants infected with 1mg fresh + 10 mg stored P. graminis lolii spores. Per homozygous recombinant 15+-3 seedlings were used, for nr. 103 only 7 seedlings were used. The error bars represent the

Only homozygous recombinant 1148 had a clear distinctive, more susceptible, phenotype compared to the other tested homozygous recombinants, and it seems that the resistance gene is located at the downstream of marker BOPA2_12_21479 (94.7 cM). However, recombinant 242 contradicts this hypothesis as the plants show a resistant phenotype, while having a susceptible L94 genotype in subregion 3. As SNPs were present on the righthand side of marker SCRI_RS_143884 according to the report of Salunke et al. (2010), four new markers developed on the right hand side (distal side) of SCRI_RS_143884 (Figure 4).

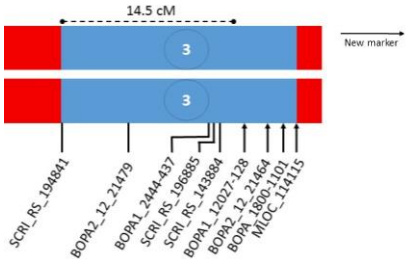


Figure 4 Overview of markers in subregion 3. Newly designed markers are indicated with an arrow. The location of the new markers is not exactly known in the used consensus map.

Newly developed markers BOPA1_12027-128, BOPA2_12_21464 and BOPA1_1800-1101 did not show different peaks for Vada and L94 allele due to high GC content and thus could not be used for genotyping using the LightScanner. Another marker was designed (MLOC114115) based on a SNP found in genome by sequencing (GBS) data (Cynara Romero, unpublished). However, the genotype of the homozygous recombinants is similar for the flanking marker and SCRI_RS_143884. Based on this data it was not possible to conclude were the resistance gene is located, except that it is on the righthand side of marker BOPA2_12_21479 (94.7 cM).

No *P. graminis lolii* resistance detected when using SusPtrit-*Rnhq* x SusPtrit mapping population

To fine-map *P. graminis lolii* resistance in the SusPtrit-*Rnhq* x SusPtrit mapping population eight disease tests were performed with in total 240 BC_1S_1 plants. The plants were still segregating for the *Rnhq* QTL as they were BC_1S_1. For phenotyping both the VIS/cm^2 and the IF/cm^2 were determined but little difference was observed between the two measures (Appendix 3A). Therefore only the VIS data were used. To be able to compare the results of the eight replications, the VIS was converted to RVIS, original genotype and phenotype data is presented in Appendix 3B. For genotyping five SNP markers in subregion 3 (SCRI_RS_194841, BOPA2_12_21479, BOPA1_2444-437, SCRI_RS_196885, SCRI_RS_143884) were selected to fine-map the resistance gene. The average RVIS was calculated per Vada and SusPtrit marker allele. The average RVIS for the five Vada marker alleles in subregion 3 showed little difference between the markers within each experiment (Figure 4). The same was observed for the SusPtrit marker alleles. However, when one of the markers would be closely linked to the resistance gene you would expect a peak for the RVIS of the SusPtrit allele and a trough for the RVIS of the Vada allele. This pattern was not observed in any of the eight replications, but still per marker a higher RVIS was observed for plants containing the SusPtrit allele compared to Vada allele. In six out of the eight replications this difference

was a statistical significant for two to all five markers (α=0.05). Only in experiment 7 and 8 no statistical significant difference was found between RVIS of markers having either the Vada or SusPtrit allele.

Figure 4 also shows that the RVIS values in experiment 3, 5 and 6 are quite low (RVIS<0.5), as you expect a RVIS around 1.0 for plants having the SusPtrit allele. Experiment 5 and 6 were performed using seeds out of the same seed bag. Therefore it is possible that this lower susceptibility is due to a line effect, where

something in the background causes this decrease in susceptibility. The seeds in experiment 3 were derived from a different seed bag, but nevertheless a similar effect might be observed.

As overall no large differences between the RVIS values between Vada and SusPtrit alleles and no peaks and troughs were observed, it was concluded that the SusPtrit-*Rnhq* x SusPtrit is not suitable for *P. graminis lolii* resistance fine-mapping in subregion 3.

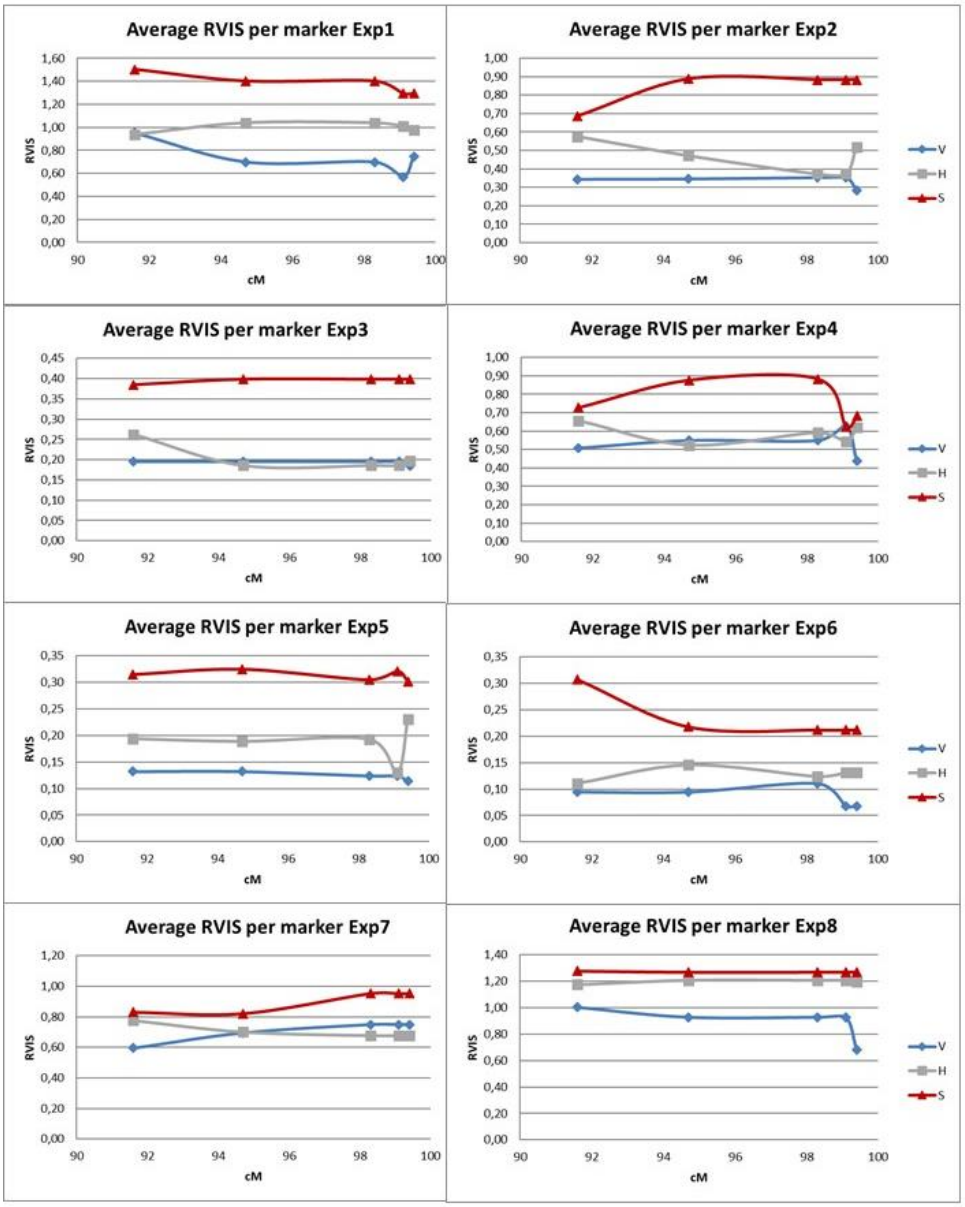


Figure 5 Average relative visible infection sites (RVIS) per marker within subregion 3 after inoculation with P. graminis lolii. *The VIS is relative to susceptible parent SusPtrit, thus the SusPtrit control would be a straight line at 1.0. The result of each experiment is presented in a separate graph. V=Vada, H=Heterozygous, S=SusPtrit.*

Contradictory indications to where *Puccinia hordei-secalini* resistance in *Rnhq* region is located

Two attempts were done to fine-map resistance against *P. hordei-secalini* using the BC_1S_1 mapping population. The (supposedly) flanking markers of subregion 1, 2 and 3 were used to determine the location of a possible resistance gene. The genotype and phenotype of the tested plant in experiment 1 are presented in Table 9. From the SusPtrit control line only one seed germinated and had a high VIS/cm² (32,6). The BC_1S_1 offspring had considerably less disease symptoms (except for plant 65), therefore the RVIS is quite low. No clear link between phenotype and genotype is visible (Table 9). Plants with the resistant Vada genotype in the *Rnhq* region (plant 94, 96, 93) have a similar number of VIS/cm² as plants having the susceptible SusPtrit genotype (plant 71, 78).

Figure 5 shows the average RVIS per marker of experiment 1. No big peak and trough is visible, but when looking at the related LOD scores (converted p-value) a significant difference was detected between the average RVIS of plants having Vada allele or SusPtrit allele in subregion 1 (α=0.05, similar to LOD score=1.3) (Figure 6). Despite the detected significance, this significance cannot be used to draw conclusions on the location of a QTL as for fine-mapping a LOD score of at least 2.7 is used.

Table 9 Genotype and phenotype of plants tested during the first experiment to fine-map P. hordei-secalini.

	Subregion 1		*Subregion 2*		*Subregion 3*			
Plant number	**63.3**	**66.2**	**84.0**	**85.7**	**91.6**	**99.4**	**VIS/cm2**	**RVIS**
94	V	V	V	V	V	V	5,38	0,17
96	V	V	V	V	V	V	3,17	0,10
93	V	V	V	V	V	V	4,21	0,13
79	H	V	V	V	V	V	6,39	0,20
76	H	H	V	V	V	V	3,97	0,12
88	H	H	V	V	V	H	0,77	0,02
72	H	H	V	V	H	V	2,29	0,07
74	V	V	H	H	H	H	2,42	0,07
84	V	V	H	H	H	H	1,15	0,04
90	V	V	H	H	H	H	2,97	0,09
97	V	V	H	H	H	H	2,46	0,08
69	V	V	H	H	H	S	4,59	0,14
70	V	V	H	H	H	S	2,86	0,09
73	V	H	H	H	H	H	4,37	0,13
77	V	H	H	H	H	H	2,29	0,07
64	H	H	H	H	H	V	5,19	0,16
67	H	H	H	H	H	V	5,97	0,18
68	H	H	H	H	H	V	1,18	0,04
91	H	H	H	H	H	V	3,64	0,11
92	H	H	H	H	H	V	2,48	0,08
95	H	H	H	H	H	V	0,20	0,01
66	H	H	H	H	H	H	2,84	0,09
87	H	H	H	H	H	H	2,12	0,07
85	H	S	H	H	H	H	5,22	0,16
75	S	S	V	V	V	V	2,03	0,06
83	H	H	H	S	S	S	4,65	0,14
81	H	S	H	H	S	S	4,24	0,13
86	H	H	S	S	S	S	4,36	0,13
89	H	H	S	S	S	S	3,38	0,10
82	H	S	S	S	S	S	1,06	0,03
80	S	H	S	S	H	V	1,35	0,04
65	S	S	S	S	S	H	21,61	0,66
71	S	S	S	S	S	S	2,02	0,06
78	S	S	S	S	S	S	4,71	0,14

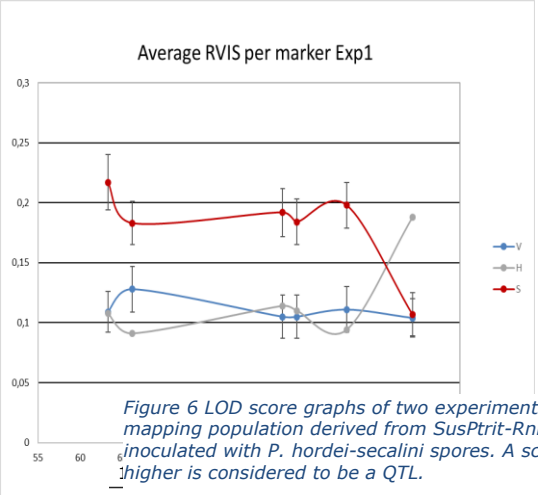
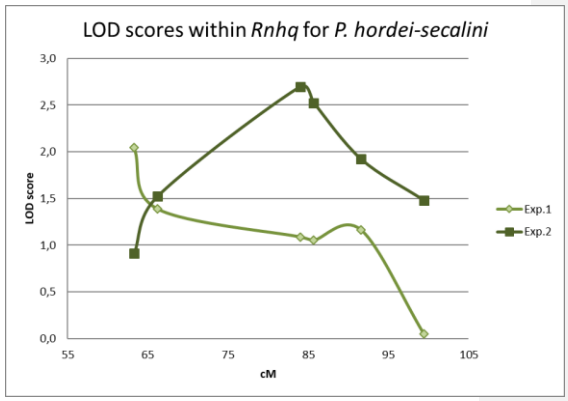


Figure 6 LOD score graphs of two experiments using BC1S1 mapping population derived from SusPtrit-Rnhq x SusPtrit inoculated with P. hordei-secalini spores. A score of 2.7 or higher is considered to be a QTL.



The results of the second experiment are shown in Table 10. In this table the plants that are Vada for the whole *Rnhq* are clearly less susceptible than plants having the SusPtrit genotype for the whole *Rnhq.* SusQnh has an intermediate phenotype which suggests that the resistance gene is not located within subregion 1, otherwise the phenotype would be expected to be more similar to the susceptible SusPtrit phenotype. Figure 7 shows the average RVIS per genotype per marker. In subregion 2 (underlined) a small peak and trough is visible, indicating that in this region there seems to be a correlation a low RVIS (relatively resistant phenotype) and Vada genotype within subregion 2 or high RVIS (susceptible phenotype) and SusPtrit genotype. This observation is supported by the results of the ANOVA test, which are presented as LOD scores in Figure 6. Plants having a SusPtrit marker allele within *Rnhq,* except for the proximal (left) flanking marker of subregion 1, had a statistically significant higher RVIS than plants having the Vada marker allele. The flanking markers of subregion 2 have high LOD scores meaning that there is a high correlation between the phenotypes and genotypes. LOD scores of 2.7 or higher are considered represent a QTL. Thus these results indicate that the resistance gene to *P. hordei-secalini* is likely to be located near the proximal flanking marker of subregion 2.

Figure 5 Average RVIS per marker of BC1S1 plants inoculated with 2mg of P. hordei-secalini*. Subregions are indicated with a line and respective number. Error bars represent standard error.*

The graph of the average RVIS of plants being heterozygous for one or several markers was included to check for dominance of the resistance gene (Figure 5, 7). In Figure 5 it seems that the gene is dominant as the heterozygous graph is close to the Vada graph. However, at the end of the heterozygous graph the RVIS is even higher than for SusPtrit making it difficult to draw real conclusions. During the second experiment the phenotype of plants being heterozygous was in between the phenotype of Vada and SusPtrit. This seems like incomplete dominance. However, more experiments should be performed to draw a definite conclusion about dominance.

Table 10 Genotype and phenotype of plants tested during the first experiment to fine-map P. hordei-secalini. For genotyping the flanking markers of the three subregions were used. For the control groups the average is given.

Subregion 1 *Subregion 2* *Subregion 3*

Plant number	63.3	66.2	84.0	85.7	91.6	99.4	VIS/cm2	RVIS
SusPtrit	S	S	S	S	S	S	1,51	1,00
SusQnh	S	S	V	V	V	V	0,53	0,35
Vada	V	V	V	V	V	V	0,05	0,03
371	V	V	V	V	V	V	0,00	0,00
373	V	V	V	V	V	V	0,57	0,38
386	V	V	V	V	V	V	0,68	0,45
387	V	V	V	V	V	V	0,45	0,30
375	V	V	V	V	V	H	0,13	0,09
372	V	V	V	V	V	H	0,59	0,39
380	V	V	H	H	H	H	0,28	0,18
385	V	V	H	H	H	H	2,35	1,55
378	V	H	S	S	S	S	3,05	2,02
383	H	V	V	V	V	V	0,36	0,24
365	H	H	V	V	V	V	0,25	0,17
382	H	H	V	H	H	V	0,12	0,08
368	H	H	H	H	V	V	0,71	0,47
377	H	H	H	H	H	H	1,76	1,16
381	H	H	H	H	H	H	0,83	0,55
367	H	H	H	H	H	V	2,72	1,80
374	H	H	H	H	S	S	0,73	0,48
384	H	H	H	H	S	S	1,00	0,66
388	H	H	H	H	S	S	0,46	0,31
369	H	H	S	S	H	H	0,82	0,54
370	S	S	S	S	H	S	1,68	1,11
366	S	S	S	S	S	S	1,69	1,12
379	S	S	S	S	S	S	1,91	1,26
376	S	S	U	S	S	S	1,32	0,87

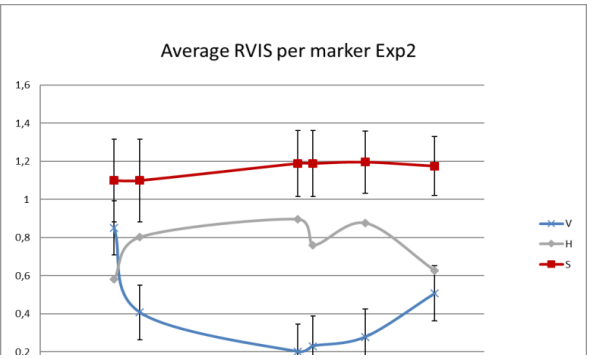


Figure 7 Average RVIS for the flanking markers of the three subregions per genotype. Data from inoculation of BC1S1 plants with 3 mg (frozen) P. hordei-secalini spores. Error bars represent standard error

A new fine-mapping experiment was performed once the BC_1S_2 mapping population was available. Figure 8 (left) shows the average RVIS per genotype including the standard deviation. Different symbols above the bars indicate that those RVIS are statistically significantly different. The raw data is included in Appendix 4. The BC_1S_2 seedlings containing SusPtrit alleles for the whole *Rnhq* region had a high RVIS comparable to the SusPtrit control. The remaining genotypes (VVVVVV, VHSSSV, VSSSSV, SSVVVV) had a RVIS that is statistically significantly the same as the resistant SusPtrit-*Rnhq* control (Figure 8, left). These results reveal that only plants having a SusPtrit allele at the distal end of subregion 3 are statistically significantly more susceptible than SusPtrit-*Rnhq*, suggesting that the resistance gene might be located at the distal end of subregion 3. The LOD score graph shows clearly a high peak for the distal marker of subregion 3 (LOD score = 5.8), but additionally a smaller peak at the proximal marker of subregion 1 (Figure 8, right). Thus this indicates that the resistance gene can be at either end of the *Rnhq* region.

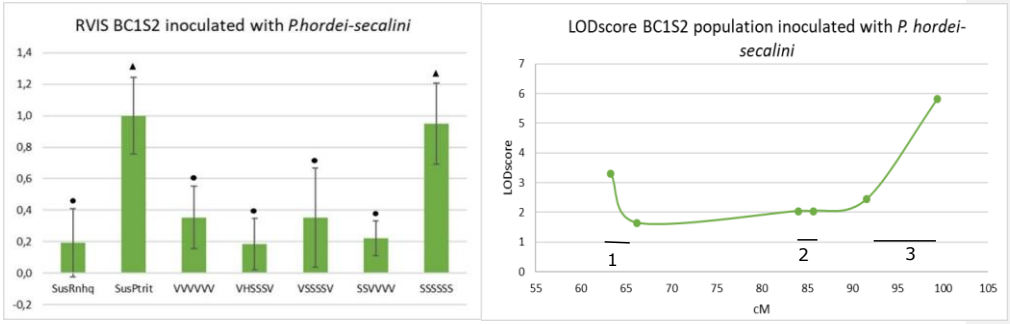


Figure 8 Left: Average RVIS per genotype of BC_1S_2 population (n=4-5, except VHSSSV; n=2. Error bars represent standard deviation). Similar symbols indicate that RVIS is not statistically significantly different (Tukey, a=0.05). Right: LOD score graph of BC_1S_2 population inoculated with P. hordei-secalini. Each dot represents either the proximal or distal flanking marker of subregion 1, 2, and 3 respectively. *A LOD score of 2.7 or higher is considered to mark a QTL.*

Per experiment the suggestion of where the resistance gene might be located is different. As the used sample sizes are quite small repeating the experiment using more seedlings per genotype of the BC_1S_2 mapping population should give a clearer results. It would also be better to have more complementary genotypes, not only complementary for the first two subregions as in this experiment but for the whole *Rnhq* region. When all complementary genotypes are present the distribution of Vada and SusPtrit alleles per marker will be nearly equal, which gives the statistical analysis more power.

Evidence that *Puccinia persistens* resistance gene is located in subregion 2 of the *Rnhq* region

Two experiments were performed using the BC_1S_1 SusPtrit-*Rnhq* x SusPtrit population for fine-mapping the location of a possible resistance gene against *P. persistens* in the *Rnhq* region. In the first experiment 2 mg of inoculum was used resulting in VIS/cm^2 values of the (non-parental) seedlings ranging from 0.0 to 3.3 and two outliers of 4.1 and 10.7 VIS/cm^2). The parental VIS/cm^2 values ranged from 0.0 to 0.52 for Vada and from 2.05 to 12.56 for SusPtrit (raw data in Appendix 5). Due to the low VIS/cm^2 values for the non-parental seedlings the RVIS (compared to SusPtrit as explained earlier) is quite low. Despite the small difference in average RVIS for the Vada and SusPtrit alleles (Figure 9; left), the difference was statistically significant for both flanking markers of subregion 2 (p=0.012 at 84.0 cM and p=0.006 at 85.7 cM) and the proximal flanking marker of subregion 3 (p=0.019 at 91.6 cM) (Figure 10). Even though the difference was statistically significant (α=0.05) it could not be considered a QTL, as it can only be considered a QTL if p<0.002. However as the used sample size was quite small for fine-mapping, it would be worthwhile to test a bigger sample size to see if a QTL located between or within subregion 2 and 3 can be found as Figure 9 (left) suggests.

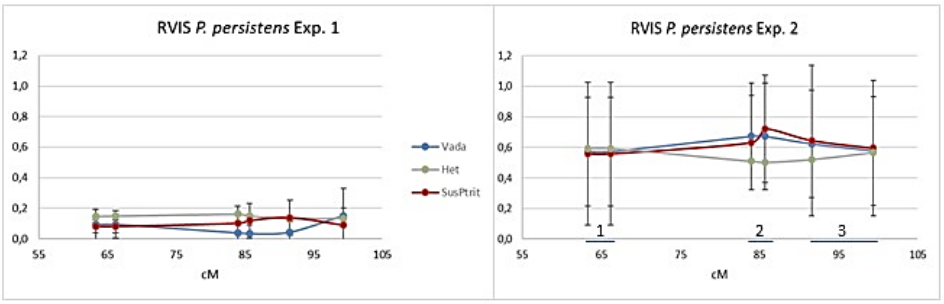


Figure 9 Average RVIS per marker genotype for P. persistens *tests. During the first test 2 mg of* P. persistens *spores were used. During the second experiment 4 mg of spores were used. Error bars represent standard deviation.*

The second experiment was conducted using 4 mg spores for inoculation to get a higher infection frequency. The VIS/cm^2 values of the non-parental seedlings ranged from 0.88 to 16.45, Vada from 0.90 to 1.88, SusQnh from 6.37 to 14.09 and SusPtrit from 9.54 to 14.95. Thus the higher inoculation density in the second experiment resulted in more visible infection sites. Figure 9 (right) shows that the average RVIS per genotype was higher when using 4 mg of spores instead of 2 mg. However, no significant differences in average RVIS between Vada and SusPtrit were found (Figure 10). The average RVIS was lowest when the seedling had a heterozygous genotype, but this was not statistical significant.

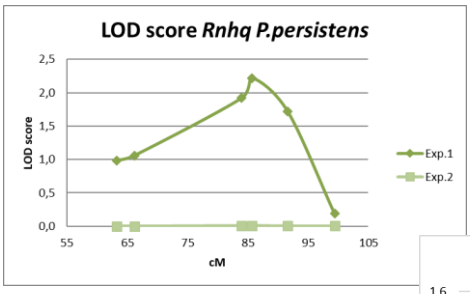


Figure 10 LOD scores of both P. persist*experiments using BC₁S₁ population. A LOD sc of 1.3 is similar to a p-value of 0.05. A LOD sc of 2.7 (p=0.002) or higher is considered to b QTL.*

Whereas the results of the non-parental seedlings do not reveal where the resistance gene might be located, the control lines do reveal the possible location. Table 11 shows the genotype and average phenotype of the control lines Vada, SusQnh and SusPtrit. SusQnh has a similar phenotype as SusPtrit, therefore these results suggest that the resistance is located on the proximal side of subregion 2.

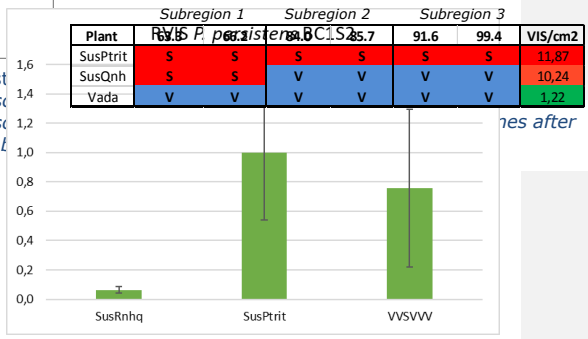


Figure 11 Average RVIS per genotype SusRnhq is homozygous Vada for the whole Rnhq *region. Error bars represent standard deviation. For SusRnhq and SusPtrit n=5, for VVSVVV n=8.*

To test the hypothesis that the resistance gene is located within or between subregion 2 and 3 as the results of the first experiment suggest, an experiment using the BC_1S_2 mapping population was performed. Best would be to use complementary genotypes for subregion 2 and 3 as shown in Appendix 2B. However only one homozygous recombinant was available (VVSVVV). Some parental (BC_1S_1) lines appeared to have a different genotype than was previously assigned (Appendix 2B), resulting in less variability in genotypes. But still the only homozygous recombinant genotype was suitable to confirm whether the resistance is close to the proximal marker of subregion 2 (around 84 cm) as suggested by the data of the first experiment. The RVIS of the homozygous recombinant is significantly higher than Sus*Rnhq* (p=0.019), but not significantly different from SusPtrit (p=0.67) (Figure 11). These results indicate that the resistance gene might be located close to the proximal marker of subregion 2.

Overall the results of the first BC_1S_1 and the BC_1S_2 experiment together indicate that the resistance gene is located in subregion 2. More specifically according to the BC_1S_2 experiment it seems that the resistance gene is located near the proximal end of subregion 2. It is worthwhile to develop and screen more markers in between subregion 1 and 2 as there is a big distance between those subregions and the resistance gene might be located just on the proximal side of subregion 2.

Segregation BC_1S_1 mapping population

The segregation was, according to the Chi-square test, in agreement with the expectation, except for marker SCRI_RS_143884 which is located at the distal side of subregion 3 (Table 12). The X^2 value of this particular marker is 10.50 while X_{crit}= 5.99 (df=2, a=0.05) meaning that the segregation deviates from the expected 1:2:1 ratio. Thus more plants being homozygous Vada were detected than expected and less heterozygous genotypes than expected. Genotyping using the LightScanner™ is based on comparing the melting peak of a BC_1S_1 plant to melting peaks of Vada and SusPtrit by eye. This causes that some errors might be made in assigning which genotype a certain plant has. However a heterozygous peak is clearly different from the homozygous peaks thus it is unlikely that the heterozygous genotype was mistaken for a homozygous genotype.

*Table 12 Chi square test to check if segregation of marker alleles is according to Mendelians law in SusPtrit-Rnhq BC_1S_1 mapping population. Xcrit=5.99; df=2; a=0.05). 'N' is the number of alleles tested for the corresponding marker (=number of plants * 2).*

Subregion	Marker name	Consensus Map	X2 value	N
1	SCRI_RS_136556	63.0	1.06	62
	BOPA1_12239-662	63.3	2.74	273
	BOPA2_12_31357	66.2	0.25	335
2	SCRI_RS_2914	84.0	0.20	335
	SCRI_RS_133026	85.7	0.03	335
3	SCRI_RS_194841	91.6	3.46	334
	BOPA2_12_21479	94.7	0.05	330
	BOPA1_2444-437	98.3	1.08	335
	SCRI_RS_196885	99.1	1.06	333
	SCRI_RS_143884	99.4	10.50	336

Discussion

Previous research by Fernandez et al. (2000) using a Vada xL94 RIL population indicated that a nonhost QTL (*Rnhq*) was located on chromosome 7H. Later, a NIL were developed by Niks et al. (unpublished), which was used by Van Dijk et al. (2007) and Salunke et al. (2010). They found that the *Rnhq* consists of three subregions and that these confer resistance to *P. graminis lolii* (subregion 3), *P. hordei-secalini* and *P. persistens*. Daniau continued the research on *Rnhq* using the L94-*Rnhq* x L94 mapping population and focussed on subregion 3 which confers resistance to *P. graminis lolii.* However, problems were encountered as many necrotic spots were present during phenotyping. Therefore the SusPtrit-*Rnhq* BC_1S_1 mapping population was developed and used to continue fine-mapping of the *P. graminis lolii* resistance gene.

The SusPtrit-*Rnhq* BC_1S_1 population was used during eight *P. graminis lolii* disease tests. The results showed that plants were slightly more resistant when the Vada allele was present in the *Rnhq* region. However, from these experiments no clear conclusions could be drawn on the location of the resistance gene, which was probably due to the presence of other QTLs present in the SusPtrit background (Jafary et al., 2006). Therefore it was concluded that the SusPtrit-*Rnhq* BC_1S_1 mapping population was not suitable for mapping the *P. graminis lolii* resistance gene in subregion 3.

To continue with fine-mapping of nonhost resistance gene against *P. graminis lolii* in subregion 3, the previous mapping population was used again (L94-Rnhq x L94). The genotype of two homozygous recombinants was different than expected based on available data about the parental genotypes. These errors are likely human errors. When genotyping using the LightScanner™, it can be hard to distuinguish the Vada and L94 peak. Incorrect assignment of a peak can explain the different genotype found for Rec. 242. For Rec. 1148 it is more likely that the seed bag was mislabelled or that the wrong plant was transplanted as four out of five marker genotypes are different.

For phenotyping, no problems were encountered as only few necrotic spots were present at 14 DPI. However several days later, all disease symptoms were transformed into necrotic spots. This increase in number of necrotic spots can occur if the hypersensitive response is dependent on the developmental stage of the plant. Therefore it is of great interest to inoculate seedlings at different time points to determine the optimal time point for inoculation to get the best phenotypic results for fine-mapping.

Even though clear differences were observed between homozygous recombinant genotypes regarding the phenotype, it was not possible to fine-map the resistance gene as one genotype did not match the expected phenotype (Rec. 242). Although Salunke et al. (2010) found that the region between 94-99 cM in subregion 3 conferred resistance to *P. graminis lolii,* this cannot be confirmed with the results of this thesis. As the resistance gene might be located just on the distal side of 99 cM developing more markers around 99 cM with focus on the distal side should give an answer to the question whether the resistance gene is located at the distal end of subregion 3.

Jafary et. al (2006) reported that the region between 65-75 cM in the Vada x SusPtrit mapping population (presumably subregion 1) conferred resistance to *P. hordei-secalini*. Salunke et al. (2010) reported that the region conferring resistance to *P. hordei-secalini* was located around 63 cM when using the L94-Rnhq x L94 mapping population, but Van Dijk et al. (2007) mentioned the region around 85 cM as possible location. During this thesis the new SusPtrit-*Rnhq* x SusPtrit mapping population was used but per experiment a different possible location for the resistance gene against *P. hordei-secalini* was found. Once, at 63.3 cM in subregion 1 a correlation between genotype and phenotype was found that could be considered a QTL (LOD score = 3.3), which is in agreement with Salunke et al. (2010). However, there are also indications that the resistant gene is located near 85 cM (subregion 2; LOD score = 2.7) as Van Dijk et al. (2007) suggest or near 99 cM (subregion 3; LOD score = 5.8). It might be possible that instead of one resistance gene in the *Rnhq* region there are multiple resistance genes present, which would partially explain that different possible locations are found. An experiment with more homozygous recombinant seedlings per genotype and more complementary genotypes should be performed in order to locate the position of the resistance gene(s).

In addition to the *P. hordei-secalini* resistance, Jafary et al. (2006) also found a QTL located between 85-100 cM (presumably subregion 2 and 3) conferring resistance to *P. persistens.* The first BC_1S_1 and the BC_1S_2 experiments could confirm that the resistance gene is likely to be located near 85 cM. During the second BC_1S_1 experiment no significant differences were found between plants having Vada or SusPtrit

alleles. This can be caused by the background of the tested line. As the BC_1S_1 mapping population has still some L94 in the background, there might be some resistance genes from L94 making it impossible to detect a difference in susceptibility. Similar observations were done by Van Dijk et al. (2007) when he was trying to fine-map *P. persistens* resistance using the L94-*Rnhq* x L94 population. He could not detect a QTL around 85 cM because L94 has other resistance genes outside of the *Rnhq* region. For future experiments it is therefore important that another line is selected than line 1318 (Appendix 2A). The BC_1S_2 experiment could only be performed using one homozygous recombinant line. Parental lines that were sown appeared to have a different genotype than previously was determined using the LightScanner™ (Appendix 2B). For one plant all marker genotypes were different, thus it seems that the wrong plant was transplanted. Furthermore two lines showed an error for marker SCRI06. This marker could not always detect the parental Vada and SusPtrit alleles even on pure DNA, indicating that the primers do not function optimally. Additionally the difference in melting temperature between the two primers is less than 1°C which can result in errors as the peaks will be hard to distinguish (Herrmann et al., 2006). Designing new primers close to the current ones might solve the problem of errors in the assigned genotype.

A Chi-square test revealed that for one marker the alleles were not segregating according to the Hardy-Weinberg principle (1:2:1). This marker, SCRI_RS_143884, is located near the distal end of subregion 3. Less heterozygous genotypes were observed than expected, but more homozygous Vada genotypes than expected. It is unlikely that this deviation is due to incorrect assignment of the genotype, as the LightScanner™ curve for heterozygous genotypes is distinct from the homozygous curves (Appendix 6 for example of LightScanner™ output). A skewed segregation might appear when plants are selected for beneficial traits (which are linked to a gene). Normally a BC_1 population consists of two genotypes; a uniform heterozygous genotype and a homozygous genotype similar to the backcross parent. As the homozygous genotype does not segregate it is not useful for the mapping population and therefore these plants were removed. On the remaining heterozygous plants from the BC_1 population no phenotypical selection has been performed as all plants had the same genotype for the region of interest. Therefore skewed segregation due to selection is not the cause. A likely possibility is that the DNA, which is extracted using the 'quick and dirty' protocol, is of too low quality (Pompanon et al., 2005) and as a consequence only one of the two alleles of the heterozygous sample is detected leading to incorrect genotyping as homozygote (Taberlet et al, 1996). Even though incorrect genotyping is not favourable, the 'quick and dirty' protocol is fast and cheap compared to high quality DNA extraction and sequencing. It might be worthwhile to extract DNA from one plant using both the 'quick and dirty' method and high quality DNA method and to compare the sequences to determine if a different extraction method would improve genotyping.

Future recommendations

For all fine-mapping experiments it is useful to design new primers to replace the primers of marker SCRI_RS_194841. For this marker several errors were found and additionally it does not always amplify the parental Vada and SusPtrit DNA which makes it hard to assign the peak of the offspring. Thus new primers with a melting temperature difference of 1°C should result in less errors during genotyping.

The results of the fine-mapping experiments of *P. graminis lolii* in the L94-Rnhq x L94 mapping population do not confirm previous research. Therefore the interval of SCRI_RS_194841 (91.6 cM) to SCRI_RS_143884 (99.4 cM) was incorrect. To continue fine-mapping more markers need to be developed on both the proximal and distal side of the flanking marker. On the proximal side as there might have been recombination between SCRI_RS_143884 and the flanking marker resulting in a small Vada introgression between these markers. And on the distal side of the flanking marker using the GBS data of Cynara.

For fine-mapping of the *P. hordei-secalini* there is evidence that the resistance is located near 63 cM. However to confirm this marker SCRI_RS_136556 (position 63.0 cM) should be tested and an additional marker proximal of 63.0 cM. Also the experiment with homozygous recombinants should be repeated using more plants (for example n=10 per genotype) and preferably with all complementary genotypes as shown in Appendix 2B. These experiments should confirm if the resistance is located in subregion 1 or as suggested in the second and third experiment to be in subregion 2 or 3 respectively.

P. persistens resistance is likely to be located near 85 cM (Jafary et al., 2006). It would be of great value to have at least one recombinant between each pair of adjacent markers and to have to complementary recombinant genotype as well (visible in Appendix 2B). More BC_1S_2 seeds should be sown and genotyped to obtain the complementary homozygous recombinant genotypes. Once all homozygous recombinant genotypes are available it should be possible to confirm whether the resistance is near 85 cM.

Once the region which is linked to resistance is narrowed down to 0.5-0.8 cM the research can be continued by looking for possible resistance genes within this region. A bacterial artificial chromosome (BAC) library screening For barley it is expected that around 10-15 genes are present within 1cM. Databases like Ensembl-Plant (TGAC+ version) and IPK Barley can be used to determine which (predicted) genes are present in the region of interest. This information can be used to check for polymorphism between the two parents that might explain a loss of function or the information can be used to design primers for the genes that are located in the region of interest. These primers can consequently be used for qPCR. With qPCR it is possible to determine if one of the genes within the region are upregulated during infection and thus might be involved in nonhost resistance (Material and Methods of qPCR in Appendix 7). If the qPCR shows that a gene is upregulated then that gene is a candidate nonhost resistance gene. To test if this gene is a nonhost resistance gene it can either be silenced in barley which would make barley more susceptible to the corresponding heterologous rust or it could be used to transform the host plant which should then become more resistant.

References

Atienza, S. G., et al. (2004). "Accumulation of genes for susceptibility to rust fungi for which barley is nearly a nonhost results in two barley lines with extreme multiple susceptibility." Planta **220**(1): 71-79.

Bakkeren, G., et al. (2012). "Functional genomic approaches in cereal rusts." Canadian Journal of Plant Pathology **34**(1): 3-12.

Conry, M. and B. Dunne (1993). "Effects of nitrogen timing and frequency of fungicide applications on grain yields of winter barley in Ireland." The Journal of Agricultural Science **120**(02): 181-186.

Flor, H. H. (1971). "Current status of the gene-for-gene concept." Annual review of phytopathology **9**(1): 275-296.

Heath, M. C. (1981). "A generalized concept of host-parasite specificity." Phytopathology **71**(11): 1121-1123.

Jafary, H., et al. (2006). "Innate nonhost immunity in barley to different heterologous rust fungi is controlled by sets of resistance genes with different and overlapping specificities." Molecular plant-microbe interactions **19**(11): 1270-1279.

Johnston, P. A., et al. (2013). "Rph22: mapping of a novel leaf rust resistance gene introgressed from the non-host Hordeum bulbosum L. into cultivated barley (Hordeum vulgare L.)." Theoretical and Applied Genetics **126**(6): 1613-1625.

Herrmann, M. G., Durtschi, J. D., Bromley, L. K., Wittwer, C. T., & Voelkerding, K. V. (2006). "Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes." Clinical chemistry, **52**(3), 494-503.

Lim, L. G. and R. E. Gaunt (1986). "The effect of powdery mildew (Erysiphe graminis f. sp. hordei) and leaf rust (Puccinia hordei) on spring barley in New Zealand. I. Epidemic development, green leaf area and yield." Plant Pathology **35**(1): 44-53.

Niks, R. (1988). "Nonhost plant species as donors for resistance to pathogens with narrow host range. II. Concepts and evidence on the genetic basis of nonhost resistance." Euphytica **37**(1): 89-99.

Niks, R. E., et al. (2000). "Specificity of QTLs for partial and non-host resistance of barley to leaf rust fungi." Acta Phytopathologica et Entomologica Hungarica **35**(1/4): 13-21.

Parlevliet, J. (1978). "Further evidence of polygenic inheritance of partial resistance in barley to leaf rust, Puccinia hordei." Euphytica **27**(2): 369-379.

Pompanon, F., Bonin, A., Bellemain, E., & Taberlet, P. (2005). Genotyping errors: causes, consequences and solutions. *Nature Reviews Genetics*, *6*(11), 847-846.

Pozsaar, B. I. and Z. Kiraaly (1966). "Phloem-Transport in Rust Infected Plants and the Cytokinin-Directed Long-Distance Movement of Nutrients." Journal of Phytopathology **56**(3): 297-309.

Qi, X., et al. (1998). "Identification of QTLs for partial resistance to leaf rust (Puccinia hordei) in barley." Theoretical and Applied Genetics **96**(8): 1205-1215.

Qi, X., et al. (1998). "Use of locus-specific AFLP markers to construct a high-density molecular map in barley." Theoretical and Applied Genetics **96**(3): 376-384.

Salunke, A., et al. (2010). "Fine Mapping of Quantitative Trait Loci for Non-host Resistance to Rusts in Barley."

Singh, R. P., et al. (2011). "The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production." Annual review of phytopathology **49**: 465-481.

Taberlet, P., Griffin, S., Goossens, B., Questiau, S., Manceau, V., Escaravage, N., ... & Bouvet, J. (1996). Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic acids research*, *24*(16), 3189-3194.

USDA (2017). World Agricultural Production. Circular Series WAP 01-17, Foreign Agricultural Service/ USDA**:** 29.

Van Dijk, T., et al. (2007). Characterization of a QTL for Non-Host Resistance in Barley, MSc, Thesis, Wageningen University, Plant Breeding Department, The Netherlands.

Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G., & Pryor, R. J. (2003). High-resolution genotyping by amplicon melting analysis using LCGreen. *Clinical chemistry*, *49*(6), 853-860.

Appendix

1. Primer sequences

The markers used during this study, their position on the consensus map and sequence are represented in the table below.

New	Marker	Location (cM)	Subregion	5' … 3'
	SCRI_RS_136556	63.0	1	FW; AGCTTATCTTCGGCCTTTTCATATGGA RE; CGAAAACCATCGCCATGGAC
	BOPA1_12239-662	63.3		FW; GCCTATATGTCCCGAATATTCATCTAAGT RE; GGTGCCGGTATGCAACATC
	BOPA2_12_31357	66.2		FW; CTACTCGGCCAGAAGGTATGAATG RE; ACCTGGATAACTGGAAGACTGGG
x	BOPA1_4054-1326	72.4		FW; CATCCAGTACTTCTTCAGTTCC RE; ATGTACCAGCGAGCTCTGT
	SCRI_RS_2914	84.0	2	FW; CAGATATTGGGTCACATCAGGCAC RE; CAGTAACCAAGCAGCTTCACAATG
	SCRI_RS_133026	85.7		FW; TTCATCTCTTCATGTAGTTCAGCATAGACA RE; TCCAGACAATTGAAGTTCTAAACAACTGATT
	SCRI_RS_194841	91.6	3	FW; AGATCTGAACAACGCCGCC RE; GGCGTGCATGTGATGTGAG
	BOPA2_12_21479	94.7		FW; CAAGCTTACATTCTCAAGGAGAAAGAG RE; TCACTGTAATGCTGCTATATTCTCTTGT
	BOPA1_2444-437	98.3		FW; GTGCAATAGGGTGAAGAAGAAGATCTAA RE; ACGACCTTGGCTTCTCCCTC
	SCRI_RS_196885	99.0		FW; TCACGACGAGGACGGTG RE; GTGATCTCGCCGAAACTGTAGGTA
	SCRI_RS_143884	99.4		FW; GACTGAAGGCGGCCAAGA RE; GTGTTGAGGCTCAGGCCCAA
x	BOPA1_12027-128	99.6		FW; TCACGTAGGATTCTGGCTTG RE; TGTCCACAGATTCCACACC
x	BOPA2_12_21464	128.6 *		FW; ATGAGGGTGTACGCGAGC RE; GACCAGTCCGTCCTCACC
x	BOPA1_1800-1101	128.6 *		FW; CGTACTACATTTACATCATCCGAC RE; CGCATGAACCATGAGATGT
x	MLOC_114115	Flanking		FW; GTCCTTCCACTCGACGCC RE; GCAACGCCGAATATAACGACC

2A. Seed bags used for experiments

Table A2 gives an overview of the seed bags (BC_1S_1) used per experiment. Also the amount of rust used in the respective experiment is included in the table. Rust collected from the -80ºC freezer is assumed to be half as effective as fresh spores. Therefore roughly double the amount was used compared to when using fresh spores.

Rust	Experiment	Amount of rust (mg)	Seed bag number	Plant numbers
P. graminis lolii	1	8	1312	1-28
	2	8	1036	29-63
	3	6	1026	163-189
	4	6	1036	190-223
	5	13 (freezer)	1325	224-253
	6	13 (freezer)	1325	254-283
	7	6	1304	284-314
	8	6	1318	315-337
P. hordei-secalini	1	2	1311	64-97
	2	3 (freezer)	1304	365-388
P. persistens	1	2	1311	98-132
	2	4	1318	338-364

2B. Parental BC_1S_1 plant used to develop BC_1S_2 population

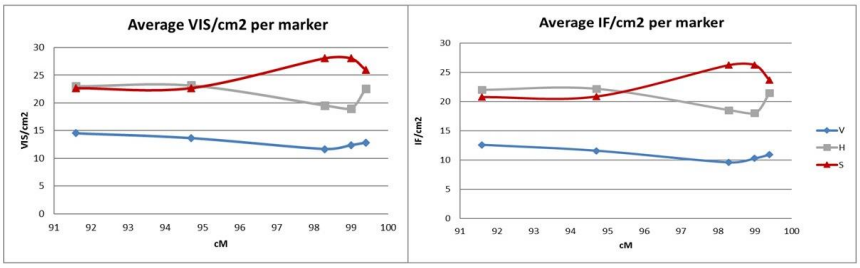
The genotype of region of interest of the parental plants used for selfing to get BC_1S_2 are represented under 'expected' in Table A3. As the offspring of some lines did not segregation at all, even though this was expected, it was concluded that the genotype of some parental lines could not be correct. The likely genotype of the parental lines derived from the information about the genotypes of the offspring is presented below 'Based in tested offspring'. As a reminder the genotypes that were preferred to use are presented on the right below 'wanted'. The offspring of which the seeds (BC_1S_2) were used for experiments with *P. hordei-secalini* and *P. persistens.*

	Expected				Based on tested offspring				Wanted			
	Subregion 1		Subregion 2		Subregion 1		Subregion 2		Subregion 1		Subregion 2	
Plant nr.	63.3	66.2	84.0	85.7	63.3	66.2	84.0	85.7	63.3	66.2	84.0	85.7
58	V	V	H		V	H	H		V	V	V	
47	V	V	H		V	H	H		V	V	S	
51	H	S	S		H	S	S		V	S	S	
3	H	H	V		H	H	V		S	V	V	
17	S	S	V		S	S	V		S	S	V	
19	S	S	S		S	S	S		S	S	S	

	Expected				Based on tested offspring				Wanted			
	Subregion 2		Subregion 3		Subregion 2		Subregion 3		Subregion 2		Subregion 3	
Plant nr.	84.0	85.7	91.6	99.4	84.0	85.7	91.6	99.4	84.0	85.7	91.6	99.4
58	V	V	V		V	V	V		V	V	V	
47	V	V	H		V	V	V		V	V	S	
51	H	S	H		V	V	V		V	S	S	
3	H	V	V		H	V	V		S	V	V	
17	S	S	H		S	S	S		S	S	V	
19	S	S	H		S	S	S		S	S	S	

3A. Difference between VIS and IF measure

Average VIS/cm^2 and IF/cm^2 per marker genotype in subregion 3. Results from BC_1S_1 population inoculated with 8mg *P. graminis lolii*. Only little difference can be observed between the two measures.



3B. Raw data *P. graminis lolii* experiments using BC_1S_1 population

Raw data of the eight BC_1S_1 experiments that were inoculated with *P. graminis lolii.* The flanking markers of subregion 1 and 2 are presented as well as the flanking markers of subregion 3 including some in between markers.

Experiment 1; Inoculation density 8 mg.

Plant number	63.0	66.2	84.0	85.7	91.6	94.7	98.3	99.0	99.4	VIS/cm2
Vada	V	V	V	V	V	V	V	V	V	1,26
Vada	V	V	V	V	V	V	V	V	V	5,70
Vada	V	V	V	V	V	V	V	V	V	1,57
Vada	V	V	V	V	V	V	V	V	V	1,90
Vada	V	V	V	V	V	V	V	V	V	6,67
SusPtrit	S	S	S	S	S	S	S	S	S	20,80
SusPtrit	S	S	S	S	S	S	S	S	S	20,75
SusPtrit	S	S	S	S	S	S	S	S	S	15,24
SusPtrit	S	S	S	S	S	S	S	S	S	32,11
7	V	V	V	V	V	V	V	V	V	30,00
26	V	V	V	V	V	V	V	V	V	10,15
27	H	H	V	V	V	V	V	V	V	11,39
25	H	H	H	H	V	V	V	V	V	17,68
18	V	V	V	V	V	V	V	H	H	16,86
3	V	V	H	V	V	V	V	H	H	25,75
11	V	V	V	V	V	H	H	H	H	24,82
1	H	H	H	H	H	V	V	V	V	7,95
14	S	S	H	H	H	H	H	H	V	24,70
13	V	V	V	H	H	H	H	H	H	17,87
2	V	V	H	H	H	H	H	H	H	21,30
20	V	H	H	H	H	H	H	H	H	25,19
6	H	H	H	H	H	H	H	H	H	17,53
28	H	H	H	H	H	H	H	H	H	22,05
24	H	H	S	S	H	H	H	H	H	11,54
22	S	H	H	H	H	H	H	H	H	20,62
16	S	S	H	H	H	H	H	H	H	16,98
23	S	S	H	H	H	H	H	H	H	16,17
12	H	H	H	H	H	H	H	S	S	23,96
21	H	H	H	H	H	S	S	S	S	24,57
4	H	H	S	S	H	S	S	S	S	14,85
17	H	H	S	S	H	S	S	S	S	39,52
10	H	S	S	S	H	S	S	S	S	34,49
19	S	S	S	S	H	S	S	S	S	36,67
8	H	H	S	S	H	U	S	S	S	43,75
5	H	S	H	H	H	U	S	S	S	8,94
9	H	H	S	S	S	S	S	S	S	34,55
15	H	H	S	S	S	S	S	S	S	32,28

Experiment 2; Inoculation density 8 mg

Plant number	63.0	66.2	84.0	85.7	91.6	94.7	98.3	99.0	99.4	VIS/cm2
Vada	V	V	V	V	V	V	V	V	V	1,47
Vada	V	V	V	V	V	V	V	V	V	0,44
Vada	V	V	V	V	V	V	V	V	V	2,11
Vada	V	V	V	V	V	V	V	V	V	2,03
SusPtrit	S	S	S	S	S	S	S	S	S	15,43
SusPtrit	S	S	S	S	S	S	S	S	S	12,10
SusPtrit	S	S	S	S	S	S	S	S	S	12,79
29	V	V	V	V	V	V	V	V	V	7,50
36	V	V	V	V	V	V	V	V	V	2,50
41	V	V	V	V	V	V	V	V	V	7,13
54	V	V	V	V	V	V	V	V	V	4,48
57	V	V	V	V	V	V	V	V	V	1,94
30	V	V	H	H	V	V	V	V	V	8,74
50	H	H	V	V	V	V	V	V	V	3,78
58	V	V	V	V	V	H	H	H	H	4,10
47	V	V	V	V	H	V	V	V	V	3,04
62	V	V	H	H	H	V	V	V	V	3,52
44	H	H	V	V	H	V	V	V	V	5,89
48	H	H	H	H	H	V	H	H	V	1,07
33	H	H	H	H	H	V	S	S	S	11,77
55	H	H	H	H	H	H	V	V	V	4,93
34	V	V	V	V	H	H	H	H	H	6,67
31	H	H	H	H	H	H	H	H	H	15,08
46	H	H	H	H	H	H	H	H	H	11,95
52	H	H	H	H	H	H	H	H	H	7,25
56	H	H	H	H	H	H	H	H	H	4,60
59	H	H	H	H	H	H	H	H	H	1,89
63	H	H	H	H	H	H	H	H	H	9,75
38	S	S	H	H	H	H	H	H	H	8,28
39	S	S	H	H	H	H	H	H	H	4,19
49	S	S	H	H	H	H	H	H	H	16,30
60	S	S	S	S	H	H	H	H	H	8,63
61	S	S	S	S	H	H	H	H	H	23,61
51	V	V	H	S	H	S	S	S	S	13,24
43	H	H	S	S	H	S	S	S	S	28,15
53	H	H	S	S	H	S	S	S	S	12,09
42	H	H	H	H	H	S	U	S	S	5,00
37	S	S	V	U	H	U	U	V	V	1,25
32	S	S	S	S	S	S	S	S	S	10,43
40	S	S	S	S	S	S	S	S	S	6,84
45	S	S	S	S	S	S	S	S	S	10,40
35	-	-	-	-	-	-	-	-	-	6,06

Experiment 3; Inoculation density 6 mg.

Plant number	63.3	66.2	84.0	85.7	91.6	94.7	98.3	99.0	99.4	
Vada	V	V	V	V	V	V	V	V	V	0,48
Vada	V	V	V	V	V	V	V	V	V	3,41
Vada	V	V	V	V	V	V	V	V	V	3,70
Vada	V	V	V	V	V	V	V	V	V	2,26
Vada	V	V	V	V	V	V	V	V	V	2,71
SusPtrit	S	S	S	S	S	S	S	S	S	17,76
SusPtrit	S	S	S	S	S	S	S	S	S	19,80
SusPtrit	S	S	S	S	S	S	S	S	S	12,00
SusPtrit	S	S	S	S	S	S	S	S	S	14,29
176	S	H	H	H	V	V	V	V	V	3,56
178	H	H	H	V	V	V	V	V	V	3,86
187	S	S	H	V	V	V	V	V	V	3,71
182	H	H	V	V	V	V	V	V	V	2,00
169	H	V	V	V	V	V	V	V	V	2,72
172	V	V	V	V	V	V	V	V	V	2,62
175	V	V	V	V	V	V	V	V	V	3,80
183	V	V	V	V	V	V	V	V	V	2,67
167	H	H	H	H	H	H	H	H	V	3,40
181	H	H	H	H	H	H	H	H	V	1,81
185	H	H	H	H	H	H	H	H	V	3,16
170	S	S	S	S	H	S	S	S	S	6,60
189	H	H	H	H	H	H	H	H	H	1,68
188	S	S	H	H	H	H	H	H	H	4,59
166	H	H	S	U	S	S	S	S	U	6,84
174	H	V	H	H	S	S	S	S	S	5,31
173	H	H	S	S	S	S	S	S	S	6,86
186	H	H	S	S	S	S	S	S	S	10,35
163	H	S	S	S	S	S	S	S	S	10,51
171	H	S	S	S	S	S	S	S	S	6,05
180	H	S	S	S	S	S	S	S	S	8,13
164	S	S	S	S	S	S	S	S	S	3,74
165	S	S	S	S	S	S	S	S	S	3,92
168	S	S	S	S	S	S	S	S	S	5,33
177	S	S	S	S	S	S	S	S	S	5,74
179	S	S	S	S	S	S	S	S	S	3,92
184	S	V	S	S	S	S	S	S	S	3,16

Experiment 4; Inoculation density 6 mg.

Plant number	63.3	66.2	84.0	85.7	91.6	94.7	98.3	99.0	99.4	VIS/cm2
Vada	V	V	V	V	V	V	V	V	V	0,91
Vada	V	V	V	V	V	V	V	V	V	0,14
Vada	V	V	V	V	V	V	V	V	V	1,27
Vada	V	V	V	V	V	V	V	V	V	0,27
SusPtrit	S	S	S	S	S	S	S	S	S	12,97
SusPtrit	S	S	S	S	S	S	S	S	S	12,66
SusPtrit	S	S	S	S	S	S	S	S	S	15,97
SusPtrit	S	S	S	S	S	S	S	S	S	16,43
197	H	H	H	H	V	V	V	V	V	5,81
200	H	H	H	H	V	V	V	V	V	4,36
204	V	V	V	V	V	V	V	V	V	3,94
209	H	H	V	V	V	V	V	V	V	6,81
210	H	H	V	V	V	V	V	V	V	8,33
219	H	H	V	V	V	V	V	V	V	14,27
191	H	H	H	V	V	H	H	H	H	8,92
208	H	V	V	V	V	H	H	H	H	8,73
215	S	H	H	H	V	H	H	H	H	4,04
222	H	H	V	V	V	H	H	H	H	9,49
223	V	V	H	H	V	H	H	H	H	6,21
203	H	H	H	H	H	V	V	V	V	8,67
195	H	H	V	H	H	H	H	H	V	8,63
199	H	H	H	H	H	H	H	H	V	10,36
218	V	V	H	H	H	H	H	H	V	9,06
190	H	H	H	H	H	H	H	H	H	9,40
194	H	S	H	H	H	H	H	H	H	9,32
198	V	H	S	S	H	H	H	H	H	3,79
206	H	H	H	H	H	H	H	H	H	5,07
211	V	V	H	H	H	H	H	H	H	6,09
212	H	H	S	S	H	H	H	H	H	5,16
216	S	S	S	S	H	H	H	H	H	5,47
217	S	S	H	H	H	H	H	H	H	16,41
221	S	U	V	H	H	H	H	H	H	7,32
193	S	S	S	S	H	S	S	S	S	21,67
196	H	H	S	S	H	S	S	S	S	11,18
201	V	V	H	H	H	S	S	S	S	10,40
202	H	H	H	H	H	S	S	S	S	6,55
220	H	H	U	S	H	S	S	S	S	11,82
205	S	S	S	S	S	H	H	H	H	5,95
213	H	H	S	S	S	S	H	H	H	12,51
192	H	H	S	S	S	S	S	S	S	21,94
207	H	H	V	V	S	S	S	S	S	11,08
214	S	S	S	H	S	S	S	S	S	6,49

Experiment 5; Inoculation density 13 mg from -80°C/liquid nitrogen.

Plant number	63.3	66.2	84.0	85.7	91.6	94.7	98.3	99.0	99.4	VIS/cm2
Vada	V	V	V	V	V	V	V	V	V	0,83
Vada	V	V	V	V	V	V	V	V	V	0,82
Vada	V	V	V	V	V	V	V	V	V	0,89
Vada	V	V	V	V	V	V	V	V	V	0,74
Vada	V	V	V	V	V	V	V	V	V	0,46
SusPtrit	S	S	S	S	S	S	S	S	S	8,85
SusPtrit	S	S	S	S	S	S	S	S	S	5,37
SusPtrit	S	S	S	S	S	S	S	S	S	11,03
238	V	V	V	V	V	V	V	V	V	2,29
243	H	H	H	H	V	V	V	V	V	1,09
249	H	H	V	V	V	V	V	V	V	0,90
253	H	H	V	V	V	V	V	V	V	0,15
237	H	H	H	H	H	H	V	V	V	0,98
233	S	S	H	H	H	H	H	H	V	0,79
224	H	H	V	V	H	H	H	H	H	0,43
226	S	S	H	H	H	H	H	H	H	1,48
228	H	H	H	H	H	H	H	H	H	1,41
229	H	H	H	H	H	H	H	H	H	0,86
230	H	H	H	H	H	H	H	H	H	0,76
231	S	H	H	H	H	H	H	H	H	1,19
235	H	H	H	H	H	H	H	H	H	0,63
239	V	V	H	H	H	H	H	H	H	1,59
240	H	U	H	H	H	H	H	H	H	2,16
241	H	H	H	H	H	H	H	H	H	0,69
244	V	V	H	H	H	H	H	H	H	0,96
245	V	V	H	H	H	H	H	H	H	2,64
246	S	S	S	S	H	H	H	H	H	2,50
247	H	S	S	H	H	H	H	H	H	1,63
251	V	V	V	V	H	H	H	H	H	0,36
252	S	S	S	S	H	H	H	H	H	1,39
236	H	H	H	H	H	H	H	S	S	3,23
242	H	H	H	H	H	H	S	S	S	2,06
225	S	S	H	H	H	S	S	S	S	1,53
250	H	H	S	S	S	H	H	H	H	1,13
232	S	S	S	S	S	S	S	S	S	2,79
248	H	S	S	S	S	S	S	S	S	3,92
227	S	S	S	S	S	H	H	H	H	1,40
234	S	S	S	S	S	S	S	S	H	3,33

Experiment 6; Inoculation density 13 mg from -80°C/liquid nitrogen.

Plant number	63.3	66.2	84.0	85.7	91.6	94.7	98.3	99.0	99.4	VIS/cm2
Vada	V	V	V	V	V	V	V	V	V	0,93
Vada	V	V	V	V	V	V	V	V	V	0,77
Vada	V	V	V	V	V	V	V	V	V	1,71
Vada	V	V	V	V	V	V	V	V	V	0,65
SusPtrit	S	S	S	S	S	S	S	S	S	5,98
SusPtrit	S	S	S	S	S	S	S	S	S	9,39
SusPtrit	S	S	S	S	S	S	S	S	S	9,28
SusPtrit	S	S	S	S	S	S	S	S	S	8,02
SusPtrit	S	S	S	S	S	S	S	S	S	6,20
264	V	V	V	V	V	V	V	V	V	0,13
280	V	V	V	V	V	V	V	V	V	0,35
273	H	H	H	H	V	V	V	V	V	1,09
281	H	H	H	H	V	V	V	V	V	0,54
279	V	V	V	V	V	V	V	H	H	0,89
272	H	H	H	H	V	V	V	H	H	1,47
262	V	V	V	V	V	V	H	H	H	0,49
257	H	H	V	H	H	H	H	H	H	0,98
271	H	H	V	H	H	H	H	H	H	3,25
277	V	V	H	H	H	H	H	H	H	1,51
261	V	H	H	H	H	H	H	H	H	1,03
283	V	H	H	H	H	H	H	H	H	0,22
255	H	H	H	H	H	H	H	H	H	0,69
259	H	H	H	H	H	H	H	H	H	2,07
265	H	H	H	H	H	H	H	H	H	0,26
266	H	H	H	H	H	H	H	H	H	3,18
274	H	H	H	H	H	H	H	H	H	0,68
278	H	H	H	H	H	H	H	H	H	0,07
282	H	H	H	H	H	H	H	H	H	0,83
258	S	S	S	S	H	H	H	H	H	2,67
270	H	H	H	H	S	S	H	H	H	1,06
276	S	H	H	H	S	S	S	H	S	1,47
260	H	H	H	H	H	H	S	S	S	1,10
268	V	V	H	S	H	H	S	S	S	0,76
263	H	H	H	H	H	S	S	S	S	0,31
254	V	V	S	S	S	S	S	S	S	3,60
256	H	H	S	S	S	S	S	S	S	8,73
269	H	S	S	S	S	S	S	S	S	4,93
267	S	S	S	S	S	S	S	S	S	0,57
275	S	S	S	S	S	S	S	S	S	2,91

Experiment 7; Inoculation density 6 mg.

Plant number	63.3	66.2	84.0	85.7	91.6	94.7	98.3	99.0	99.4	VIS/cm2
Vada	V	V	V	V	V	V	V	V	V	0,76
Vada	V	V	V	V	V	V	V	V	V	1,25
Vada	V	V	V	V	V	V	V	V	V	4,57
Vada	V	V	V	V	V	V	V	V	V	1,13
Vada	V	V	V	V	V	V	V	V	V	1,13
SusQnh	S	S	V	V	V	V	V	V	V	9,35
SusQnh	S	S	V	V	V	V	V	V	V	5,90
SusQnh	S	S	V	V	V	V	V	V	V	10,38
SusQnh	S	S	V	V	V	V	V	V	V	5,83
SusQnh	S	S	V	V	V	V	V	V	V	4,37
SusPtrit	S	S	S	S	S	S	S	S	S	15,88
SusPtrit	S	S	S	S	S	S	S	S	S	17,28
SusPtrit	S	S	S	S	S	S	S	S	S	9,39
SusPtrit	S	S	S	S	S	S	S	S	S	5,03
296	V	H	H	V	V	V	V	V	V	8,73
297	S	S	H	H	V	V	V	V	V	8,67
299	H	V	V	V	V	V	V	V	V	6,25
302	H	H	H	H	V	V	V	V	V	10,14
303	H	H	V	V	V	V	V	V	V	6,98
304	H	H	H	H	V	V	V	V	V	11,27
312	V	V	V	V	V	V	V	V	V	6,69
294	V	V	V	V	V	V	H	H	H	6,78
311	V	V	V	V	V	V	H	H	H	9,52
305	V	V	V	V	V	H	H	H	H	4,74
292	S	S	H	H	H	H	V	V	V	9,17
306	S	S	H	H	H	H	V	V	V	9,71
288	H	H	H	H	H	H	H	H	H	8,96
289	H	H	H	H	H	H	H	H	H	15,19
291	H	H	V	H	H	H	H	H	H	12,80
293	H	H	H	H	H	H	H	H	H	11,81
298	S	S	H	H	H	H	H	H	H	10,64
300	H	H	H	H	H	H	H	H	H	6,21
301	S	S	S	S	H	H	H	H	H	5,51
309	H	H	H	H	H	H	H	H	H	7,53
310	H	H	H	H	H	H	H	H	H	6,64
313	H	H	H	H	H	H	H	H	H	5,14
290	H	H	H	H	S	H	H	H	H	10,10
308	H	H	H	H	S	S	H	H	H	8,17
285	S	S	S	S	S	S	S	S	S	22,80
286	V	V	S	S	S	S	S	S	S	12,31
287	S	S	S	S	S	S	S	S	S	11,36
295	H	H	S	S	S	S	S	S	S	10,37
307	S	S	S	S	S	S	S	S	S	4,19
314	H	H	S	S	S	S	S	S	S	7,08

Experiment 8; Inoculation density 6 mg.

Plant number	63.3	66.2	84.0	85.7	91.6	94.7	98.3	99.0	99.4	VIS/cm2
Vada	V	V	V	V	V	V	V	V	V	0,97
Vada	V	V	V	V	V	V	V	V	V	1,88
Vada	V	V	V	V	V	V	V	V	V	1,43
Vada	V	V	V	V	V	V	V	V	V	2,13
Vada	V	V	V	V	V	V	V	V	V	0,93
SusQnh	S	S	V	V	V	V	V	V	V	9,34
SusQnh	S	S	V	V	V	V	V	V	V	13,66
SusQnh	S	S	V	V	V	V	V	V	V	3,68
SusQnh	S	S	V	V	V	V	V	V	V	2,34
SusQnh	S	S	V	V	V	V	V	V	V	4,18
SusPtrit	S	S	S	S	S	S	S	S	S	7,18
SusPtrit	S	S	S	S	S	S	S	S	S	7,01
SusPtrit	S	S	S	S	S	S	S	S	S	6,86
SusPtrit	S	S	S	S	S	S	S	S	S	11,25
SusPtrit	S	S	S	S	S	S	S	S	S	5,38
319	V	V	V	V	V	V	V	V	V	5,33
329	V	V	V	V	V	V	V	V	V	4,77
330	V	V	V	V	V	V	V	V	V	7,34
331	V	V	V	V	V	V	V	V	V	3,65
334	V	V	V	V	V	V	V	V	V	4,39
318	S	S	V	V	V	V	V	V	V	5,43
336	V	V	V	V	V	V	V	V	H	8,85
325	V	V	V	V	V	H	H	H	H	9,71
324	H	H	H	H	V	H	H	H	H	7,68
332	H	H	V	V	H	H	H	H	H	5,40
316	H	H	H	H	H	H	H	H	H	5,29
317	H	H	H	H	H	H	H	H	H	7,05
320	H	H	H	H	H	H	H	H	H	9,45
323	H	H	H	H	H	H	H	H	H	8,60
333	H	H	H	H	H	H	H	H	H	7,52
322	S	S	S	H	H	H	H	H	H	8,75
326	S	S	S	H	H	H	H	H	H	13,71
335	S	S	S	S	H	H	H	H	H	13,90
321	S	S	S	S	S	H	H	H	H	9,71
337	H	H	S	S	S	S	S	S	S	6,36
327	S	S	S	S	S	S	S	S	S	9,11
328	S	S	S	S	S	S	S	S	S	13,19

4. Raw data *P. hordei-secalini* experiment using BC_1S_2 population

Raw data of BC_1S_2 plants inoculated with 2.8 mg of *P. hordei-secalini* spores.

Plant number	63.3	66.2	84.0	85.7	91.6	99.4	VIS/cm2	RVIS
SusP-Rnhq	V	V	V	V	V	V	0,17	0,08
SusP-Rnhq	V	V	V	V	V	V	0,00	0,00
SusP-Rnhq	V	V	V	V	V	V	1,20	0,54
SusP-Rnhq	V	V	V	V	V	V	0,21	0,09
SusP-Rnhq	V	V	V	V	V	V	0,54	0,25
SusPtrit	S	S	S	S	S	S	2,65	1,20
SusPtrit	S	S	S	S	S	S	1,43	0,65
SusPtrit	S	S	S	S	S	S	2,00	0,91
SusPtrit	S	S	S	S	S	S	2,19	0,99
SusPtrit	S	S	S	S	S	S	2,75	1,25
62-17	V	H	S	S	S	V	0,15	0,07
62-32	V	H	S	S	S	V	0,66	0,30
44-2	V	V	V	V	V	V	1,41	0,64
44-4	V	V	V	V	V	V	0,67	0,30
44-6	V	V	V	V	V	V	0,42	0,19
44-12	V	V	V	V	V	V	0,63	0,28
44-7	S	S	V	V	V	V	0,40	0,18
44-13	S	S	V	V	V	V	0,19	0,09
44-16	S	S	V	V	V	V	0,65	0,29
44-31	S	S	V	V	V	V	0,72	0,33
62-5	V	S	S	S	S	V	1,81	0,82
62-6	V	S	S	S	S	V	0,18	0,08
62-7	V	S	S	S	S	V	0,94	0,43
62-13	V	S	S	S	H	V	0,10	0,04
62-15	V	S	S	S	S	V	0,86	0,39
19-4	S	S	S	S	S	S	2,24	1,01
19-5	S	S	S	S	S	S	2,86	1,30
19-6	S	S	S	S	S	S	1,31	0,59
19-7	S	S	S	S	S	S	1,86	0,85
19-8	S	S	S	S	S	S	2,21	1,00

5. Raw data *P. persistens* experiments

Experiment 1; BC_1S_1 mapping population. Inoculation density 2 mg. The genotypic data is sorted with focus on the second subregion (84-85.7 cM).

Plant number	63.3	66.2	84.0	85.7	91.6	99.4	VIS/cm2
Vada	V	V	V	V	V	V	0,00
Vada	V	V	V	V	V	V	0,14
Vada	V	V	V	V	V	V	0,41
Vada	V	V	V	V	V	V	0,52
SusPtrit	S	S	S	S	S	S	12,56
SusPtrit	S	S	S	S	S	S	8,16
SusPtrit	S	S	S	S	S	S	7,53
SusPtrit	S	S	S	S	S	S	2,05
SusPtrit	S	S	S	S	S	S	4,57
106	V	V	V	V	V	V	0,09
110	V	V	V	H	H	V	0,49
108	H	H	V	V	V	V	0,00
112	H	H	V	V	H	S	0,00
123	H	H	V	V	V	V	0,10
119	H	H	V	V	H	H	0,18
104	H	H	V	V	V	V	1,01
122	V	V	H	H	H	H	0,28
99	H	V	H	H	H	H	0,65
118	V	V	H	H	H	H	0,80
107	V	V	H	H	H	H	1,96
132	H	H	H	H	H	S	0,07
126	H	H	H	H	H	S	0,14
125	H	H	H	H	H	H	0,18
131	H	H	H	H	H	H	0,23
114	H	H	H	H	H	H	0,72
130	H	H	H	H	H	V	0,79
129	H	H	H	H	H	H	0,92
103	H	H	H	H	S	S	1,73
105	H	H	H	H	H	H	1,79
101	H	H	H	H	H	V	2,60
109	H	H	H	H	H	V	3,30
121	H	H	H	H	H	H	4,06
111	S	S	H	H	H	H	0,19
128	S	S	H	H	H	H	0,52
102	S	S	H	H	H	H	0,55
98	S	S	H	H	H	H	0,95
120	V	V	S	S	S	S	0,25
127	H	H	S	H	H	S	0,00
113	S	S	S	S	S	S	0,19
115	S	S	S	S	S	S	0,79
117	S	S	S	S	S	S	0,79
100	S	S	S	S	S	H	10,65

Experiment 2; BC_1S_1 mapping population. Inoculation density 4 mg.

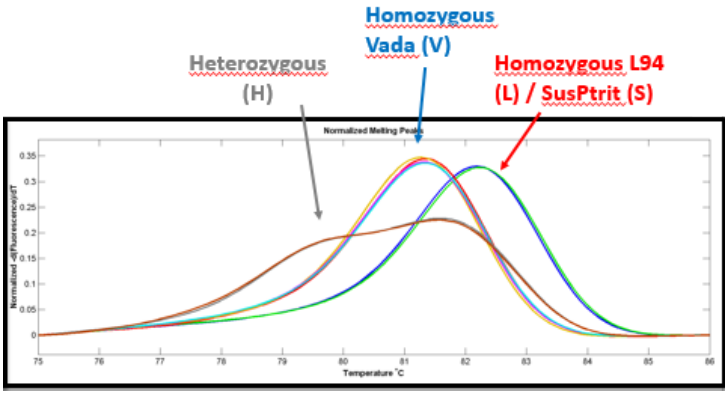
Plant number	63.3	66.2	84.0	85.7	91.6	99.4	VIS/cm2
Vada	V	V	V	V	V	V	1,88
Vada	V	V	V	V	V	V	0,90
Vada	V	V	V	V	V	V	1,00
Vada	V	V	V	V	V	V	1,38
Vada	V	V	V	V	V	V	0,92
Sus-NH	S	S	V	V	V	V	6,37
Sus-NH	S	S	V	V	V	V	11,64
Sus-NH	S	S	V	V	V	V	14,09
Sus-NH	S	S	V	V	V	V	8,84
SusPtrit	S	S	S	S	S	S	11,11
SusPtrit	S	S	S	S	S	S	14,95
SusPtrit	S	S	S	S	S	S	9,54
347	V	V	V	V	V	V	1,84
355	V	V	V	V	V	V	5,70
359	V	V	V	V	V	V	10,69
358	V	V	V	V	V	V	11,93
349	V	V	V	V	V	H	4,72
339	H	H	V	V	V	V	13,05
342	S	S	V	V	V	V	7,96
348	V	V	H	H	H	S	2,08
346	H	H	H	H	H	V	2,62
354	H	H	H	H	H	H	3,47
364	H	H	H	H	H	H	3,63
357	H	H	H	H	H	S	4,42
341	H	H	H	H	H	H	6,15
356	H	H	H	H	H	H	6,59
360	H	H	H	H	H	H	6,73
350	H	H	H	H	H	H	11,84
340	H	H	H	H	S	S	5,44
353	H	H	H	H	H	S	11,11
344	S	S	H	H	V	V	3,28
362	S	S	H	H	S	S	0,88
363	S	S	H	H	S	S	16,45
338	H	H	S	H	H	H	6,37
343	S	S	S	H	H	V	4,40
351	V	V	S	S	S	S	10,48
361	S	S	S	S	H	H	11,08
345	S	S	S	S	S	S	2,36
352	H	H	S	U	S	S	10,36
103	H	H	H	H	S	S	1,73
105	H	H	H	H	H	H	1,79
101	H	H	H	H	H	V	2,60
109	H	H	H	H	H	V	3,30
121	H	H	H	H	H	H	4,06
111	S	S	H	H	H	H	0,19
128	S	S	H	H	H	H	0,52
102	S	S	H	H	H	H	0,55
98	S	S	H	H	H	H	0,95
120	V	V	S	S	S	S	0,25
127	H	H	S	H	H	S	0,00
113	S	S	S	S	S	S	0,19
115	S	S	S	S	S	S	0,79
117	S	S	S	S	S	S	0,79
100	S	S	S	S	S	H	10,65

Experiment 3; BC_1S_2 mapping population. Inoculation density 3.2 mg.

Plant number	63.3	66.2	84.0	85.7	91.6	99.4	VIS/cm2
SusP-Rnhq	V	V	V	V	V	V	0,03
SusP-Rnhq	V	V	V	V	V	V	0,07
SusP-Rnhq	V	V	V	V	V	V	0,10
SusP-Rnhq	V	V	V	V	V	V	0,06
SusP-Rnhq	V	V	V	V	V	V	0,06
SusPtrit	S	S	S	S	S	S	1,56
SusPtrit	S	S	S	S	S	S	0,36
SusPtrit	S	S	S	S	S	S	1,30
SusPtrit	S	S	S	S	S	S	0,83
SusPtrit	S	S	S	S	S	S	0,95
3-1	V	V	S	V	V	V	0,26
3-2	V	V	S	V	V	U	0,16
3-10	V	V	S	V	V	H	1,15
3-12	V	V	S	V	V	V	0,80
3-19	V	V	S	V	V	V	1,07
3-22	V	V	S	V	V	V	0,59
3-27	V	V	S	V	V	V	1,72
3-29	V	V	S	V	V	V	0,30

6. LightScanner output

Example LightScanner™ output. Clearly the heterozygous peak deviates from the homozygous peaks as the heterozygous one has a kind of shoulder next to the peak.



7. qPCR material and methods

Two boxes containing 15 seedlings of both L94 and L94-*Rnhq* each were inoculated 12 days after sowing. Completely unfolded primary leaves were placed in a horizontal position with the adaxial side facing upwards and other leaves were removed. One box was inoculated with 20 mg *P.graminis lolii* spores mixed with Lycopodium spores and one box with Lycopodium spores only (mock). At 0, 6, 12, 24, 48, 72 hours post infection (HPI) and 10 DPI 2 cm of the primary leaf of each of the four treatments was sampled and immediately snap-frozen in liquid nitrogen and stored at -80°C.

RNA extraction was performed using the RNeasy Plant Mini Kit (Qiagen) according to the protocol provided by the manufacturer. The RNA samples were stored at -20°C. DNA was removed from the RNA samples using the TURBO DNA-*free*™ kit (Invitrogen). 20ul RNA and 30ul reaction mixture containing 1ul TURBO DNase was mixed and incubated for 30 min at 37°C. 5ul of DNase Inactivation Reagent was added and incubated for 5 min at room temperature. The samples were centrifuged at 13.3 rpm for 1.5 min (Heraeus Fresco 17 centrifuge; Thermo electron corporation) and the supernatant containing the RNA was collected and stored at -20°C. The concentration of RNA was measured using NanoDrop® Spectrophotometer ND-1000 (Iosgen lifescience). The samples were run on a 1.5% agarose gel in TBE buffer to check if the both 18S and 28S ribosomal RNA bands were visible.