Fine mapping of two sub-regions within QTL *Rnhq* that confer resistance to three non-adapted rusts in barley (*Hordeum vulgare*)

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Final report

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Fine mapping of two sub-regions within QTL *Rnhq* that confer resistance to three non-adapted rusts in barley (*Hordeum vulgare*)

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Cover picture	**Barley recombinants with the presence or absence of the QTL *Rnhq.* They are inoculated with *Pt*-INRA, which is a non-adapted pathogen.**

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Abstract

Non-host resistance occurs in the entire host plant species and acts against all isolates of a pathogen species. It would be very efficient if this type of resistance in non-host species could be transferred into host species. This would create major opportunities in agriculture. However, the genetics behind non-host resistance are poorly understood. There are non-adapted rust fungi known in barley that are able to infect some barley genotypes. These non-adapted rusts of marginal hosts could be used to study the genetics behind non-host resistance. A QTL (*Rnhq*) of approximately 30cM was found on chromosome 7H that confers resistance to four non-adapted rusts. This region is supposed to consist of three sub-regions, from which sub-region 1 confers resistance to *Phm* and *Phs*, sub-region 2 to *Pt,* and sub-region 3 to *Pgl*. The aim of this thesis was to continue fine-mapping the QTL region of sub-region 1 and 2. This was performed by phenotypically and genotypically evaluating independent recombinants, developing new markers within the QTL region, and finding new recombinants in which crossing over occur in this region. Furthermore, histological analysis was carried out to determine the effect of the QTL on *Pt* infection units.

In this study, it is concluded that the gene responsible for *Phm* resistance is not located in sub-region 1. In order to find the exact location of *Phm* resistance, it is suggested to redo QTL mapping on the L94-*Rnhq* NIL. It is also recommended to phenotypically evaluate the recombinants of sub-region 1 inoculated with *Phs,* since these recombinants where only tested with *Phm*. Moreover, the interval of the region containing the responsible gene for *Pt* resistance is reduced from 3.4cM down to 0.6cM in sub-region 2. To pin down this gene, it is recommended to continue marker development and to find and evaluate new rare recombinants which have their recombination point within this interval. Finally, evidence is found that the introgressed gene of Vada in L94 is responsible for quantitative resistance, that reduces the ability of the fungus to form haustoria. In this study, Vada, L94-*Rnhq*, and rec204 showed high levels of pre-haustorial resistance to *Pt* compared to the susceptible landrace L94. The use of this pre-haustorial resistance in breeding programmes of barley could result in a durable resistance to *Pt*.

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1. Introduction

Barley (*Hordeum vulgare*) is the fourth most important cereal crop world-wide, cultivated for feed and food industry (Thomas, 2003; Park *et al*., 2015). Within the food industry, barley is best known for its malting and brewing qualities. Barley is a widely adapted cereal grain with a good cold, drought, and salt tolerance. It can grow under higher latitudes and altitudes and farther into deserts than any other cereal crop (Ullrich, 2010). In 2012, a total of 133 million tons of barley was produced over an area of 50 million hectares (FAOSTAT, 2015). Barley is a strictly autogamous diploid (2n = 14) crop having a haploid genome size of 5.1 Gb (Arumuganthan & Earle, 1991).

There are four rust diseases that can affect barley, namely crown rust caused by *Puccinia coronata* var. *hordei*, leaf rust caused by *Puccinia hordei*, stem rust caused by *Puccinia graminis*, and stripe rust caused by *Puccinia striiformis* f. sp. *hordei* (Park *et al*., 2015). Among these diseases, leaf rust is considered to be the most destructive one, which can cause economically losses up to 30%. When barley is infected by *P. hordei*, pale spots will emerge on the adaxial leaf side in which afterwards orange-brown pustules will appear containing fungal spores. As one of the consequences, photosynthetic areas will be reduced causing a reduction of plant growth and grain filling leading to yield losses (Park *et al*., 2015). The most common way for controlling leaf rust is through the use of fungicides. However, chemicals have a negative impact on human health and the environment, resulting in an increasing demand for finding alternatives that reduce infection. A better approach to manage *P. hordei* is by using genetic resistance (Niks *et al*., 2000).

Plants have developed constitutive and induced defence mechanisms. Constitutive defence mechanisms are pre-formed physical and chemical barriers on the plant cell its surface that may prevent pathogens from penetrating the cell wall. The plant initiates inducible defences when pattern-recognition receptors (PRRs) of the plant recognizes the presence of non-self molecules, the so-called pathogen-associated molecular patterns (PAMPs; Ingle *et al*., 2006; Mackey & McFall, 2006). This plant-pathogen interaction results in the activation of PAMP-triggered immunity (PTI), which can stop most of the potential pathogen infections. In turn, pathogens will produce effectors in order to prevent PAMP recognition by PRRs or to interfere with PTI (Pel & Pieterse, 2012), as a result that they can successfully colonize within the plant (Cook *et al*., 2015). These effectors have specific operative targets in the plant. A pathogen is able to infect a plant when the structure of the operative targets in the plants are compatible with the effectors of the pathogen. In this way, PTI is supressed by effectors, which called effector-triggered susceptibility (ETS).

However, some effectors of the pathogen can be recognized by intracellular receptors encoded by resistant genes (R-genes) of the plant. These effectors are known as avirulence proteins (Avr effectors). Recognition of Avr-effectors will result in the activation of effector-triggered immunity (ETI). This type of defence is cultivar- and accession specific and functions on a gene-for-gene relationship between host and pathogen (Flor, 1971). However, these genes are not durably effective since only one simple loss-of-function mutation within the pathogens *Avr* effector is required. Pathogens with this loss-of-function-mutation have a higher reproduction compared to pathogens with the Avr-effectors (Niks & Marcel, 2009). For example, in wheat, a new virulent strain of stem rust called Ug99 emerged, which can overcome the resistance conferred by a broad range of R-genes (Stokstad, 2007).

A plant species is a host to a pathogen species, when the pathogen can overcome the basal defense, such as PTI, that plant species mounted against them. This means that basic compatibility is achieved (Heath, 1997). A plant is called a non-host when the resistance occurs in the entire host plant species and act against all isolates of a pathogen species (so-called non-adapted pathogens; Heath, 1981a; 2000; Mysore & Ryu, 2004; Niks & Marcel, 2009). Therefore, non-host resistance is race non-specific and durably effective to a wide range of pathogens (Niks, 1987; Mysore & Ryu, 2004). This type of resistance in plants is very efficient and creates major opportunities in agriculture, only if this type of resistance in non-host species could be transferred into host species (Niks, 1987; Mysore & Ryu, 2004). Unfortunately, it is poorly understood why a specialized plant pathogen infect a certain host plant species, but not a non-host even in a closely related species.

Constitutive defence mechanisms are more likely to contribute to pathogens of plant species that are taxonomically different (e.g. *Arabidopsis* to wheat leaf rust) than to pathogens of plant species related to the non-host (e.g. barley to wheat leaf rust; Heath, 2000; Niks & Marcel, 2009). For plant species that are closely related, non-host resistance is more likely to be based on the activation of induced defences (Niks & Marcel, 2009). For example, when the majority of the barley accessions (e.g. Cebada Capa and Vada) are infected with haustoria forming pathogens, like *Puccinia* rusts, most of the infection units aborted after the formation of one to six haustorial mother cells (HMC) and before the development of a haustorium (Niks, 1987). This defence is associated with cell wall reinforcements through the deposition of papilla (O' Connell & Panstruga, 2006). However, it is presumed that host pathogens (e.g. *P. hordei*) and related non-host pathogens (e.g. non-adapted rust fungi) do have very similar or identical PAMPs, as a result that they are too conserved to explain difference between host and non-host pathogens (Niks & Marcel, 2009; Niks, 2014).

It is assumed that the structure of operative targets of effectors in plants probably will determine the host status of a plant (Yeo, 2014). The degree to which PTI is supressed will be determined by the success rate of effectors to manipulate the operative targets. Effectors of non-adapted rusts to which barley is a non-host cannot suppress the PTI in barley (Yeo, 2014). However, there are also plant species, the so-called marginal host species, which are moderately susceptible when infected by a certain pathogen (Niks, 1987). In barley, there are some non-adapted rust fungi, for example *P. hordei-murini* and *P. triticina*, that are able to infect some barley genotypes, such as the landrace 'L94' (Niks *et al*., 1996). The effectors of these non-adapted rusts may supress only a part of the PTI, resulting in some level of susceptibility in these barley genotypes. Hence, PTI is apparently easier to supress in some barley genotypes than in others by these non-adapted rusts. This suggests that there is genetic variation between barley accessions in how easy the PTI can be expressed.

It is difficult to do research on the genetics of non-host resistance, as there is no susceptible cultivar to cross with (Hoogkamp *et al*., 1998). Inter-species crosses between a susceptible host and resistant non-host species could solve this problem. However, these are difficult to make and suffer from unwanted effects, such as embryo abortion, lack of fertility, and abnormal growth (Niks, 1988; Jeuken & Lindhout, 2002). A way to study the genetics of non-host resistant is by using the marginal host species. In this way, intra-specific crosses can be made, which allow regular inheritance studies.

With quantitative inheritance studies, the operative targets in the plant that determine the host status could be identified. In barley, more than twenty QTLs have been found for the resistance to barley leaf rust (Qi *et al.,* 1998; Marcel *et al*., 2007a, 2008; Marcel & Niks, unpublished). These loci seem not to

coincide with loci for hypersensitive resistance (Qi *et al.,* 1998), but with loci responsible for the resistance to non-adapted rust fungi (Jafary *et al*., 2008). Some of the QTLs were typically effective to one or two rusts, and some to at least four non-adapted rusts (Jafary *et al*., 2006). This suggests that non-host resistance to rust pathogens is controlled by a mix of non-specificity and specificity of the genes (Niks, 2014). Furthermore, non-host resistance QTLs tend to co-localize with partial resistance QTLs. Partial resistance is characterized as a low infection level, while it has a susceptible infection type (Parlevliet & van Ommeren, 1975). This reduction in epidemic development is due to several components, like latency period, infection frequency, and spore production per uredosorus. Partial resistance shares some mechanisms with non-host resistance of barley, although it is less complete (Niks, 1983a,b). It is suggested to be a weak form of non-host resistance, based on PTI (Niks & Marcel, 2009; Niks *et al*., 2011).

The genes responsible for the effect of the QTLs of barley to non-adapted rusts need to be identified and isolated in order to understand the molecular aspects of non-host resistance. For this, near-isogenic lines (NILs) are used, which are developed by introgressing the resistance QTL of the donor into a susceptible parent by repeated backcrosses followed by selfing. In this way, each QTL can be evaluated in a uniform genetic background in which the resistance QTL and the genes flanking the QTL are the only genetic variation. The region of the identified QTLs is normally large (> 10 cM) and it is essential to reduce this region in order to find and isolate the target genes (Yeo, 2014). To reduce a QTL, sub-NILs are used that carry different recombination areas in the interval between the markers flanking the QTL (Han *et al*., 1999; Marcel *et al*., 2007b; Xue *et al*., 2010; Zhou *et al*., 2010). The markers within the QTL interval are used to genotype all individuals of the sub-NILs for the identification of new recombinants. Afterwards, new markers will be developed within the interesting interval to determine all recombination points. All independent recombinants are phenotyped and genotyped to reduce the marker interval in which the responsible gene is located. When the QTL is fine-mapped to a genetic distance <0.5 cM, the candidate genes present in this region can be validated through transient overexpression and by stable transformation in the host species (Lee *et al*., 2012).

In a RIL population derived from a cross between the susceptible landrace ‘L94’ and the resistant cultivar ‘Vada’, a QTL (designated *Rnhq*) of approximately 30 cM was found on chromosome 7H that confers resistance to four non-adapted rust pathogens, namely *P. hordei-murini* (*Phm*), *P. hordei-secalini* (*Phs*), *P. triticina* (*Pt*), and *P. graminis-lolii* (*Pgl*; Niks *et al*., 2000). The *Rnhq* region of ‘Vada’ was introgressed into ‘L94’ by repeated backcrosses followed by selfing (Niks *et al*., unpublished). In 2012, Salunke found evidence that this region did not consist of one gene conferring broad resistance to all four rusts, but was divided into three sub-regions. Sub-region 1 is supposed to confer resistance to *Phm* and *Phs*, sub-region 2 to *Pt*, and sub-region 3 to *Pgl* (*figure 1.1*).

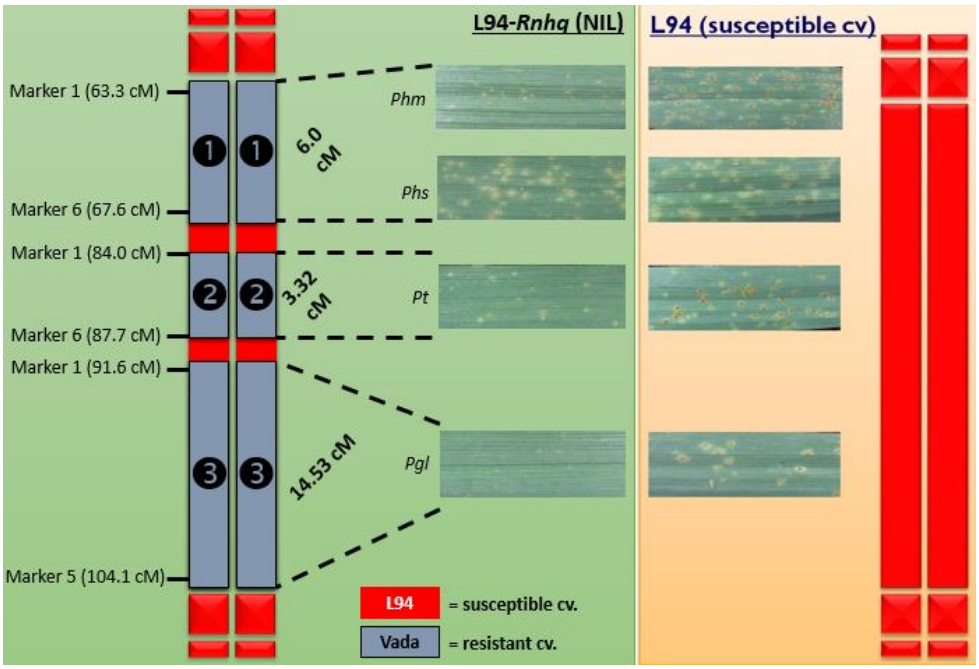

Figure 1.1 The Rnhq region found on chromosome 1H of barley, conferring resistance to four non-adapted rusts. This region of Vada (blue) introgressed in landrace L94 (red) contains three sub-regions. The marker intervals in which these sub-regions are located are 63.3-67.6 cM, 84.0-87.7 cM, and 91.6-104.1 cM respectively for sub-region 1, 2, and 3. Sub-region 1 is supposed to confer resistance to P. hordei-murini (Phm) and P. hordei-secalini (Phs), sub-region 2 to P. triticina (Pt), and sub-region 3 to P. graminis f. sp. lolli (Pgl). Furthermore, the infection level of L94-Rnhq plants and L94 plants inoculated with Phm, Phs, Pt, and Pgl are shown (Daniau, unpublished).

In 2016, 17 single-nucleotide polymorphism (SNP) markers were developed over three sub-regions (*appendix 1*). Later the genetic distance between each marker in each sub-region was calculated by using the recombination frequencies of all independent recombinants. Based on a population of 360 plants for sub-region 1, it appeared that markers M1.1 and M1.2, and markers M1.4 and M1.5 had the same genetic position. A similar observation has been done for marker M2.3 and M2.4 of sub-region 2 in a population of 742 plants (*figure 1.2*). Previous phenotyping and marker genotyping experiments by Daniau (unpublished) suggest that within sub-region 1, the resistance to *Phm* and *Phs* is expected to be located between marker M1.5 and M1.6. For sub-region 2, the resistance to *Pt* is likely to be between marker M2.2 and M2.5 and the resistance to *Pgl* within sub-region 3 is expected to be located between marker M3.2 and M3.5 (*figure 1.2*).

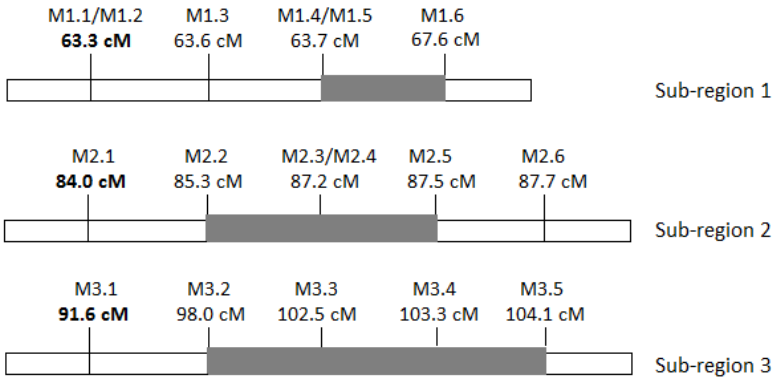

Figure 1.2 The genetic location of each marker of sub-region 1-3. The locations in bold are based on the data of the consensus map. The not in bold genetic locations were calculated by using recombination frequencies of all independent recombinants. The resistance is expected in the grey areas.

The aim of this study was to continue fine-mapping the QTL region of sub-region 1 and 2. The research on sub-region 3 was not continued due to the unavailability of the *Pgl* inoculum. First, the reduced intervals for sub-region 1 and 2 needed to be confirmed by retesting some recombinant progenies, but also by screening new independent recombinant progenies. Afterwards, new SNP markers were developed within the reduced marker intervals and these markers were evaluated on all independent recombinants found in this interval. Furthermore, new recombinants were generated in order to find new recombination points within the reduced interval. Finally, histological analysis of the infection process of *Pt* in barley seedlings was performed to determine the mechanism of the resistance at tissue level.

2. Materials & Methods

2.1 Starting material

In previous studies, progenies of 14 heterozygous recombinants were tested for *Phm* resistance (*appendix 2*) and progenies of 23 heterozygous recombinants for *Pt* resistance (*appendix 3*). In this study, the progenies of five recombinants and one non-recombinant for sub-region 1 (*table 2.1*) and the progenies of nine recombinants and one non-recombinant for sub-region 2 (*table 2.2*) were evaluated in order to reduce the marker interval. Some of these recombinants were retested and some were tested for the first time. For each evaluated homozygous recombinant, 10 seeds were sown. For each heterozygous recombinant, a maximum of thirty seeds were sown, depending on the amount of available seeds. After sowing the progeny of heterozygous recombinants, the plants were genotyped and only the homozygous recombinants were transplanted and used for phenotyping. In parallel with the tested recombinants, five seeds of L94 and L94-*Rnhq* were sown as respectively susceptible and resistant control in each tray. All trays were placed for twelve days at 20 °C into a greenhouse compartment at Unifarm, Wageningen.

Table 2.1 Progenies of five recombinants and one non-recombinant of barley that were genotyped for six markers within sub-region 1. A marker is homozygous for the Vada (V; blue background) or L94 (L; red background) allele, or is heterozygous (H; grey background). For each recombinant that was retested, the phenotype when tested for the first time by Daniau is shown.

Parent	**SNP markers**				**Phenotype**
	M1.1/M1.2	M1.3	M1.4/M1.5	M1.6	
G02006305_23_rec476*	L	L	L	L	Intermediate
G02006305_23_rec476*	V	L	L	L	Intermediate
G02006305_22_rec328a*	V	L	L	L	Intermediate
G02006305_26*	V	V	L	L	Resistant
G02006305_23_rec472*	L	L	V	V	Resistant
G02006305_24_rec498	L	L	H	H	-

*: progenies that were retested

Table 2.2 Progenies of nine recombinants and one non-recombinant of barley that were genotyped for sub-region 2 based on six SNP markers. Blue background indicates that these regions originate from Vada (V), and the regions having a red background originate from L94 (L). Regions having a grey background are heterozygous (H). For each recombinant that was retested, the phenotype when tested for the first time by Daniau is presented.

Parent	**SNP markers**					**Phenotype**
	M2.1	M2.2	M2.3/M2.4	M2.5	M2.6	
G02006305_22_rec461_rec1766*	V	V	V	L	L	Segregation
G02006309_23_rec615	H	H	L	L	L	-
G02006309_32_rec2086	V	V	L	L	L	-
G02006308_96_rec597*	V	V	L	L	L	Resistant
G02006307_06_rec283_rec2937*	V	V	L	L	L	Resistant
G02006305_24_rec249_rec1946	V	V	H	H	H	-
G02006308_09_rec401_rec2025	V	V	H	H	H	-
G02006305_24_rec252	L	L	H	H	H	-
G02006307_11_rec305	L	L	H	H	H	-
G02006308_96_rec597*	L	L	L	L	L	Resistant

*: progenies that were retested

2.2 DNA isolation and genotyping

Nine to twelve days after sowing, two cm^2 of the second leaf of each seedling was collected for DNA isolation and subsequent genotyping. From these leaves, DNA was extracted according to the quick and dirty method protocol (*appendix 4*). Afterwards, amplification of each marker for the LightScanner® System was carried out by using a 10µl master mix containing 4.64µl MQ water, 2.00µl 5X buffer, 1.00µl dNTPs, 0.80µl LC-green, 0.25µl of both primers (10µM), 0.06µl 5X phire enzyme, and10-20ng/µl DNA. The reaction was topped up with 10µl mineral oil. For each SNP marker that was amplified, a different primer set was used (*appendix 1*). Each marker was amplified in a T-Gradient thermal cycler with the following program: initial denaturation at 98 °C for 2 minutes, 40 cycles of denaturation at 98 °C for 10 seconds, annealing at 60 °C for 15 seconds, and extension at 72 °C for 20 seconds, and terminated by a final extension at 72 °C for 1 minute and 94 °C for 40 seconds.

PCR products were analysed by using the LightScanner® System to look for polymorphism. This system is based on the interaction of a fluorescent double-stranded DNA (dsDNA) binding dye (LCGreen) and the PCR product. Sample profiles were compared to the profiles of the controls (L94, Vada, and heterozygous) in order to determine the genotype of each recombinant. Isolated DNA of the progeny of each recombinant were kept in the freezer. This DNA was used later on for further genotyping with the newly developed markers.

2.3 Inoculation

Twelve days after sowing, the first leaf of each seedling was inoculated whereas the other leaves were removed. The first leaf was fixed horizontally in the tray with the adaxial side up. The inoculum consisted of 4.5 mg rust urediniospores mixed with approximately 60 mg lycopodium powder, using spores of *Phm* (Rhenen isolate) and *Pt* (INRA isolate). The inoculum was blown into a settling tower, in which the tray was placed containing the plant material. During the inoculation process, the tray rotated, resulting in a uniform distribution of the rust spores. Furthermore, each tray contained a microscope slide to check if the urediniospores successfully germinate. Once the leaves were inoculated, the tray was placed into a humidity chamber under a relative humidity of 100% at 20 °C overnight. The next day, plants were transferred into a greenhouse compartment at 20 °C.

2.4 Phenotyping

At twelve days post-inoculation (dpi), the number of sporulated pustules was counted on the first leaf of each seedling. Furthermore, the leaf length and width were measured. Based on these measurements, infection frequency (IF) was calculated, which is the number of sporulating pustules divided by the leaf surface. IF was converted to values relative to corresponding infection data on L94-*Rnhq* and L94, which was set at 0% and 100%, respectively (see formula below). Recombinants with a relative infection frequency (RIF) ≤ 25% were categorized as resistant, 26-74% as intermediate, and ≥ 75% as susceptible.

$$\mathrm{RIFrec} = \frac{(\mathrm{IF\ rec} - \mathrm{IF\ L94}Rhhq)}{(\mathrm{IF\ L94} - \mathrm{IF\ L94}Rnhq)} \times 100\%$$

All phenotypic data of the recombinants were analysed by using One Way ANOVA with LSD to detect significant differences for IF between the recombinants and the controls (L94 + L94-*Rnhq*). Statistical analysis was performed with SPSS Statistics 22.

2.5 Marker development in new interval

When the genetic interval of the QTL was reduced, new markers were developed within the new region. First, we searched for genes that were located within the marker interval in the barley reference Morex. Homologues of these barley genes were identified in the brachypodium and rice genomes which are two model species related to barley. This was done by using EnsemblPlants (http://plants.ensembl.org-/index.html) and IPK Barley Blast (http://webblast.ipkgatersleben.de-/barley_ibsc/). The conserved genes between the three genomes were prioritized for developing new markers. The longest intron of these genes was amplified by PCR by using primer sequences of the intron. This intron was sequenced for L94 and Vada. Subsequently, the sequence of Vada and L94 were compared with SnapGene in order to look for SNPs. Primer pairs of 18-30 nucleotides per primer were designed with Primer3Plus next to the identified SNPs, resulting in new markers. Finally, the new markers were tested on L94 and Vada by using the LightScanner to confirm their polymorphism.

A second method to develop new markers was used when the amplified intron by PCR was non-specific or when no clear sequence was obtained for Vada and L94. In this method, the Morex sequence of all genes located within the marker interval were blasted against Barke and Bowman, of which the genomes are similar to Vada and L94, respectively. The sequence of Morex, Barke, and Bowman were compared to look for SNPs. Afterwards, primer pairs were designed on either side of the identified SNPs, resulting in a new marker. Finally, the new markers were analysed on L94 and Vada by using the LightScanner to confirm their polymorphism.

For sub-region 1, Daniau (unpublished) previously screened 360 plants and found 40 recombinants between marker M1.1 and M1.6. For sub-region 2, 55 recombinants were found out of 742 plants between marker M2.1 and M2.6. All new developed markers within sub-region 1 or sub-region 2 were tested on all the independent recombinants of sub-region 1 or sub-region 2 of which DNA was kept in the freezer.

2.6 Development of new independent recombinants

For the development of new independent recombinants within sub-region 2, 469 seeds were sown coming from seven different parents, which were heterozygous for sub-region 2. Plant material of each seedling was collected and DNA was isolated in the same way as previously described. All 469 plants were genotyped for marker M2.2 and M2.5. The heterozygous and homozygous recombinants for either markers were transplanted and tested with the other markers present in the interval. The recombination points for all recombinants were determined. Finally, the genetic distances between markers were calculated by using the recombination frequencies between SNP markers.

2.7 Histological analysis

To visualize the development of rust fungal structures, eight seeds of susceptible (L94 and G02006312_68_rec658) and resistant (Vada, L94-*Rnhq*, G02006307_19_rec204) genotypes were sown. Twelve days after sowing, seedlings were inoculated with 4.5 mg *Pt*-INRA spores (= approximately 270 urediniospores per cm^2) in the same way as previously described in paragraph 2.3. At seven dpi, four middle-leaf segments of 3 cm^2 were collected and stained using Uvitex 2B according to the protocol described by Dugyala *et al*., 2015.

After staining, fifty infection units on each leaf segment were scored for stage of development under the UV microscope. Each infection unit was assessed based on four categories: early abortion without

necrosis (EA - N), early abortion with necrosis (EA + N), established colonies without necrosis (Est - N), and established colonies with necrosis (Est + N; *appendix 4*). A colony was categorized as early aborted when less than six haustorial mother cells (HMC) were formed and when there were little or no branched infection hyphae.

All data were analysed with SPSS by using One Way ANOVA with LSD to detect significant differences for the four categories between the three parental lines and two recombinants.

3. Results

3.1 Sub-region 1

From previous research by Daniau, the resistance to *Phm* is expected to be located between marker M1.5 (63.7 cM) and M1.6 (67.6 cM) in sub-region 1. However, three out of fourteen independent recombinants and one non-recombinant showed previously a phenotype for infection severity that was in conflict with this hypothetical location of resistance (*table 3.1*). Also after retesting these conflicting lines, the expect location of resistance was not confirmed. Furthermore, recombinant G02006305_24_rec498 tested in this study, showed a susceptible phenotype (RIF ≥ 75%), where it was expected to be resistant (RIF ≤ 25%) on the basis of Daniau's earlier work. Subsequently, G02006305_23_rec472 which was previously resistant, was retested since the infection severity is expected to be the same as G02006305_24_rec498, because they belong to the same genotypic group. After retesting this recombinant, it has a susceptible score, which is in conflict with hypothetical location of resistance.

The data of 15 independent recombinants and one non-recombinant suggest that the responsible gene is not located within sub-region 1 (*table 3.1*).

Table 3.1 Marker genotypes of six SNP markers and the mean relative infection frequency (RIF) of fifteen independent recombinants, one non-recombinant, and the controls of barley. Blue background indicates that the region originates from Vada (V) and regions having a red background originates from L94 (L). The RIF of the parents were classified as susceptible (RIF ≥ 75%; red background), intermediate (RIF 26-74%; orange), or resistant (RIF ≤ 25%; green). Based on previous research, the resistance was expected to be at the right side of marker M1.5 and left side of marker M1.6 (bold line).

Parents	**M1.1 /1.2**	**M1.3**	**M1.4 /1.5**	**M1.6**	**RIF of previously study***	**RIF of this study**
G02006305_23_rec476	L	L	L	L	27	24
G02006305_22_rec328a	V	L	L	L	68	76
G02006305_23_rec476	V	L	L	L	27	49
G02006305_26	V	V	L	L	0	0
G02006305_22_rec328b	V	V	V	L	6	-
G02008072_46_rec264_rec1495	V	V	V	L	11	-
G02006307_06_rec381	V	V	V	L	19	-
G02006305_23_rec348	V	V	V	L	31	-
G02006308_95_rec43_rec1131	V	V	V	L	8	-
G02006307_06_rec383	L	L	L	V	54	-
G02006305_23_rec365	L	L	L	V	70	-
G02006307_24_rec253_rec1466	L	L	L	V	28	-
G02006307_14	L	L	L	V	11	-
G02006307_09_rec289_rec1506	L	L	L	V	0	-
G02006305_23_rec472	L	L	V	V	0	83
G02006305_24_rec498	L	L	V	V	-	87
L94 control	L	L	L	L	100	100
L94-*Rnhq* control	V	V	V	V	0	0

*: tested by Daniau

3.2 Sub-region 2

3.2.1 Fine mapping

The data from previous research by Daniau suggest that the responsible gene for *Pt* resistance is located between marker M2.2 (85.3 cM) and M2.5 (87.5 cM) in sub-region 2, with the exception of the results on three recombinants and one non-recombinant (*table 3.2*). After re-phenotyping these recombinants and by evaluating six new independent recombinants, the hypothetical location of *Pt* resistance was confirmed.

For a better saturation of the target region, it was necessary to develop more markers within sub-region 2 localized on the right side of marker M2.2 and left side of marker M2.5. In this region, 13 new primer pairs were designed over nine candidate genes, from which four were polymorphic between L94 and Vada (L2.1-2.4; *appendix 5*). Since marker L2.1 was out of the target region, only marker L2.2, L2.3, and L2.4 were evaluated on all independent recombinants. Based on nine SNP markers, the 29 tested recombinants were divided into seven genotypic groups (*table 3.2*). Generally, the data suggest the responsible gene to be located on the right side of marker L2.2 and left side of marker L2.4. For one of the recombinants, the marker genotypes of marker L2.2, L2.3, and L2.4 were unknown.

Table 3.2 Genotypes of nine SNP markers and the mean relative infection frequency (RIF) of 29 independent recombinants, one non-recombinant, and the controls of barley. A marker is homozygous for the Vada allele (V; blue background) or for L94 (L; red background). The RIF of the parents are classified as susceptible (RIF ≥ 75%; red background), intermediate (RIF 26-74%; orange), or resistant (RIF ≤ 25%; green). Based on previous research, the resistance is expected to be at the right side of marker M2.2 and left side of marker M2.5 (bold line).

Parents	M2.1	M2.2	L2.2	L2.3	M2.3	M2.4	L2.4	M2.5	M2.6	RIF Daniau*	RIF of this study
G02006308_96_rec597	L	L	L	L	L	L	L	L	L	8	100
G02006305_22_rec461_rec1771	V	L	L	L	L	L	L	L	L	100	-
G02008071_106	V	L	L	L	L	L	L	L	L	94	-
G02006311_53	V	V	L	L	L	L	L	L	L	100	-
G02006312_75	V	V	L	L	L	L	L	L	L	100	-
G02006306_116	V	V	L	L	L	L	L	L	L	100	-
G02013093_82_rec2141	V	V	L	L	L	L	L	L	L	83	-
G02006312_69	V	V	L	L	L	L	L	L	L	100	-
G02006308_96_rec597	V	V	L	L	L	L	L	L	L	0	100
G02006307_06_rec2937	V	V	L	L	L	L	L	L	L	7	100
G02006307_09_rec401_rec2025	V	V	L	L	L	L	L	L	L	-	100
Go2006305_24_rec249_rec1946	V	V	L	L	L	L	L	L	L	-	100
G02006309_32_rec2086	V	V	L	L	L	L	L	L	L	-	100
G02006309_23_rec615	V	V	L	L	L	L	L	L	L	-	100
G02006305_22_rec461_rec1766	V	V	V	V	V	V	L	L	L	segregation	21
G02006307_09_rec289_rec1705	V	V	V	V	V	V	L	L	L	10	-
G02006307_09_rec392	V	V	V	V	V	V	L	L	L	13	-
G02006305_22_rec461_rec1793_rec2283	V	V	V	V	V	V	L	L	L	11	-
G02006305_22_rec461_rec1771_rec2239	V	V	V	V	V	V	L	L	L	4	-
G02006305_22_rec461_rec1786_rec2267	L	L	V	V	V	V	L	L	L	0	-
G02006305_22_rec465_rec1968	L	L	L	V	V	V	V	V	V	0	-
G02006307_19	L	L	V	V	V	V	V	V	V	0	-
G02006305_22_rec334	L	L	V	V	V	V	V	V	V	0	-
G02006305_22_rec339	L	L	-	-	V	V	-	V	V	6	-
G02006311_61_rec850	L	L	V	V	V	V	V	V	V	0	-
G02006311_61_rec857	L	L	V	V	V	V	V	V	V	0	-
G02006305_24_rec252	L	L	V	V	V	V	V	V	V	-	0
G02006307_11_rec305	L	L	V	V	V	V	V	V	V	-	0
G02006306_113	L	V	V	V	V	V	V	V	V	3	-
G02006308_91	L	V	V	V	V	V	V	V	V	0	-
L94 control	L	L	L	L	L	L	L	L	L	100	100
L94-*Rnhq* control	V	V	V	V	V	V	V	V	V	0	0

*RIF of tested progenies in a previously study by Daniau

3.2.2 Development of independent recombinants

To increase the number of recombinants having a recombination point between marker M2.2 and M2.5 of sub-region 2, 469 plants derived from seven heterozygous parents were genotyped. From these plants, 18 heterozygous recombinants were found, from which recombination points occur between every marker pair in this region, except between marker L2.4 and M2.5. For one recombinant, the crossing over occurs at the right side of marker L3, but it is unknown if this occurs between marker L2.3 and M2.3 or between marker M2.3 and M2.4 (*table 3.3*).

Table 3.3 The marker genotypes of seven SNP markers for 18 heterozygous recombinants of barley

Number of independent recombinants	M2.2	L2.2	L2.3	M2.3	M2.4	L2.4	M2.5
3	L	H	H	H	H	H	H
3	H	V	V	V	V	V	V
1	H	L	L	L	L	L	L
5	V	H	H	H	H	H	H
1	V	V	H	H	H	H	H
1	H	H	H	V	V	V	V
1	H	H	H	H	V	V	V
1	H	H	H	H	L	L	L
1	H	H	H	-	L	-	-
1	V	V	V	V	V	H	H

Our additional 18 recombinants bring the total of independent recombinants in sub-region 2 to 73, found in a total of 1211 plants. The calculation of the genetic distance between the flanking markers M2.1 and M2.6 showed that sub-region 2 was 3.44 cM (*figure 3.1*). Crossing over occur between every marker pair in this region, except between marker L2.4 and M2.5. However, for eight recombinants (not included in this figure), it was unclear between which markers the crossing over occur, due to unavailable DNA or low DNA quality. 29 out of 73 recombinants were phenotypically evaluated, suggesting that the responsible gene is located on the right side of marker L2.2 and left side of marker L2.4. Within this new marker interval, seven recombinants were not previously phenotyped. These recombinants could be tested in order to reduce the marker interval.

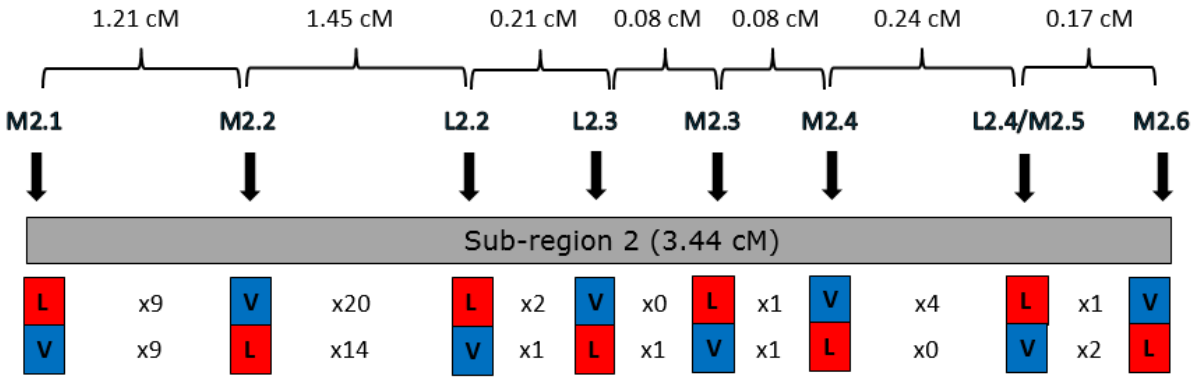

Figure 3.1 Number of independent recombinants found between each marker of sub-region 2 illustrated at the bottom of this figure for either Vada (V) or L94 (L). Based on the identified recombinants, the genetic distance for each marker was calculated which is shown at the top of this figure.

3.2.3 Histological analysis

To visualize the development of rust fungal structures, the infection units of susceptible (L94 and rec658) and resistant (Vada, L94-*Rnhq*, rec204) genotypes were assessed based on four categories: early abortion without necrosis (EA - N), early abortion with necrosis (EA + N), established colonies without necrosis (Est - N), and established colonies with necrosis (Est + N). Leaf segments of Vada had, with a percentage of 94%, the significantly highest percentage of early aborted colonies, followed by L94-*Rnhq* and rec204, with a percentage of 75% and 80%, respectively ($p < 0.05$; *table 3.4*). The percentage of early aborted colonies on leaf segments of L94 was almost three times lower than on leaf segments of Vada. This was in accordance with macroscopic observations in this study, from which L94-*Rnhq* and rec204 showed a resistant phenotype, while L94 was susceptible. These data suggest that the resistance to *Pt* is mainly based on pre-haustorial resistance.

Table 3.4 Percentages of early aborted (EA) and established colonies (Est) with (+N) or without (-N) necrosis of Pt-INRA for three parental lines and two recombinants of barley. Numbers with different letters are significantly different ($p < 0.05$).

Parent	**%EA (of which +N)**	**%Est (of which +N)**
L94	32.9^{a} (15.0)	67.1^{a} (37.5)
G02006312_68_rec658	54.0^{b} (16.6)	46.0^{b} (56.2)
L94-*Rnhq*	75.5^{c} (1.1)	24.5^{c} (74.7)
G02006307_19_rec204	81.0^{c} (9.4)	19.0^{c} (58.8)
Vada	94.0^{d} (7.8)	6.0^{d} (25.0)

4. Discussion

4.1 Sub-region 1

One of the aims of this study was to reduce the interval size of sub-region 1 (4.3 cM), which is supposed to confer resistance to *P. hordei-murini* (*Phm*) and *P. hordei-secalini* (*Phs*). *Phm* resistance was expected to be located on the right side of marker M1.5 and left side of marker M1.6. However in this study, five out of fifteen recombinants were in conflict with this hypothetical location. The resistance to *Phm* did not only not fit with marker M1.5 and marker M1.6, but did not fit with any of the markers located in sub-region 1. In addition, one non-recombinant (G02006305_23_rec476) was found from which the whole sub-region was homozygous for the susceptible L94 allele, while it showed a resistant phenotype. Based on these results, there can be concluded that the gene responsible for *Phm* resistance is not located within sub-region 1.

In 2007, van Dijk characterized resistance to *Phm* and *Phs* on L94-*Rnhq* NILs. QTL mapping was performed by using marker and phenotypic data on 23 homozygous F4 recombinants. One QTL for both *Phm* and *Phs* was identified next to CAPS marker SKT1 (85.8 cM), which is located in sub-region 2. However, van Dijk also phenotyped 22 F3 recombinants, in which he found evidence that the infection frequencies of *Phm* did not co-segregate with marker SKT1 (*appendix 7*). Thus, based on these results, it is difficult to confirm whether the gene explaining resistance to *Phm* is located next to marker SKT1 or not. Salunke (2012) continued fine-mapping by developing new markers within the *Rnhq* region. He phenotypically and genotypically evaluated the same 22 recombinants as used in van Dijk's study. Based on these results, Salunke found evidence that the QTL conferring resistance to *Phm* was not close to marker SKT1, but close to marker BOPA1_12239_662 in sub-region 1 (marker M1.1, 65.8 cM). However, when converting his observed infection frequencies (IF) of *Phm* to relative infection frequencies corresponding to L94 and L94-*Rnhq* (RIF), *Phm* phenotypes did not clearly fit with any of the marker genotypes (*appendix 8*). Additionally during this thesis as well as in fine-mapping studies by Daniau, no *Phm* resistance was found close to marker M1.1 in sub-region 1.

Although *Phm* resistance is not identified in sub-region 1, it should be present within the *Rnhq* region. This because L94-*Rnhq* had a lower infection level than L94 when inoculated with *Phm*. Therefore, it is recommended to start over and redo QTL mapping on the L94-*Rnhq* NIL to find out which gene is responsible for *Phm* resistance. For *Phs* resistance, Salunke (2012) found that the IF of recombinants inoculated with *Phs* clearly co-segregates with marker BOPA1_12239_662 (*appendix 8*). Hence, it is suggested to test the available recombinants of sub-region 1 with *Phs,* since the recombinants in our study were only tested for *Phm*. If *Phs* resistance will co-segregate with marker BOPA1_12239_662, it can be concluded that the resistance to *Phm* and *Phs* are governed by genes at different loci.

4.2 Sub-region 2

4.2.1 Fine-mapping

The aim of this of this study was to fine map the gene for resistance to *P. triticina* (*Pt*) in sub-region 2 (3.44 cM). Fine mapping of sub-region 2 was performed by Daniau, in which the resistance to *Pt* was expected to be located on the right side of marker M2.2 and left side of marker M2.5. However, the progeny of three homozygous recombinants and one non-recombinant phenotyped by Daniau were in contradiction with this hypothetical location of resistance, as a result that they were retested. When retesting, the resistance was confirmed to be between marker M2.2 and marker M2.5, corresponding to a 2.1 cM interval.

Four new markers were developed in this reduced interval from which three markers (L2-L4) were evaluated on all independent recombinants. Six independent recombinants contain the Vada region on the left side of marker L2.4 and showed a resistant phenotype. Furthermore, one recombinant for which markers L2.3 to M2.6 were homozygous for the Vada allele had a resistant phenotype (*table 3.2*). Based on these results, the *Pt* resistance is located at the right side of marker L2.2 (86.7cM) and left side of marker L2.4 (87.3 cM). Our results are in accordance with Salunke (2012), who found that *Pt* resistance was located close to marker SKT1 (*appendix 8*). Although the new interval is reduced from 2.1 cM down to 0.6cM, the genomic interval is still relatively large for using it in map-based cloning. In further studies, it is recommended to develop more markers between marker L2.2 and L2.4 to saturate the new target region. In addition, there are ten independent recombinants available for this interval (*figure 3.1*), from which three were previously tested. The remaining recombinants should be tested for further fine-mapping experiments. If the interval is not reduced based on the evaluation of these recombinants, it is recommended to sow more seeds from heterozygous parents for sub-region 2 in order to find new rare recombinants with recombination points between these markers. Additionally, eight recombinants were not genotyped for all markers due to unavailable or low-quality DNA, as a result that it is unclear between which markers the crossing over did occur. The seeds of six recombinants are available and should therefore be sown and genotyped again.

Once the QTL region is smaller than 0.5 cM, it is recommended to use the flow sorting chromosome technique, from which chromosome 7H of Vada and L94 will be isolated. The sequence of Vada and L94 will be obtained, from which we can identify the genes present in QTL region of interest between our flanking markers. In this way, the sequence of this region can be compared between Vada and L94 in order to look for polymorphism and to try to identify which gene is more likely responsible for resistance. Subsequently, functional studies of candidate genes can be performed by stable transformation. With stable transformation, the transgene is transmitted through grains from one generation to the next, as a result that several identical plants are available containing the transgene. However, the efficiency of this technique depends on the barley genotype that is used (Hensel *et al*., 2008). Golden Promise, which is resistant to non-adapted pathogens, is known to be a suitable cultivar for stable transformation. Yeo *et al*. (2014) developed the barley line 'Golden SusPtrit', which combines the high susceptibility to non-adapted rust fungi, like *Pt*, and the high amenability to *Agrobacterium*-mediated transformation of Golden Promise. To validate the candidate genes found in our region, they can be cloned individually and transferred into Golden SusPtrit. Subsequently, they will be overexpressed in Golden SusPtrit to identify the gene involved in *Pt* resistance.

4.1.2 Histological analysis

Histology is a useful technique to determine the effect of the QTL on *Pt* infection at tissue level. By using Uvitex 2B for staining the leaf segments, is was possible to clearly observe and distinguish the different infection units. Based on histological analyses, two types of resistance could be defined, namely pre-haustorial and post-haustorial resistance. Pre-haustorial resistance prevents the development of haustoria of infection units and is very common in non-host plant-pathogen interactions (Heath, 1977; 1981b; 1985). By post-haustorial resistance, a hypersensitive response will be elicited within the host cell when the infection unit formed haustoria (Heath, 1981b; Niks, 1983a; Niks & Dekens, 1991). If both resistance mechanisms are not expressed, the infection unit will colonize the plant tissue (Anker & Niks, 2001).

In this study, large differences in mean percentages of early aborted colonies were observed between three parental lines and two recombinants (from 32-94%). On leaf segments of Vada, L94-*Rnhq*, and rec204, ≥ 74 % of the infection units were early aborted without necrosis at 7dpi. This showed that these genotypes have a high level of pre-haustorial resistance. This was in accordance with macroscopic observations, were L94-*Rnhq* and rec204 showed a resistant phenotype. The majority of the infection units on L94 leaf segments were not early aborted and developed into established colonies. This was also observed at macroscopic level, in which L94 showed a high infection level. This indicates that L94 has a low level of pre-haustorial resistance, which has also been observed by Neu *et al* (2003). These data confirm the importance of pre-haustorial resistance in non-host resistance and indicate that some barley lines might be susceptible to non-adapted rusts due to a deficiency in pre-haustorial resistance.

Moreover, we found that the majority of the established colonies on the leaf segments of L94-*Rnhq,* rec204, and rec658 were associated with necrosis. However, no necrosis was observed during macroscopic evaluations, except a few small necrotic flecks on the inoculated leaves. This suggest that the hypersensitive response appeared to be ineffective and the hyphae were able to escape. Based on this study, there can be concluded that the gene of Vada within region *Rnhq* conferring resistance to *Pt* is responsible for quantitative resistance in order to reduce haustoria formation. This means that the gene of Vada do not rely on post-haustorial resistance.

Furthermore, the mean percentage of established colonies on the leaf segments of rec658 was 46%, from which the majority is these colonies were associated with necrosis. However, a higher percentage of established colonies was expected, similar to L94. This because the region between marker L2.2 and L2.4 was homozygous for the L94 allele for this recombinant, while the resistance to *Pt* is located in this interval. The markers on the left side of marker L2.2 were homozygous for the Vada allele. It could be that the resistant gene of *Pt* is closely located to marker L2.2, as a result that this recombinant showed an intermediate infection level. In further studies, it is recommended to phenotypically evaluate this recombinant in order to confirm its level of resistance.

5. Conclusions and recommendations

In this study, it is concluded that the gene responsible for *Phm* resistance is not located in sub-region 1. However, when L94-*Rnhq* was inoculated with *Phm,* it had a lower infection level than L94, indicating that the gene explaining resistance to *Phm* is located in within the *Rnhq* region. Further QTL mapping studies on the L94-*Rnhq* NIL are needed in order to find the exact location of *Phm* resistance. Since the independent recombinants of sub-region 1 were only tested with *Phm*, it is also suggested to phenotypically evaluate them with *Phs* to check whether resistance to *Phs* co-segregates or not with resistance to *Phm*.

For sub-region 2, there is evidence that the gene explaining resistance to *Pt* is located at the right side of marker L2.2 (86.7cM) and left side of marker L2.4 (87.3cM). To pin down the responsible gene, it is recommended to continue marker development and to find and test new rare recombinants which have their recombination point within this interval. Once the QTL region is smaller than 0.5cM, candidate genes will be identified which are likely responsible for resistance by using flow sorting techniques. These candidate genes will be cloned and subsequently overexpressed in Golden SusPtrit to identify the gene explaining *Pt* resistance.

Based on histological analysis, the effectiveness and/or mechanisms of the plant its defence against non-adapted pathogens varies over plant genotypes. Vada, L94-*Rnhq*, and rec204 have a high level of pre-haustorial resistance compared to the susceptible landrace L94, indicating that this type of defence mechanism is of great importance in non-host resistance against rust pathogens. Hence, the gene of Vada that in introgressed in L94 is responsible for quantitative resistance to *Pt*, from which it reduces the ability of the fungus to form haustoria. No hypersensitive response will be elicited after the formation of haustorium, indicating that the gene of Vada do not rely on post-haustorial resistance. The use of pre-haustorial resistance in breeding programmes of barley could make the resistance against *Pt* more durable.

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Appendix 1: Developed markers of each sub-region

Table 1 The forward and reverse sequence of primer pairs that were used to amplify a marker of each sub-region

Marker	Name of primer pair	Forward (F) and reverse (R) sequence (5'-3')
M1.2	SCRI_RS_146382	F: ATCGGTTGCCATGTAAACCAAGAAC R: GGCAACTATAGAAAGCCGATGTACG
M1.3	SCRI15	F: AGCTTATCTTCGGCCTTTTCATATGGA R: CGAAAACCATCGCCATGGAC
M1.4	Mk6-02	F: AGTATTACTAAACTCTCCGATTTGCAGAAGA R: GGTCGCTCGATCGTGGCA
M1.6	BOPA12_31357	F: CTACTCGGCCAGAAGGTATGAATG R: ACCTGGATAACTGGAAGACTGGG
M2.1	SCRI_RS_2914	F: TTCATCTCTTCATGTAGTTCAGCATAGACA R: TCCAGACAATTGAAGTTCTAAACAACTGATT
M2.2	Synt2119	F: GTAAGTTTAATGTGCCTGAATTGCCCT R: TGTTCCAAATATCGGCCAATTAATCACCAT
M2.3	Synt61085	F: GTCACGGAAGCAGCATATCATAAGTAAG R: GTGAGTCGTCCAAGACATCTCGTTTA
M2.5	Synt18066	F: TGGAATGAGTTACTGCTGGTGA R: GGCCCAGTTTTAAGGCGGAA
M3.1	SCRI_RS_194841	F: AGATCTGAACAACGCCGCC R: GGCGTGCATGTGATGTGAG
M3.2	BOPA2_12_21479	F: CAAGCTTACATTCTCAAGGAGAAAGAG R: TCACTGTAATGCTGCTATATTCTCTTGT
M3.3	BOPA1_2444-437	F: GTGCAATAGGGTGAAGAAGAAGATCTAA R: ACGACCTTGGCTTCTCCCTC
M3.4	SCRI_RS_143884	F: GACTGAAGGCGXCCAAGA R: GTGTTGAGGCTCAGGCCCAA
M3.5	SCRI_RS_196885	F: TCACGACGAGGACGGTG R: GTGATCTCGCCGAAACTGTAGGTA

Appendix 2: Previous tested recombinants for sub-region 1

Table 1: List of 14 independent recombinants and one non-recombinant from which their progenies were previously tested. Blue background indicates that these regions originate from Vada (V) and the regions having a red background originate from L94 (L). The RIF of the parents are classified as susceptible (RIF ≥ 75%; red background), intermediate (RIF 26-74%; orange), or resistant (RIF ≤ 25%; green). The resistance is expected to be at the right side of marker M1.5 and left side of marker M1.6 (bold line). The independent recombinants coloured in red were retested in this study.

Parents	M1.1/M1.2 (63.3 cM)	M1.3 (63.6 cM)	M1.4/M1.5 (63.7 cM)	M1.6 (67.6 cM)	RIF
G02006305_23_rec476	L	L	L	L	27
G02006305_22_rec328a	V	L	L	L	68
G02006305_23_rec476	V	L	L	L	27
G02006305_26	V	V	L	L	0
G02006305_22_rec328b	V	V	V	L	6
G02008072_46_rec264_rec1495	V	V	V	L	11
G02006307_06_rec381	V	V	V	L	19
G02006305_23_rec348	V	V	V	L	31
G02006308_95_rec43_rec1131	V	V	V	L	8
G02006307_06_rec383	L	L	L	V	54
G02006305_23_rec365	L	L	L	V	70
G02006305_24_rec253_rec1466	L	L	L	V	28
G02006307_14	L	L	L	V	11
G02006307_09_rec289_rec1506	L	L	L	V	0
G02006305_23_rec472	L	L	V	V	0

Appendix 3: Previous tested recombinants for sub-region 2

Table 2. List of 23 independent recombinants and one non-recombinant from which their progeny was previously tested. Blue background indicates that these regions originate from Vada (V) and the regions having a red background originate from L94 (L). The RIF of the parents are classified as susceptible (RIF ≥ 75%; red background), intermediate (RIF 26-74%; orange), or resistant (RIF ≤ 25%; green). The resistance is expected to be at the right side of marker 2.2 and left side of marker 2.5 (bold line). The independent recombinants coloured in red, do not confirm the expected location of resistance.

Parents	M2.1 (84 cM)	M2.2 (85.3 cM)	M2.3/M2.4 (87.2 cM)	M2.5 (87.5 cM)	M2.6 (87.7 cM)	RIF
G02006308_96_rec597	L	L	L	L	L	8
G02006305_22_rec461_rec1771	V	L	L	L	L	100
G02008071_106	V	L	L	L	L	94
G02006311_53	V	V	L	L	L	100
G02006312_75	V	V	L	L	L	100
G02006306_116	V	V	L	L	L	100
G02013093_82_rec2141	V	V	L	L	L	83
G02006312_69	V	V	L	L	L	100
G02006308_96_rec597	V	V	L	L	L	0
G02006307_06_rec2937	V	V	L	L	L	7
G02006305_22_rec461_rec1766	V	V	V	L	L	Segregation
G02006307_09_rec289_rec1705	V	V	V	L	L	10
G02006307_09_rec392	V	V	V	L	L	13
G02006305_22_rec461_rec1793_rec2283	V	V	V	L	L	11
G02006305_22_rec461_rec1771_rec2239	V	V	V	L	L	4
G02006305_22_rec461_rec1786_rec2267	L	L	V	L	L	0
G02006305_22_rec465_rec1968	L	L	V	V	V	15
G02006307_19	L	L	V	V	V	0
G02006305_22_rec334	L	L	V	V	V	0
G02006305_22_rec339	L	L	V	V	V	6
G02006311_61_rec850	L	L	V	V	V	0
G02006311_61_rec857	L	L	V	V	V	0
G02006306_113	L	V	V	V	V	3
G02006308_91	L	V	V	V	V	0

Appendix 4: Protocol DNA isolation

■ DNA isolation procedure:

a. Sample 2 cm first leaf material;
b. Add 150 ul DNA extraction buffer;
c. Smash the samples with the Retch/Tissue Lyser for 2 min at 20 hz;
d. Centrifuge for 2 min to spin down buffer and plant material;
e. Incubate samples at 80℃ for 10 min;
f. Centrifuge for 10 min to separate plant tissue from aqueous phase containing DNA.
g. Take out 30 ul supernatant and dilute it into 60 ul water
   ⇨ These samples can be used as a stock.
h. Take out 10 ul stock and dilute it into 60 ul water.
   ⇨ These can be used as a template for further LightScanner PCR.

■ DNA extraction buffer recipe:

Tris-HCL (0.5 M， pH = 7.5)	5 ml	20 ml
NaCL	0.88 g	3.52 g
Sucrose	5.135 g	20.54 g
H2O	Adjust to 50 ml	Adjust to 200 ml

⇨ Autoclave for long storage.

Appendix 5: Categories infection units

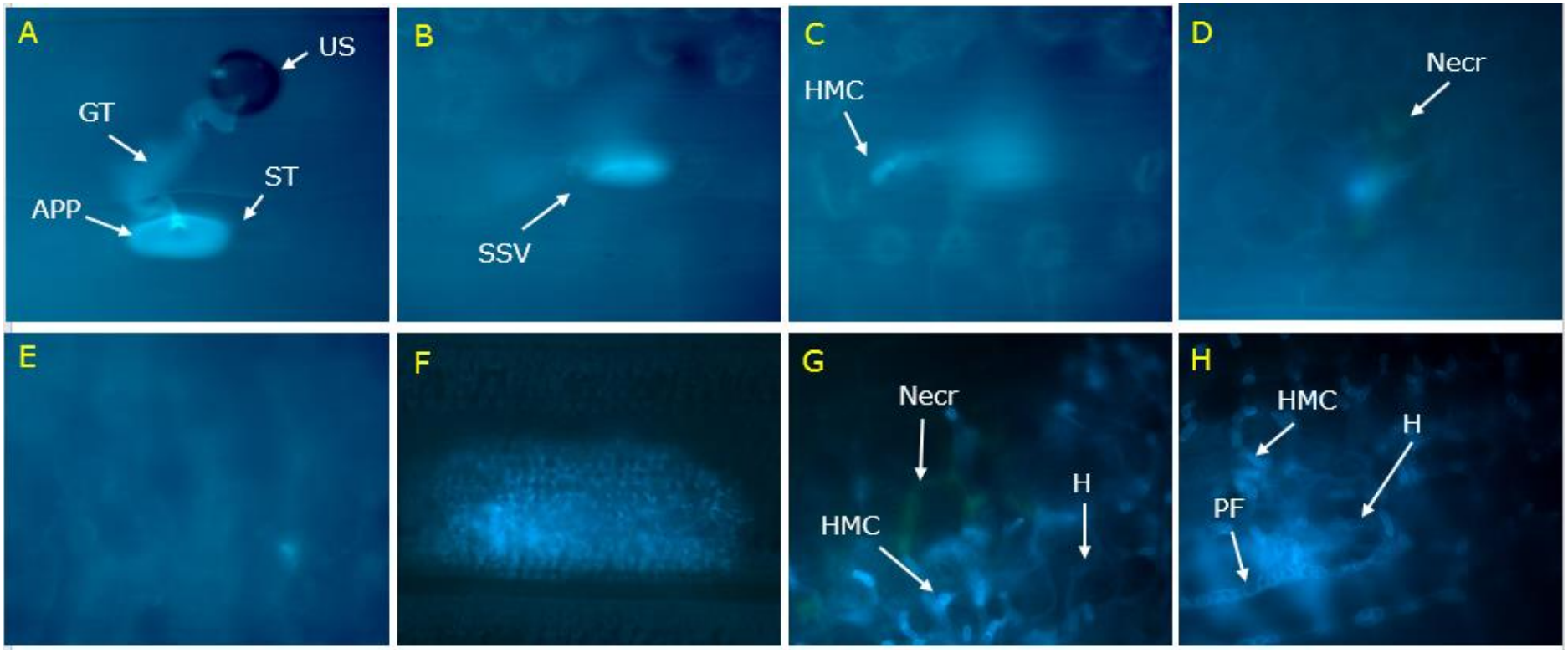

Figure 1: P. triticina infection on barley as a non-host at 7dpi under a UV microscope. A: Germinated uredinospore (US) with germtube (GT) that reach the stomata (ST) and form an appressorium (APP). B: Sub-stomatal vesicle (SSV) formation from appressorium. C: First haustorial mother cell (HMC) is developed, which cannot branch. Fungal growth is arrested. D: Early abortion with necrosis (Necr). E. Early abortion without necrosis. F. Established colony (10x). G. Established colony with necrosis. H. Established colony without necrosis and early development of pustule formation (PF).

Appendix 6: Designed primer pairs sub-region 2

Table 3 The forward and reverse sequences of 13 designed primer pairs within sub-region 2. The name of the gene in which the primer is designed and their polymorphism is also included.

Primer name	Gene	Forward (F) and reverse (R) sequence (5'-3')	Polymorphic
Synt86410-1	HORVU7Hr1G086410	F: TCTCTAGCATTATTTTGGACATTGTGAAA R: GTGATGATGTAAAACTACAAAGCCTGAA	No
Synt86410-2 (L2.1)	HORVU7Hr1G086410	F: ATTGCACCTTTGGGTTCAAGAAT R: AGCACACATATTCTCTGCACAAGA	Yes
Synt86650	HORVU7Hr1G086650	F: TGATAGACTAAACATGCATATGAGCAC R: TTGATGCCTTCATGTTAGTTTGTTAAA	No
Synt86670	HORVU7Hr1G086670	F: AGAACTGTAATTGAAGTGGAGATGGTG R: CGATCTATTCCCACATGTACTACTCCTA	No
Synt87050-1	HORVU7Hr1G087050	F: CTGACATCCTGGAGACCTGTCAGA R: AGGGTCGTAGATTTGTGTATGATCATGC	No
Synt87050-2 (L2.2)	HORVU7Hr1G087050	F: AGACGTTACACATGCATAAACATATCGA R: TGGCTGGTTCTGATGCTTGAC	Yes
Synt87070	HORVU7Hr1G087070	F: CAGTCCTATTTCAACTGCCCGTTC R: TCCCTGGAGTCGGAGCTG	No
S2_Cont50935 (L2.3)	HORVU7Hr1G087150	F: TCCCGGGTCCAAGGTACGAA R: TGGCGAGATGATCTTTGGTTTGG	Yes
Synt87210-1	HORVU7Hr1G087210	F: GAAAGCTGTACGTGGCAG R: ACGCAATCAAGGACAAGC	No
Synt87210-2	HORVU7Hr1G087210	F: ATGACCGTTCCCAGCAAA R: CCAGACAGTCATGTTAAGCTCT	No
Synt87250	HORVU7Hr1G087250	F: GCACGAGCTGACGGTGGTGGAG R: GGCTCCACCGGGTTGCCG	No
Synt87320-1 (L2.4)	HORVU7Hr1G087320	F: GCTTGGAGTTAAACAAACGG R: TAGTTAGCTGTACAGGGCAA	Yes
Synt87320-2	HORVU7Hr1G087320	F: AGCTTTACCGAATCAGTACACTT R: GCGCCATATCTGTCATCC	No

Appendix 7: Marker and phenotypic data van Dijk (2007)

Table 4: Marker genotypes of nine markers and the relative infection frequency of 22 recombinants inoculated with Phm and Phs. Blue background indicates that these regions originates from L94 (A) and regions having a red background originates from Vada (B). Green coloured RIF: recombinant is resistant (RIF ≤ 25 %). Orange coloured RIF: intermediate (RIF 26-74%). Red coloured RIF: susceptible (RIF ≥ 75%). Marker data of SKT1 is presented twice, since van Dijk found evidence that Phs co-segregates with marker SKT1.

Recombinant	WBE101	GBM1359	SKT1	P14M61_275	E33M61_85	MWG2031	SKT7	MN	GBM1303	IF Phm*	RIF Phm**	SKT1	IF Phs*	RIF Phs**
Rec17	A	A	A	A	A	A	B	B	B	16.4	87.4	A	8.6	42.6
Rec22	A	A	A	A	B	B	B	B	B	15.3	81.4	A	8.3	40.6
Rec16	A	A	A	B	B	B	B	B	B	13.8	73.2	A	10.8	56.8
Rec30	A	A	B	B	B	B	B	B	B	7.1	36.6	B	5.4	21.9
Rec29	A	A	B	B	B	B	B	B	B	11.8	62.3	B	5.4	21.9
Rec25	A	-	B	B	B	B	B	B	B	5.6	28.4	B	5.0	19.4
Rec11	A	A	B	B	B	B	B	B	B	3.8	18.6	B	2.2	1.3
Rec1	A	B	B	B	B	B	B	B	B	9.8	51.4	B	3.8	11.6
Rec4	A	B	B	-	B	B	B	B	B	14.3	76.0	B	5.0	19.4
Rec26	B	A	A	A	A	A	A	A	A	3.0	14.2	A	7.8	37.4
Rec18	B	A	A	A	A	A	A	A	A	2.3	10.4	A	9.2	46.5
Rec23	B	B	B	A	A	A	A	A	A	5.1	25.7	B	2.8	5.2
Rec9	B	B	B	B	A	A	A	A	A	0.6	1.1	B	0.3	0.0
Rec3	B	B	B	B	B	B	A	A	A	2.4	10.9	B	0.4	0.0
Rec14	B	B	B	-	B	B	A	A	A	7.2	37.2	B	2.2	1.3
Rec28	A	A	B	B	B	B	B	B	B	8.0	41.5	B	3.8	11.6
Rec10	A	-	B	B	B	B	B	B	B	3.6	17.5	B	0.0	0.0
Rec19	A	B	B	B	B	B	B	B	B	1	3.3	B	0.2	0.0
Rec20	A	A	B	B	B	B	B	B	B	4.4	21.9	B	0.2	0.0
Rec15	B	A	A	A	A	A	A	A	A	30.2	100.0	A	20.4	100.0
Rec5	B	A	A	A	A	A	A	A	A	16.5	88.0	A	26.8	100.0
Rec2	B	B	A	A	A	A	A	A	A	18.4	98.4	A	9.6	49.0
L94	A	A	A	A	A	A	A	A	A	18.7	100.0	A	17.5	100.0
L94-*Rnhq*	B	B	B	B	B	B	B	B	B	0.4	0.0	B	2.0	0.0

* : Infection frequencies (IF) and classification of van Dijk (2007)

**: IF converted to relative infection frequencies (RIF) corresponding to L94 and L94-*Rnhq*

Appendix 8: Marker and phenotypic data Salunke (2012)

Table 5: Genotypes of 23 markers and the relative infection frequency of 22 recombinants inoculated with Phm, Phs, and Pt. Nine markers were also used by van Dijk (2007, grey background). A marker can be homozygous for the Vada allele (red background; B) or for L94 (blue background; A). Green coloured RIF: recombinant is resistant (RIF ≤ 25 %). Orange coloured RIF: intermediate (RIF 26-74%). Red coloured RIF: susceptible (RIF ≥ 75%). Yellow bold line indicates the location of sub-region 1, and the black bold line indicates the location of sub-region 2.

Recombinant	IF Pt*	RIF Pt**	IF Phm*	RIF Phm**	IF Phs*	RIF Phs**	BOPA1_12239-662	BOPA1_12239-662	SCRI_RS_230478	SCRI_RS_15864	SCRI_RS_236651	BOPA2_12_30496	WBE101	SCRI_RS_2914	SCRI_RS_194085	GBM1359	SCRI_RS_150062	BOPA1_1674-468	SKT1	SCRI_RS_133026	P14M61_275	E33M61_85	MWG2031	BOPA1_11619-618	BOPA1_1676-557	SKT7	MN	GBM1303	SCRI_RS_194841
								65.8	70.1	71.6	72.4	75.1		81.8			82.4	83.6		84.2									88.0
Rec17	21	54	53	100	42	100	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B
Rec22	23	63	37	71	37	100	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B
Rec16	22	57	39	84	37	100	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B
Rec30	11	2	34	56	22	63	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B
Rec29	10	0	42	100	23	72	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B	B
Rec25	4	0	20	0	30	100	A	A	A	A	A	A	A	A	A	-	B	B	B	B	B	B	B	B	B	B	B	B	B
Rec11	11	1	22	0	-	-	A	A	A	A	A	A	A	B	B	A	B	B	B	B	B	B	B	B	B	B	B	B	B
Rec1	11	2	28	21	30	100	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Rec4	16	25	40	92	34	100	A	A	A	A	A	A	A	B	B	B	B	B	B	B	-	B	B	B	B	B	B	B	B
Rec26	27	83	16	0	8	0	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	-
Rec18	17	32	30	33	4	0	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Rec23	13	13	9	0	2	0	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A
Rec9	11	2	11	0	2	0	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A
Rec3	8	0	3	0	4	0	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A
Rec14	5	0	17	0	2	0	B	B	B	B	B	B	B	B	B	B	B	B	B	B	-	B	B	B	B	A	A	A	A
Rec28	6	0	16	0	-	-	B	B	B	B	B	B	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B
Rec10	8	0	16	0	4	0	B	B	B	A	A	A	A	A	A	-	A	B	B	B	B	B	B	B	B	B	B	B	B
Rec19	7	0	3	0	2	0	B	B	B	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Rec20	9	0	11	0	11	11	B	B	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B
Rec15	32	100	51	100	36	100	A	A	A	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Rec5	29	94	35	62	34	100	A	A	A	A	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Rec2	21	54	43	100	27	89	A	A	A	A	A	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A
L94	19	100	41	100	29	100	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
L94-*Rnhq*	11	0	25	0	9	0	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B

* : Infection frequencies (IF) and classification of Salunke (unpublished)

**: IF converted to relative infection frequencies (RIF) corresponding to L94 and L94-*Rnhq*