Genetic analysis of partial resistance to barley leaf rust (*Puccinia hordei)* in the Oregon Wolfe Barleys

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MSc Thesis Plant Breeding

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Abstract

The foundation work made by Marcel *et al.* (2007) and Yeo Kuok San (2007) on the genetic dissection of QTLs for partial resistance against *Puccinia hordei* in the Oregon Wolfe Barleys has been continued in this study.

Two new putative QTLs for partial resistance have been identified at the adult plant stage by combining data from Yeo Kuok San (2007) and new data collected in this study. At a different position on chromosome 5H as *Rphq16*, definitely not effective at the adult plant stage, one QTL for partial resistance has been detected by using data from the counting of the flag leaves and the F-1 leaves, showing the relevance of the F-1 to assess the level of partial resistance. The second QTL was mapped on chromosome 2H, at a similar locus as *Rphq6* found in L94xVada population (Qi *et al.*, 1999), and overlapping with *Rphq18*, a QTL found by Marcel *et al.* (2007) in the OWB population at seedling stage. The two other QTLs for partial resistance, detected on chromosome 6H (new location) and 7H (closed from *Rphq1* and *Rphq8*) could be considered as minor QTLs effective only under specific condition since they have been detected only in the first replicate.
A pleiotropic effect of the inoculation groups (linked to the heading date of the genotypes) on the level of partial resistance was observed since two QTLs for earliness were overlapping with QTLs for partial resistance on chromosome 4H and on chromosome 5H.

The fine-mapping of *Rphq16*, the most effective QTL at seedling stage in the OWBs, put forward a new position of the QTL upstream from the one from Yeo Kuok San (2007), now located in genetic window of 0.7 cM (out of a complete high resolution genetic map of 13.3 cM) between ABC09095 (a new marker added between MWG2249 and GBS0408, but closer from MWG2249) and MWG2249. The analysis of new recombinants in the *Rphq16* region, allowed us to bring forward the hypothesis that *Rphq16* might co-segregate with ABC09095.

Key words: QTL mapping, partial resistance, Oregon Wolfe Barleys, *Rphq16*, fine-mapping, marker saturation, pleiotropy, *Puccinia hordei*

1. General introduction

QTL mapping is a powerful tool to identify regions of the genome influencing a specific trait. It has been widely used in plant breeding, to identify for example chromosome regions that contribute to resistance of a crop to a specific pathogen.
At Wageningen UR Plant Breeding, the "Barley Group" has mapped some QTLs for partial resistance of barley to *Puccinia hordei*.

The barley studied is *Hordeum vulgare* L., the cultivated barley. This crop is very diverse by having summer and winter type forms, and six rows or two rows ears. It is an autogamous diploid crop (*2n=2x=14*) with chromosomes labelled from 1H to 7H according to their homology to wheat chromosomes (Franckowiak *et al.*, 1997, cited in Yeo Kuok San, 2007).

The barley leaf rust, *Puccinia hordei* Otth (Basidiomycota, Uredinales, Pucciniaceae)is among the harmful fungi for cultivated barley, causing worldwide high yield losses.

To avoid high costs due to heavy chemical fungicide treatments, breeding for resistant cultivars is of the utmost importance. The resistance (i.e. "the ability of the plant to reduce either the growth and/or the development of the parasite after contact has been initiated or established", Niks and Lindhout, 2006) of the new cultivars should be durable (i.e. that it "remains effective when applied at large scale in an optimal environment for the parasite after a long period of time", Niks and Lindhout, 2006). It exists different mechanisms of resistance, some are believed more durable than others. The resistance can be split into non-host and host resistances. In turn host resistance can be divided into hypersensitivity and non-hypersensitivity or partial resistance (Niks and Lindhout, 2006).

A plant specie is a non-host to a potential pathogen specie when all the genotypes of that plant specie are fully resistant to all genotypes of that pathogen specie. As barley is a host to *Puccinia hordei*, let focus of the different host-resistance type.

Major genes, named *Rph* (or R genes) are governing the hypersensitivity resistance. This resistance is isolate specific and function according to a gene-to-gene system (Niks and Lindhout, 2006). Breeders are interesting in R genes, because of their effectiveness (complete

resistance, stage independent) and easy to breed for but it is easily overcome by the pathogen, so not durable.

The other type of host-resistance is called “partial” resistance or field resistance (Parlevliet, 1975, cited in Qi *et al.,* 1999). Niks and Lindhout (2006) defined partial resistance as “the resistance that results in a reduced epidemic built-up of the natural enemy, despite a susceptible, non hypersensitive infection type”. The reduction of the infection is quite interesting since Ochoa and Parlevliet (2007, cited in Yeo Kuok San, 2007) report that high yield losses are correlated with high infection type. Van der Planck (1963, 1969, cited in Qi *et al.,* 1999) put forward that partial resistance is race non-specific because partially resistant cultivars are performing equally well to a wide range of rust isolates. A Cultivar x Isolate effect has been suggested by several researches mentioned in Qi *et al.* (1999) and by Marcel *et al.* (2008) stating that partial resistance might be race specific, and function according to Parlevliet and Zadocks (1977) on a minor gene-to-minor gene interaction system.
The main drawbacks for breeders of partial resistance are that sometimes the QTLs could be stage dependent or very difficult to detect (small effect, interacting with other QTLs) and the resistance is not complete. However QTLs for partial resistance can be accumulated (Qi *et al.,* 2000) and the barley genome contains a lot of QTLs for partial resistance (Qi *et al.,* 2000, Marcel *et al.,* 2007). A multitude of QTLs, all having a small effect on the level of partial resistance, can have a bigger influence on the development of the pathogen when put together (Niks and Lindhout, 2006). The abundance of QTLs, hampering the adaptation of the pathogen to several genes at the same time, was put forward as an explanation of the durability of partial resistance (Qi *et al.,* 2000, Marcel *et al.,* 2007). Even if more difficult to handle than hypersensitive resistance, the durability and wide cover (race-non specific) of partial resistance make it quite interesting for breeders.

Based on the future prospect established by Yeo Kuok San (2007), there are two objectives for this thesis:

- ***Objective 1***: the first objective of this study was to repeat the QTL mapping for partial resistance on adult plants, in order to gain a better statistical power and a better confidence in the QTLs detected (Yeo Kuok San, 2007). The purpose is to compare the QTLs for partial resistance at seedling stage and at adult plant stage to see if there is any overlap. In this, study the pleiotropy of QTL for heading date and for partial resistance was also studied.

- ***Objective 2***: the second and main objective of this study was to confirm the high-resolution genetic map and to precise the fine genetic position of *Rphq16*, a QTL involved in partial resistance of the OWB population against *Pucinia hordei*. The final aim of fine-mapping a QTL of interest is the isolation of its causal gene(s) (i.e. "target gene explaining the QTL") through a "map-based cloning" approach. Fine-mapping a QTL requires the development of new tools including new molecular marker like CAPS or SCAR markers

2. Mapping QTLs for partial resistance against *Puccinia hordei* at adult plant stage of the Oregon Wolfe Barleys

2.1 Introduction

The Oregon Wolfe Barley (OWB) population consists of 94 lines, which are double-haploid spring barley (Costa *et al.*, 2001) derived from F_1 by using the *Hordeum bulbosum* system. The two parents are Dom (containing dominant morphological marker stock) and Rec (containing recessive morphological marker stock) (Wolfe 1972, cited in Costa *et al.*, 2000).
This population was developed to be a powerful research tool: it has a high degree of phenotypic variation (to map all kind of different traits) and ideal for marker development since formatted for PCR plates having 96-wells format (94 DH lines in the population).

For each developmental stage (adult and seedlings), different QTLs for partial resistance against *P. hordei* were found and mapped in the OWBs. Yeo Kuok San (2007) and Marcel *et al.* (2007) reported that there was no overlap between the QTLs found at seedling stage and the QTLs found at the adult plant stage. Yeo Kuok San (2007) put forward that *Rphq16*, the most effective QTL detected in the OWB population by Marcel *et al.* (2007), mapped on chromosome 5H, appeared to be effective only at seedling stage (Figure 1).

More replications are needed to gain a better statistical power and a better confidence in the QTLs detected. Like in the previous study, the purpose of this experiment is to determine whether the QTLs found on seedlings by Marcel *et al.* (2007) are also effective at the adult plant stage.
QTLs for heading date were also mapped in this study in order to identify possible pleiotropy of the heading date on the level of partial resistance.

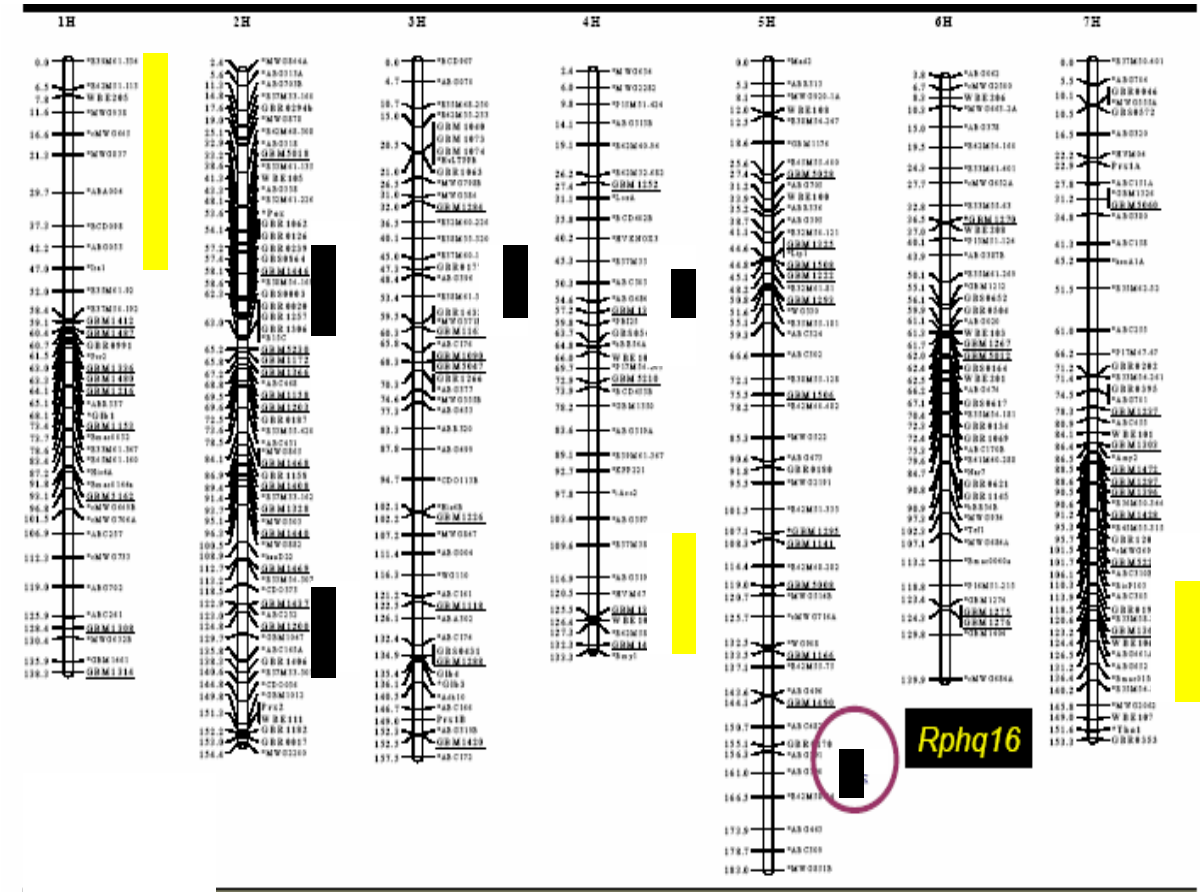

Figure 1 : QTL expressed at the different development stage of OWB (In yellow: QTLs found by Yeo Kuok San 2007a at adult stage; in black: QTLs for partial resistance found by Marcel *et al.*, 2007, at seedling stage). Map used: consensus map from Marcel *et al.*, 2007.

2.2 Material and Methods

2.2.1 Inoculation experiment

P. hordei 1.2.1 spores were multiplied on a very susceptible line, L94. The mapping population used was the OWBs (accession nr. G2004456 to G2004549 or G2007001 to G2007094 in case no plant germinated).

If for several genotypes, the flag leaves appeared at the same time, they were grouped together as an inoculation group, to be then inoculated with *Puccinia hordei*. The plants were inoculated with *P. hordei* (1.2.1) (1 mg of spores per pot) diluted 10 times with lycopodium powder. The inoculated plants were then put overnight into the inoculation chamber with 8 hours of dark and 100% relative humidity.

Three seeds were sown per pot for each parental line and each double haploid line of the mapping population.

Parental lines were sown in parallel (L94, Rec, Dom, Vada, SusPrit). They were sown several times at three day intervals in order to ensure having reference plants at the right developmental stage available for each group of inoculation.

2.2.2 Physiological trait: Days Between Inoculation Group

The physiological trait measured, Days Between Inoculation Group (DBIG), for this study was linked to the heading date of each genotype.

In each replicate, the genotypes from the first inoculation group received a DBIG of 1. The DBIG of the genotypes belonging to the other inoculation group was equal to the to the time between the inoculation date of those genotypes and the inoculation date of the first inoculation group as shown in Figure 2

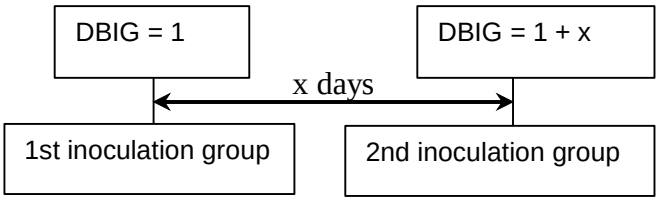

Figure 2 : calculation method to obtain the DBIG of a genotype

2.2.3 Partial resistance: latency period, relative latency period and genotypic effect

The latency period was used to assess the level of partial resistance of our genotypes. It can be defined as "the period elapsing from the moment of inoculation to the moment of becoming infectious". For this study, the LP50A was measured as the time needed for the pathogen to reach half of the maximum number of mature pustules. To measure it, the number of *P. hordei* pustules was counted every day in a delimited leaf area (containing about 50-100 infection sites) from the day at which the first symptoms were observed. The latency period was determined on the flag leaves (from 1 to 3 flag leaves per line depending on germination of the seeds) and on the F-1 leaves (idem).

The LP50A was then calculated with the following formula:

$$LP50A = T_1 + (T_2 - T_1) \times \frac{(N_{100/2} - N_1)}{(N_2 - N_1)}$$

T_1 = The time just before 50% of the pustules are mature

T_2 = The time just after 50% of the pustules are mature

N_1 = Number of mature pustules at T1

N_2 = Number of mature pustules at T2

$N_{100/2}$ = Half of the total mature pustules number

To normalize the results, the relative latency period (RLP50A) was calculated. The RLP50A of a genotype is the ratio between the LP50A of this genotype and the LP50A of a reference line. In this study Dom was selected as reference to calculate the RLP50A because it was the most constant line over replicates. It means that Dom showed a low variation of LP50A between inoculation sets in each replicate. LP50A and RLP50A were calculated for the flag leaves and the F-1 leaves. The average of RLP50A for the flag leaves, for the F-1 leaves, and for the flag leaves together with the F-1 leaves were used to detect new QTLs for partial resistance.

The Genotype Effect (GE) was also calculated with the statistical software Genstat, version 11.0. The GE indicates the deviation of each genotype compared to the mean of all the genotypes. It removes the background "noise" such as block effect or environmental effect. It was calculated by using the RLP50A from the three replicates.

2.2.4 QTL mapping

The QTL mapping was done by using the software MapQTL® version 5.0 developed by van Ooijen (2004). It began with the Interval Mapping (IM) which combined the collected phenotypic data and the genotypic data (a skeleton map of the OWBs extracted from the barley integrated map 2009). The IM allowed us to see which markers had a high (i.e. LOD>1) and/or significant (i.e. LOD>3) LOD score. Such markers were then selected as co-factors and an Automatic Co-factor Selection (ACS) was performed to retain only the most useful co-factors.

With the set of co-factors retained, Multiple QTL mapping (MQM) was run (Jansen, 1993). If new peak markers were detected, new cofactors were selected and a new ACS was performed before repeating the MQM analysis.

When no additional peak markers were noticeable (i.e. stable LOD profile), a restricted MQM was run to characterize the detected QTLs: LOD value, percentage of the variation explained.

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The map graphs were done with MapCharts 2.2, also developed by van Ooijen (2004). The position of the QTLs was determined by calculating the 2-LOD and the 1-LOD confidence intervals:

- *[LOD of the peak marker] - 2* : it gives an interval of positions in which the QTL is located with an error rate of 0.05.
- *[LOD of the peak marker] - 1* : it gives an interval of positions in which the QTL is located with an error rate of 0.1.

The map used in MapChart 2.2 was the skeleton map of the OWBs extracted from the barley integrated map 2009 (Aghnoum *et al*. submitted).

2.3 Results

2.3.1 QTLs for partial resistance against *P.Hordei* 1.2.1 in OWB

For each replicate, the population shows a normal distribution indicating a transgressive segregation for its RLP50A frequency distribution (for the flag leaf) especially clear for the third replicate and the three replicate together (Figure 3). This means that both parents are contributing for the resistance. The same phenomenon was observed with the frequency distribution of the F-1 leaf (See Appendix 2).

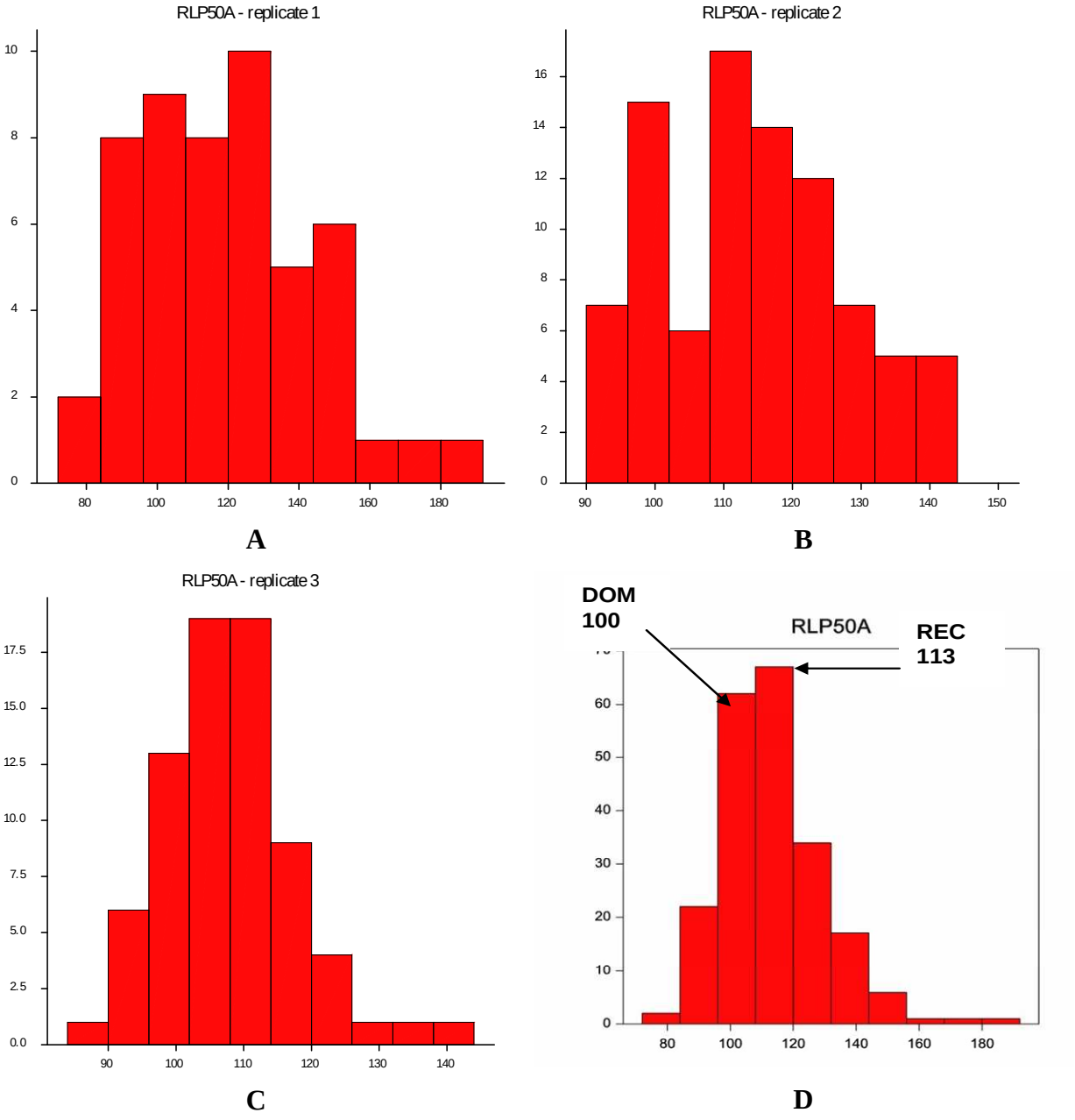
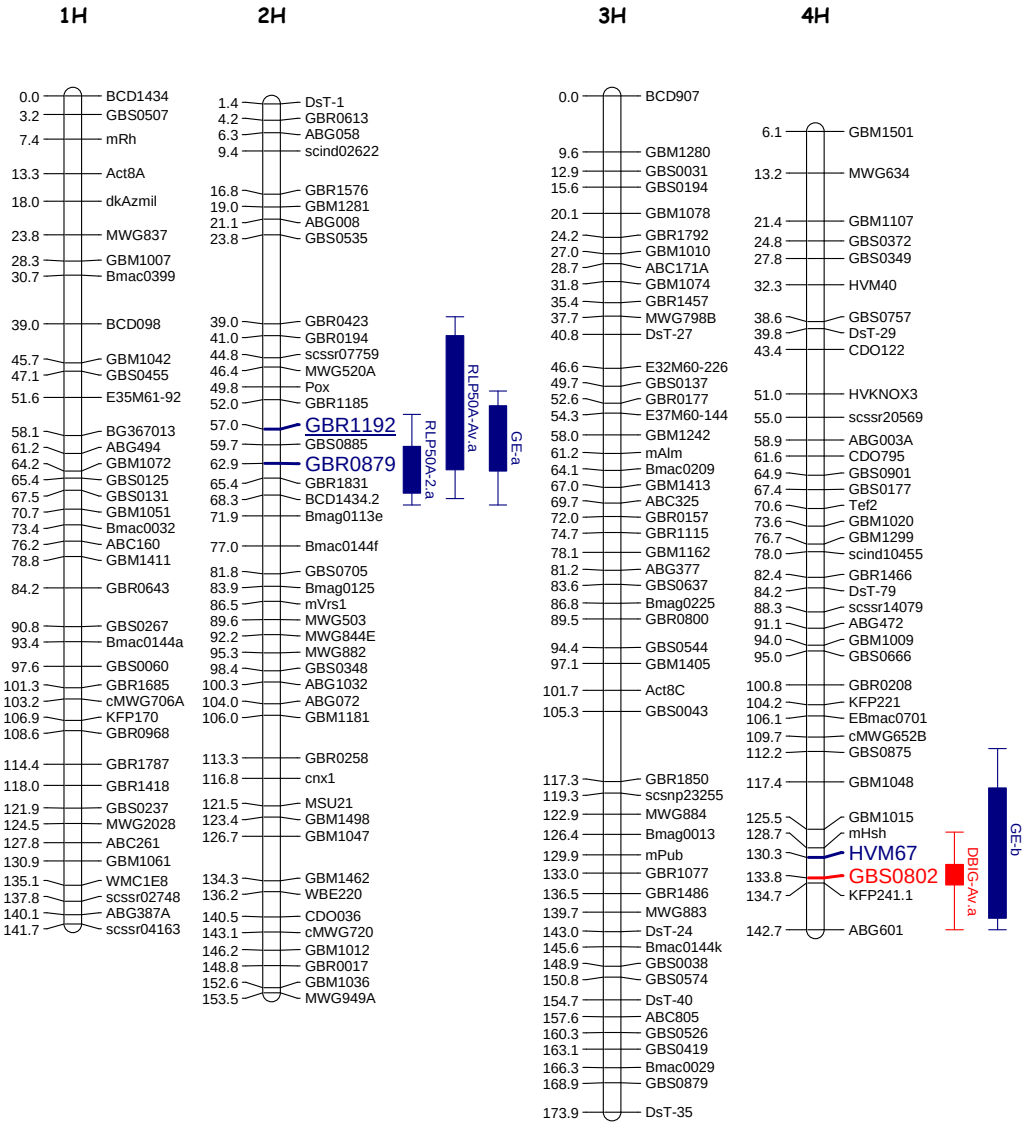


Figure 3 : frequency distribution of phenotypes for the RLP50A (for the flag leaf) in OWB for : A=first replicate; B=second replicate; C=third replicate; D= the three replicate. X-axis: RLP50A values; Y-axis= number of lines.



The QTLs identified in the Oregon Wolfe Barleys for partial resistance against *Puccinia hordei* 1.2.1 are different between replicates and differs also depending on the data set used for mapping (i.e. RLP50A or GE).

In the first replicate (data collected by Freddy Yeo Kuok San in 2007), 3 QTLs for partial resistance were detected (Table 1, Figure 4). The three QTLs explained about the same percentage of the variation, and Dom was contributing of the resistance allele (positive additive effect, Table 1). They were detected only in the first replicate and not in the other two.

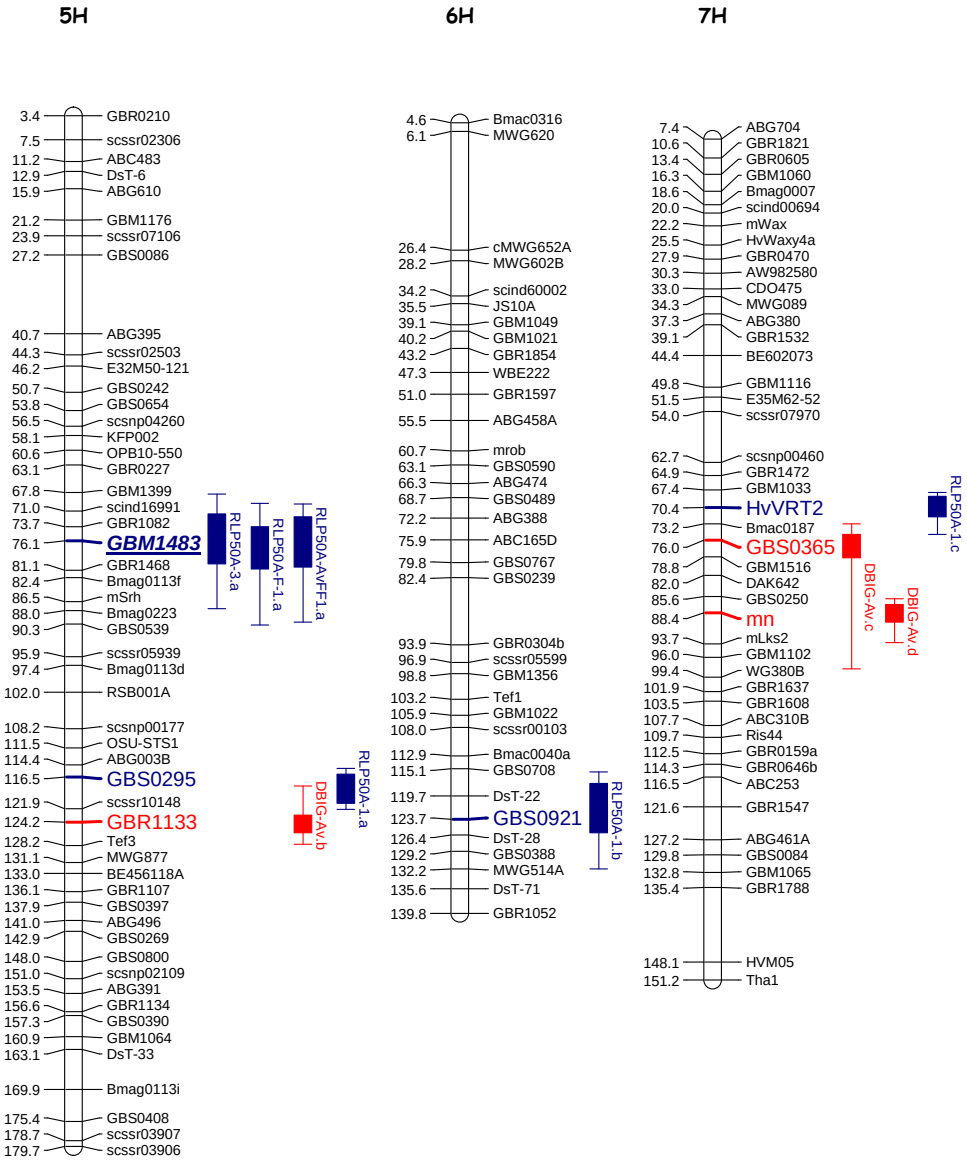


Figure 4 : Distribution of QTLs for partial resistance against *P. hordei* at adult plant stage (blue bar) and of QTLs for Day Between Inoculation Group (red bars) on the OWB skeleton map extracted from the integrated map 2009 (Aghnoum *et al.*, submitted) (Table 1). Markers in bold: peak marker. Markers in bold and underlined: peak markers for more than one QTL.

The same QTL on chromosome 2H was detected by using the data from replicate 2 (data collected by Freddy Yeo Kuok San in 2007), the RLP50A average of the three replicates and the genotypic effect (Table 1, Figure 4). Rec was contributing for the resistant allele of this QTL (negative additive effect in the three cases, Table 1).

Table 1 : QTLs for partial resistance in the OWB population detected in each replicate, with the average of the three replicates and the genotypic effect (data of the three replicates). The positions are given for the integrated OWB skeleton map 2008 (extracted from the integrated map 2009, Aghnoum *et al.*, submitted).

	Rep. or Av.	Leaf	QTL	Peak marker	Chr.	2 LOD (cM)	1 LOD (cM)	LOD	Exp%	Add.
RLP50A	1	F	RLP50A-1.a	GBS0295	5H	115 - 121.9	116 - 121	5.22	17.6	10.6
			RLP50A-1.b	GBS0921	6H	115.6 - 132.2	117.6 - 126	5.42	15.7	10.45
			RLP50A-1.c	HvRT2	7H	67.8 - 75	68.5 - 72	4.98	22.5	11.88
	2	F	RLP50A-2.a	GBR0879	2H	54.5 - 70	60 - 68	6.99	23.9	-6.48
	3	F	RLP50A-3.a	GBM1483	5H	68.1 - 87.7	71.5 - 80	6.09	26.1	-5.27
		F-1	RLP50A-F-1.a	GBM1483	5H	69.7 - 90.5	73.7 - 80.9	3.71	21.5	-3.57
		F+F-1	RLP50A-AvFF1.a	GBM1483	5H	69.8 - 90	72 - 80.6	4.2	14.6	-3.31
	Av.	F	RLP50A-Av.a	GBR1192	2H	37.8 - 68.9	41 - 64	4.1	13	-4.33
GE	Av.		GE-a	GBR1192	2H	50.5 - 70	53 - 64.2	4.4	12	-5.93
			GE-b	HVM67	4H	111.7 - end	118.4 - 140.7	3.34	8.7	-4.67

Rep.: number of the replicate; Av.: average of the three replicates Exp%: percentage variation explained by the QTL; Add.: additive effect; F: flag leaf; F-1: F-1 leaf; F+F-1: average of the data from the flag leaf and the F-1 leaf; Chr.: chromosome

Another QTL for partial resistance was detected on chromosome 5H (Figure 4) by using only data from the third replicate (data collected by the author of this thesis in 2008): RLP50A for the flag leaf, RLP50A for the F-1 leaf, average of RLP50A for the flag leaf and the F-1 leaf. Rec was contributing for the resistance allele (negative additive effect, Table 1)

2.3.2 QTLs for heading date in OWB

The DBIG was constant between replicates for every genotype since the correlation factor between replicates was of about 0.6 on average.

Four QTLs related to the development stage of the plant were found by suing the DBIG (Table 2).

The QTL DBIG-Av.a is localized at the telomeric end (long arm) of chromosome 4H (Table 2) and the QTL DBIG-AV.b on chromosome 5H and the two other QTLs were detected (since two distinct peak markers were found) on chromosome 7H overlaping with each other (Table 2, Figure 4).

Table 2 : QTLs related to the plant development stage found in the Oregon Wolfe Barleys by using the DBIG (Day Between Inoculation Group).

	Replicate or Average	QTL	Peak marker	Chr.	2 LOD (cM)	1 LOD (cM)	LOD	Exp. %	Add.
DBIG	Average	DBIG-Av.a	GBS0802	4H	126 - end	131.5 - 135	7,11	14,8	2,25
		DBIG-Av.b	GBR1133	5H	118 - 128	123 - 126	6,75	14,6	-2,23
		DBIG-Av.c	GBS0365	7H	73.2 - 98	75 - 79	3,35	8,8	-1,77
		DBIG-Av.d	mn	7H	86 - 93.5	87 - 90	3,82	10	-1,88

Exp%: percentage explained by the QTL; Add.: additive effect; Chr.: chromosome

2.4 Discussion

2.4.1 QTLs for partial resistance against barley leaf rust

In general, the QTLs for partial resistance detected in the different replicates were not overlapping each other (Table 1, Figure 4), however overlaps between QTLs were observed between the ones detected in the different replicates and the other identified with the RLP50A average of the three replicates and with the genotypic effect (of the three replicates), or between QTLs found with different dataset from the same replicate (counting of flag and F-1 leaves in replicate 3)

In this discussion, we were more confident in QTLs detected with more than one dataset than QTLs identified with only one.

On chromosome 2H, the same QTL for partial resistance has been detected three times (Table 1, Figure 4) at a similar locus as *Rphq6*, found at adult stage in L94xVada population (Qi *et al.*, 1999) and more interesting at a similar locus as *Rphq18*, identified in OWBs at seedling stage (Marcel *et al.*, 2007). So it could mean that *Rphq18* is present at both stages (adult and seedling, so stage independent) in the OWB population.

Another QTL for partial resistance was detected three times on chromosome 5H at a location where no QTLs for partial resistance have been mapped before. It has been identified with three dataset coming from the third replicate: the RLP50A of the flag leaf, the RLP50A of the F-1 leaf and the average of RLP50A for the flag and F-1 leaves. This showed the relevance of the F-1 to assess the level of partial resistance against the leaf rust. It raised the hypothesis of a homogeneous repartition of the resistance through the plant.

Rphq16 is definitely absent adult stage as shown on the rMQM profile of the chromosome 5H for the three dataset mentioned previously (Figure 5)

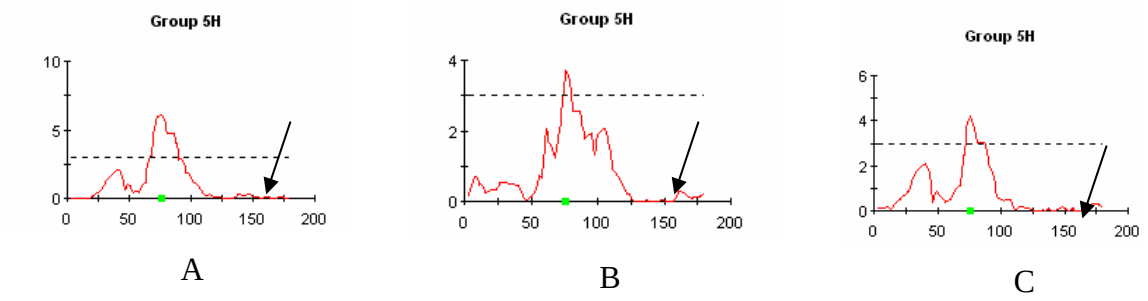


Figure 5 : LOD score profile for the rMQM of chromosome for the following dataset coming from replicate 3: A= RLP50A for the flag leaf; B=RLP50A for the F-1 leaf; C= average of RLP50A for the flag and F-1 leaves. The arrows are indicating the position of *Rphq16*. Green dot: peak marker of the QTL, GBM1483.

Two other QTLs for partial resistance were detected but only in the first replicate: one on chromosome 6H (Table 1, Figure 4) at a completely new location (i.e no QTL for partial resistance mapped there before), and one on chromosome 7H, closed to the location of *Rphq1* (mapped in L94xVada at seedling stage, Qi *et al*., 1999) and *Rphq8* (mapped adult stage in L94xVada, Qi *et al.*, 1999; at seedling stage in SteptoexMorex, Marcel *et al.*, 2007, in SusPtritxVada, Jafary *et al.*, 2006). As they were detected with only one dataset, this put forward the hypothesis that they could be minor QTLs effective under different conditions (since the replicates have been made at different time and in different greenhouse compartment).

2.4.2 Pleiotropic effect of the inoculation groups on the level of partial resistance to barley leaf rust in OWB

The presence of a pleiotropic effect was confirmed by the significant inoculation group effect (represented by the DBIG average of the three replicate) on the level of partial resistance against barley leaf rust (RLP50A), shown by the ANOVA realized with the DBIG values as factor and the RLP50A (of all individuals for the three replicates) as variable (See Appendix 1).

This pleiotropic effect was illustrated by the overlap between QTLs found with the DBIG (average of the three replicates) and the QTLs for partial resistance detected at the telomeric end of chromosome 4H (genotypic effect of the three replicate, 3.5 cM between peak markers) and the one on chromosome 5H (data from the first replicate collected by Yeo Kuok San, 2007, 7.7 cM between peak markers) (Table 1, Figure 4).

At a similar locus as the QTL for partial resistance detected on chromosome 2H, QTLs for heading date and for plant height have also been mapped by Qi *et al.* (1999) in L94xVada (one for day to heading and one for plant height) and by Yeo Kuok San (2007, GrainGenes data) in Steptoe x Morex population (after a field inoculation, also one for day to heading and one for plant height). A gene for earliness, *Pdp-H1*, has also been mapped at this locus (Emebiri *et al.*, 2005; Cuesta Marcos *et al.*, 2008a&b; Sameri *et al.*, 2006). So a pleiotropic effect could be at the origin of this QTL. But this hypothesis was wrong since no QTL have been identified in this study with the DBIG as shown by the rMQM profile of the DBIG average of the three replicates (Figure 6).

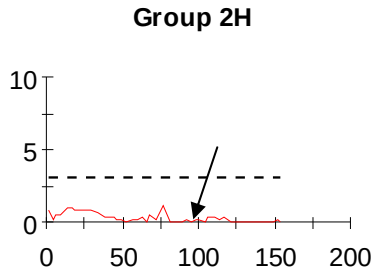


Figure 6 : rMQM of the DBIG average of the three replicates for the chromosome 2H. The arrow is indicated the position of the QTL for partial resistance detected during this study on chromosome 2H

3. Fine mapping of the QTL region *Rphq16*

3.1 Introduction

Rphq16 is a major QTL for partial resistance against *P. hordei* 1.2.1 detected by Marcel *et al.* (2007) in the OWBs. Dom contributes the resistance allele of this QTL, which explains 33% of the phenotypic variation. It is located on the telomeric end of chromosome 5H long arm with the SSR marker Bmag113i as a peak marker (Marcel *et al.*, 2007). Yeo Kuok San (2007) claimed that this QTL is developmental stage dependant and is only effective at the seedling stage.

Rphq16 was located by Yeo Kuok San (2007) in a very high recombination region of chromosome 5H (ratio physical/genetic distances = 0.2 to 0.9 Mb/cM, Künzel *et al.*, 2000), which is favourable to follow a map-based cloning approach for that QTL (Figure 7).

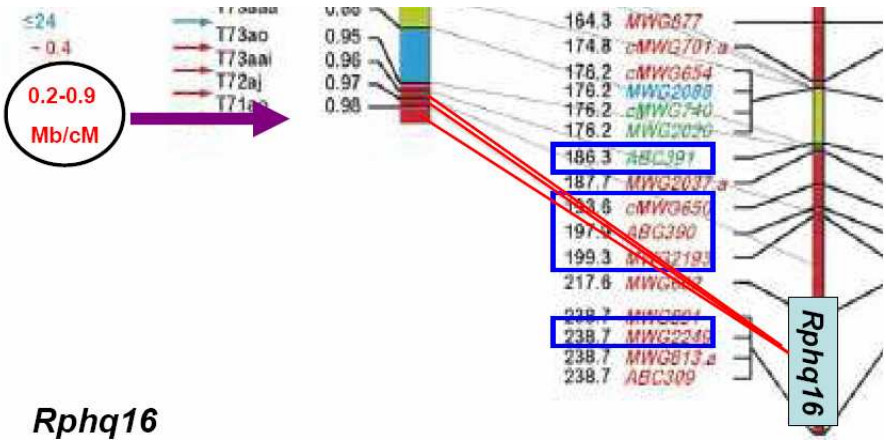


Figure 7 : physical (left) and genetic map (right) of *Rphq16* region. In blue: marker used by Yeo Kuok San (2007).

The plant material for the fine mapping of *Rphq16* was coming from a cross between Dom (donor of *Rphq16* and *Rphq17*, two QTLs for partial resistance) and SusPtrit (highly susceptible to *P.hordei* with no background QTL and to heterologous rusts). Because of its susceptibility, SusPtrit was selected as recurrent parent to make Dom x SusPtrit backcross. At the Dom x SusPtrit BC_1 stage, a positive selection was performed for *Rphq16* and a negative selection for *Rphq17*. The selected BC_1 plants were used to make marker assisted backcrosses in order to develop a *Rphq16*-NIL in SusPtrit background, which will take approximately 2-3 years to be ready (Figure 9).

The plant material used by Yeo Kuok San (2007) were part of a short-coming to traditional fine-mapping, consisting at using the segregating BC_1 plants (BC_1S_1, Figure 9) for a fine-mapping strategy, assuming that the background without *Rphq17* will not influence too much the phenotype of *Rphq16*. The NILs will be used later on to confirm the fine-mapping results, the QTL effect and to determine whether *Rphq16* may also be involved in non-host resistance to heterologous rust species (Jafary *et al.*, 2008).

Yeo Kuok San (2007) fine mapped the position of *Rphq16* in a genetic windows of 3.9 cM (between the CAPS co-dominant marker TC147930 and the SSR marker GMS002) by combining data from a seedling disease and a high resolution map of the region (Figure 8). The seedling disease test was performed on BC_1S_1 plants, indicated a QTL effect of 10 hours. He used 260 homozygous plants (derived from 26 BC_1S_1 recombinants) to generate the high resolution genetic map obtained by saturating the region with markers developed from available marker information (e.g.: RFLP sequences, EST sequences) or from the synteny (defined as the conservation of linear gene and marker order (co-linearity) between plant species, Perovic *et al.* (2004)) between rice and barley. It turned out that the synteny between the two crops was not conserved (Appendix 3). The high resolution genetic map generated was similar to OWB maps (GrainGenes: OWBs, 2005) but showed some differences with the consensus map of Marcel et al. (2007) (integration of information from different genetic map of different mapping population) (Figure 8).

The next step in the fine-mapping of *Rphq16* would be to confirm the previous results (high resolution genetic map; *Rphq16* location and the QTL effect) and to continue narrowing down the genetic windows of its location (marker development, screening for new recombinants, new disease tests).

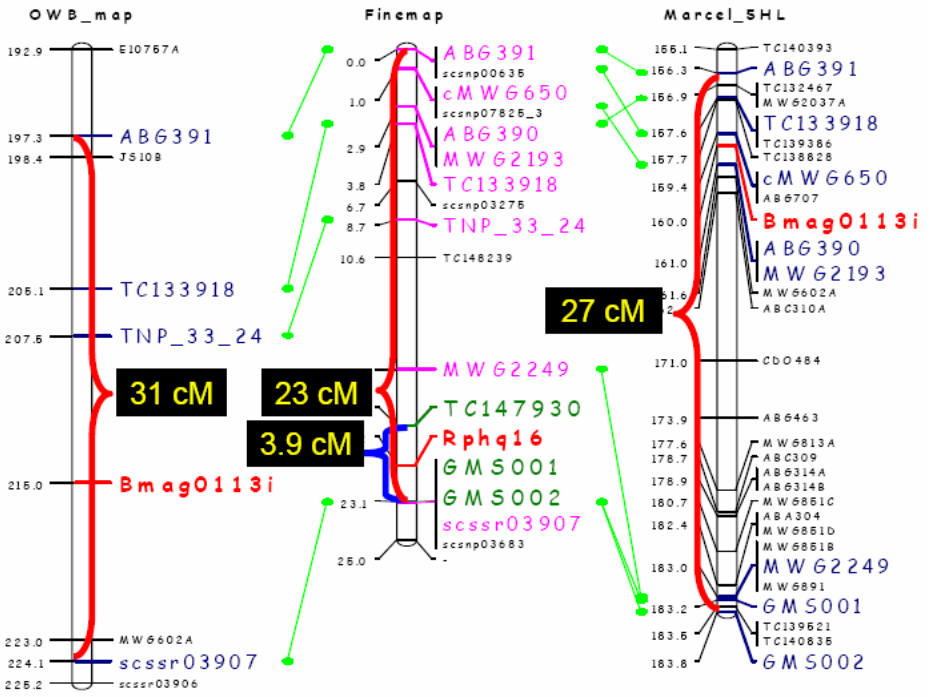


Figure 8 : fine mapping of *Rphq16* region. Alignment of the OWB maps, the genetic map obtained by Yeo Kuok San (2007), and the consensus map developed by Marcel *et al.* (2007). The blue brace indicates the new confidence interval of *Rphq16*, and the red brace indicates the distance between the flanking markers used.

3.2 Material and methods

3.2.1 Phenotyping of available homozygous lines

Thirty-four homozygous recombinants (BC_1S_3, Figure 9) for the *Rphq16* region were already available (left over from Yeo Kuok San, 2007). A seedling disease test was performed to evaluate the latency period of those recombinants. The experiment was performed three times and in each replicate, 5 seeds per line were sowed.

Four inoculation boxes were used in each replicate: three boxes contained 40 plants and the last one 45 plants. In each box, 5 reference lines (Dom, Rec, SusPtrit, Vada, L94; 1 seed per reference line) were sowed, so 20 reference lines per replicate..

Each box was inoculated with 2.5 mg of *P.hordei* 1.2.1 and the latency period of each line was measured (same formula as in part 2.2.3). The relative latency period was calculated according to the latency period of SusPtrit (lowest standard deviation between replicate: 3.8).

The phenotypic data collected were combined with the genotypic data (i.e. the genotypes of the homozygous lines in Rphq16 region) from Yeo Kuok San (2007).

Two groups were distinguished according to a Tuckey permutation test ($p<0.05$) (done with GenStat 11.0 on the RLP50S from the 3 replicates): the susceptible recombinants and the resistant ones (Appendix 5).

3.2.2 Screening for new recombinants

From a cross between Dom (donor of *Rphq16*) and SusPtrit (highly susceptible), a BC_2S_1 population segregating for *Rphq16* (Figure 9) was screened with the co-dominant flanking markers scsnp03275 (CAPS) and GMS002 (SSR) (Figure 8) The genetic distance between the two flanking markers is of about 16,8 cM (Yeo Kuok San, 2007, Figure 8).

The BC_2S_1 seeds were sown in 96 wells-boxes, each containing 92 potential recombinants, and 2 SusPtrit and 2 Dom as a parental references. The DNA was extracted with NaOH based method developed by Wang *et al.* (1993).

655 plants were screened successfully (i.e. both flanking marker genotypes are known) and 168 recombinants were found, indicating a genetic distance of 12.4 cM between the flanking markers.

3.2.3 Strategic recombinants for fine mapping

In order to locate their recombination site on the genetic map of *Rphq16*, the 168 recombinants were genotyped with all the markers present in the region (Table 3) after having extracted their DNA with the CTAB protocol.

According to the position of their recombination site, 12 strategic recombinants were selected for fine mapping. A strategic recombinant was defined as a recombinant having a recombination site closed to the putative location of *Rphq16*.

Table 3 : markers used to genotype the individuals that have recombined in the *Rphq16* region.

Name(s)	Position*	Type	Pattern	Quality	°Tm	Rest. Enzyme(s)
ABG390	157,7	CAPS	Codom	Clear	56°	*Alu I*
ABG391	153,5	CAPS	Codom	Clear	56°	*Alu I*
DsT-33	163,1	SCAR	Codom	Allele competition	45°	-
GBS0408	175,4	CAPS	Codom	Clear	56°	*Mse I*
GBS0576	163,1	CAPS	Codom	Clear	56°	*BspL I*
MWG2193	158,0	CAPS	SusPtrit	Clear	56°	*Alu I*
MWG2249	175,3	CAPS	DOM	Clear	56°	*Dde I*
scsnp03275	-	CAPS	Codom	Clear	65°	*Bgl II*
scsnp03683	176,2	CAPS	Codom	Clear	56°	*HpyCH4 IV*
GMS002	178,7	SSR	SusPtrit 123, DOM 131		Prof. A	-
GMS001	178,7	SSR	SusPtrit 125, DOM 129		Prof. A	-
scssr03907	178,7	SSR	SusPtrit 129, DOM 123		Prof. A	-
scssr09041	178,7	SSR	SusPtrit 150, DOM 167		Prof. A	-

Green color: CAPS/SCAR markers; Yellow: SSR markers; *: integrated map 2008 (Marcel *et al.* 2007)

BC_2S_1 recombinant lines were selfed and the progeny (BC_2S_2) (Figure 9) of 12 strategic recombinant families were selected for further evaluation. Per family, 30 seeds were sown, raising to 360 the total number of plants to be evaluated. Each plant was genotyped with the segregating flanking marker of *Rphq16* and phenotyped to estimate the latency period of *P. hordei* 1.2.1

The experiment was performed in three distinct inoculations. Per inoculation, four inoculation boxes containing 40 plants each were used. In each box were sown the progeny of 2 lines (15 plants per line) and the 5 reference lines Dom, Rec, SusPtrit, Vada and L94 (2 seeds each). For each line, 15 seeds were sown in two different inoculations (Table 4).

The DNA of each plant was extracted using the NaOH-based method developed by Wang *et al.* (2003), which allows isolating large numbers of DNA samples in a limited amount of time.

Only the flanking marker (ABG390 very closed from scsnp03275 or GMS002) segregating in a specific recombinant family was run on the plants of that family (Table 4).

Table 4 : flanking markers genotype of the 12 strategic recombinants as determined before selfing and their respective inoculation groups

NAME	ABG390	GMS002	IS 1	IS 2	IS 3
117.5	A	H	x		x
120.6	A	H	x	x	
121.6	A	H	x	x	
142.7	A	H	x	x	
154.7	A	H	x		x
29.2	B	H	x		x
49.3	B	H	x		x
156.7	B	H	x	x	
124.6	H	B		x	x
130.6	H	B		x	x
147.7	H	B		x	x
149.7	H	B		x	x

x=15 seeds sowed, genotyped and phenotyped. IS = inoculation set; A= Dom; B=SusPtrit; H=AB, heterozygous.

The strategic recombinant lines were phenotyped at seedling stage. Each inoculation box was inoculated with 2.5 mg spores of *P.hordei* 1.2.1 and the latency period was measured. The relative latency period was calculated, relative to the parental line presenting the lowest standard deviation among the four boxes per inoculation set.

In each strategic recombinant family, the average RLP50S for the three genotypes (homozygous with only Dom alleles, homozygous with only the SusPtrit allele, or heterozygous) was calculated. If inside one family the RLP50S was significantly different between these three possibilities, we considered that this family was segregating for the presence/absence of *Rphq16*.

The new homozygous recombinants in *Rphq16* region were selected for strategic recombinant families and transplanted for seed production. The seeds of the 156 remaining recombinants were kept to be sown later to detect new homozygous recombinants (results not available for this study). The purpose of all these new homozygous lines in *Rphq16* region was to create a small mapping population for only the *Rphq16* region.

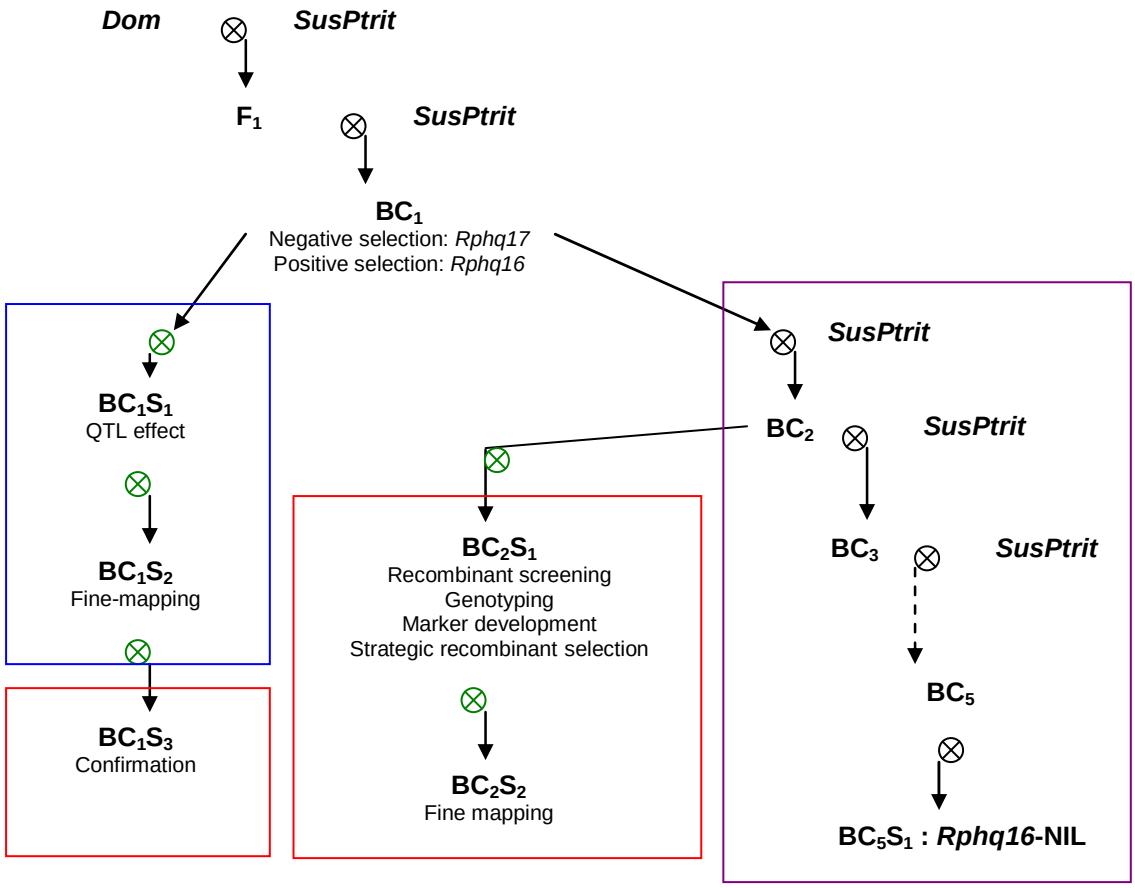


Figure 9 : flowchart of the backcross to obtain the plant material for the fine-mapping of *Rphq16*. *Caption*: blue line= backcross procedure used by Yeo Kuok San (2007); red lines= plant material used by the author of this thesis; purple line= backcross program to obtain *Rphq16*-NILs, at each step there is a marker assisted selection; black cross= crossing between two genotypes; green cross= selfing; Dashed arrow= backcross with SusPtrit until BC_5.

3.2.4 Marker development

3.2.4.1 Conversion of transcript derived markers into simple PCR markers

The transcript derived markers (TDMs) mapped by Potokina *et al.* (2008) in the Steptoe x Morex population were integrated with the OWBs mapping data on the integrated map of barley developed by Aghnoum *et al.* (submitted). Sequences of the TDMs that mapped in the region of *Rphq16* on the barley integrated map were retrieved from the SCRI barley SNP database website (http://germinate.scri.ac.uk/barley_snpdb/dbStats_contig.html) and used to develop new molecular markers.

The software PrimerSelect was used to design the new primer pairs. The optimal fragment length was between 600bp and 800bp because it was not too small (so with a high chance to find polymorphism) and not too big (so too difficult to amplify). A gradient PCR was performed on the new primer pairs: ABC11015, ABC03582, ABC03584, ABC08240, ABC08917, ABC09095 (designed primers names correspond to the sequence ID in the SCRI barley SNP database). The range of temperatures tested in the gradient PCR were: 45°C, 50°C; 55°C, 58°C, 61°C, 65°C.

If a distinct polymorphism was noticed between Dom and SusPtrit for one primer pair, it meant that a new SCAR marker was identified. Otherwise, for primers that amplified a single clear band for Dom and SusPtrit, sample PCR products of both parents were sent to sequencing at Greenomics. Sequences were analysed for the presence of SNPs or indels allowing the development of a CAPS marker.

The PCR products of some primer pairs, for which the pattern was not clear enough to be sequenced, was directly test digested with the following restriction enzymes: Bme1390I, HpyF10VI, HpyCH4IV, AluI, Hin1II, SsiI, MnII, DdeI, HinfI, HaeIII, BspLI, Eco88I.

Then, the new markers were confirmed on the parental lines Dom and SusPtrit before to be used for genotyping the identified recombinant lines.

3.2.4.2 Synteny based markers

The EST sequences corresponding to TDMs mentioned in the previous section were also used to search for synteny between barley and *Brachypodium*, and between barley and rice. These

EST sequences were BLASTed against the genome of *Brachypodium* (http://www.brachypodium.org/browse). If the best hit of most barley ESTs fell in the same region of the *Brachypodium* genome, the other *Brachypodium* genes present in that region were retrieved and in turn BLASTed against the barley EST database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=barley). Best barley hits to those *Brachypodium* genes where good candidate to develop new barley molecular markers. The procedure was the same to find synteny between rice and barley.

The genes conserved through barley, rice and *Brachypodium* were retrieved and the TC from barley closed to our region of interest were used to develop new markers following the procedure described in section 3.2.4.1.

3.2.4.3 Markers for Light Scanner machine

The light scanner was another possibility to develop new markers. It reveals the SNPs between sequences by showing differences of melting curves. The melting curves are obtained by browsing a range of temperature between 75°C and 95°C. The machine measured the fluorescence of the samples thanks to a fluorescent dye, the LCG green. The main advantages of the light scanner are that the PCR cycle is shorter (less than one hour) and the analysis of the samples with the machine lasts few minutes.

The new contigs mentioned previously were used to design primer pairs amplifying small fragments (from 250bp to 400bp) with few SNPs, more suitable for this machine, because the light scanner would be unable to see difference between samples if there are too many SNPs in the amplified fragment. They were tested directly on the light scanner. If it did not work(unclear polymorphism between melting curves or bad amplification products) , a gradient PCR was performed to check the source of the problem (bigger fragments than expected because of an intron present in the genomic sequence, or multiple fragments because of the low specificity of the primers). Several primer pairs were developed based on the sequences of the contig numbers 3282 and 3284 because the corresponding TDMs were mapped very close from the peak marker of *Rphq16*.

Another option was to convert CAPS/SCAR markers by developing an amplicon i.e. a small fragment of around 100bp containing only one SNP, requiring the exact sequence and the position of the SNPs in this sequence. In a CAPS/SCAR amplified fragments there could be many SNPs. But by knowing where the different SNPs were, it was possible to develop primer pairs flanking one of these SNPs and amplifying only a fragment of 100bp. Two

amplicons were designed based on the sequence of the two flanking markers of Rphq16: scsnp03275 and scsnp03683 in order to obtain faster tool for screening new recombinants.

3.3 Results

3.3.1 High resolution genetic map of *Rphq16*

The 168 identified recombinants were used to create a high-resolution genetic map with 13 molecular markers that have been run successfully. New elements from the markers development were added to this map to complement it.

As it has been said in part 3.2.4.1, six TDM markers mapped by Potokina *et al.* (2008) were tried to be converted into PCR based markers. Three had a single and clear band after gradient PCR amplification while the others three had multiple bands (Table 5). The sequencing of ABC03582 and ABC03584 confirmed that these two markers were derived from the same gene.

ABC09095 had a clear polymorphism that was revealed after digestion with DdeI..

A digestion with 12 restriction enzymes selected from the CAPS Kit (Bai *et al.*, 2004) was tried on the other three (showing more than one band after the gradient), but no polymorphism was found (Table 5).

Table 5 : summary of the results obtained for the conversion of the new transcript derived markers

Number of bands	Marker name	Optimal Tm	Polymorphism
One band	ABC03582	61°C	No
One band	ABC03584	65°C	No
One band	ABC09095*	58°C	Yes – MseI ; DdeI Co-dominant
More bands	ABC11015	58°C	?
More bands	ABC08230	61°C	No**)
More bands	ABC08917	61°C	Unclear - HpyCH4IV ; Eco88I Dominant?

*PCR cycle: 1 min at Tm=58°C (annealing), 2 min at T=72°C for replication. ?= no amplification. . **= after digestion with 12 restriction enzymes selected from the CAPS Kit (Bai *et al.* 2004).

The high throughput strategy with the light scanner machine was not successful. Indeed only one among the eight primer pairs gave good results: ABC03584_4. A gradient PCR was performed on all of the eight primers pairs, and all of them, except ABC03584_4, were not specific enough or most of the time amplified fragment of a big size (bigger than 400bp) containing too many SNPs. Unfortunately the sequencing of this new markers was not successful so it was impossible to know where the SNPs were.

The two amplicons showed good results at a Tm of 65°C better results were observed, since the scoring of the CTAB DNA from twelve of the heterozygous recombinants corresponds to the scoring made previously. It was also possible to differentiate SusPtrit and Dom. Unluckily, when tried on the complete population of recombinants, the results were messy and did not correspond to scoring observed.
95% of homology between the result on the light scanner and the results obtained with the classical way were observed with the amplicon derived from scsnp03683, tested on the DNA from the last inoculation set (containing the family derived from the recombinant DOM 117.5, DOM 154.7, DOM 29.2, DOM 49.3), extracted with the NaOH based method (Wang *et al.*, 1993).

The high-resolution genetic map was calculated with the maximum likelihood algorithm in JoinMap 4®. When using the regression mapping algorithm also available in JoinMap 4®, the two groups of markers on both sides of the main gap could not be merged with each other, probably because of insufficient linkage.
The markers listed as weak (ABC622) or showing an unclear polymorphism (scsnp00635, GBR0097) were tried but they did not work properly so they were excluded from the map.

The genetic map of *Rphq16* obtained was of 13.3 cM of length (23 cM for Yeo Kuok San, 2007). On the whole, the marker order was the same as the one found previously, except that MWG2193 and scsnp03275 were at the same locus. As for Yeo Kuok San (2007) two clusters of markers were found: one from ABG391 (position 0.0 cM) to GBS0576 (position 1.9 cM) and the other one from GBS0408 (position 12.4 cM) and GMS002 (position 13.3 cM). They were separated by genetic gap of 10.5 cM (8.6 cM for Yeo Kuok San 2007) with again MWG2249 in the middle (Figure 10). The new markers, ABC09095 was co-dominant and it turned out to be located between MWG2243 and GBS0408 (Figure 10). With this new marker the linkage between the two marker clusters is now sufficient to use the regression algorithm.

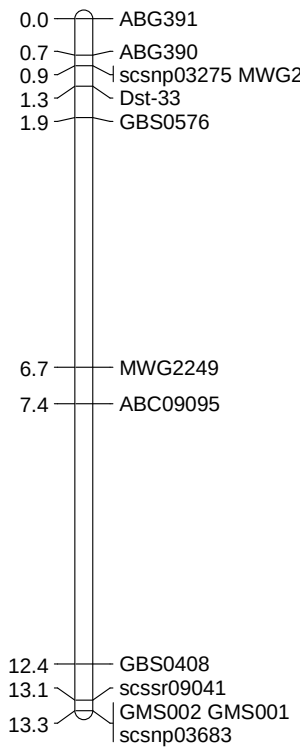


Figure 10 : fine genetic map of the *Rphq16* region after run markers present in the region on the DNA of the heterozygous recombinants population and with the position of the new markers ABC09095. The distances between markers are in cM.

3.3.1.1 Conserved synteny between barley, rice and Brachypodium in the *Rphq16* region

The new TDMs found in the *Rphq16* region (representing a region of 9.7 cM) highlighted new element of synteny between rice and barley, and between barley and *Brachypodium*. All these TDMs had high BLAST score with *Brachypodium* genes from a region of 296 Kb of the super_0 contig, containing 53 genes (52 found an equivalent in barley) (Figure 11). The TDMs all corresponded to rice sequence from a region of 277 Kb the chromosome 3 (Figure 11), containing 46 genes (all had an equivalent in barley).

Nevertheless, the order of the genes was not conserved between crops, indicating a disrupted microsynteny (Figure 11).

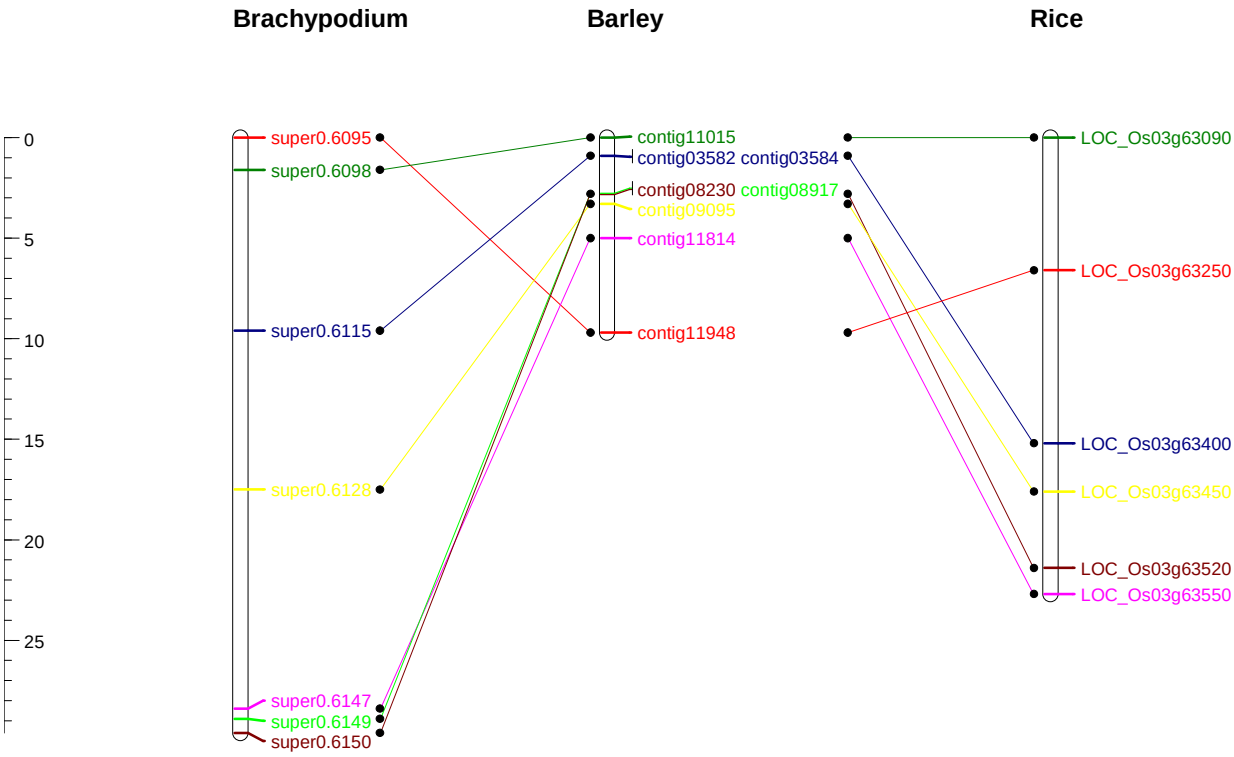


Figure 11 : synteny observed between new barley TDMs in the *Rphq16* region (Potokina *et al.*, 2008), rice and *Brachypodium*. All *Brachypodium* genes are on the super_0 contig and all rice genes are on the chromosome 3. Scale: for *Brachypodium* and rice physical map, 1 unit=10Kb; for barley genetic map, 1 unit=1cM.

Ten genes implied in the resistance were found in the Brachypodium region and 8 in the rice region. The most interesting among them were the resistance genes against powdery mildew and the one for LRR family (Leucine Rich Repeat) involved in the signalling of the pathogen attack.

Table 6 : candidate genes for their involvment in resistance found between Brachypodium and barley, and betweeen rice and barley

Plant	Gene	Barley TC	Annotation
Brachypodium	super0.6116	TC172116	Cysteine protease CP1
Brachypodium	super0.6120	TC186354	Monothiol glutaredoxin-S7 chloroplast precursor
Brachypodium	super0.6122	TC190414	NB-ARC domain containing protein
Brachypodium	super0.6123	TC177869	NB-ARC domain containing protein;
Brachypodium	super0.6134	TC178271	Putative bacterial blight resistance protein Or Leucine Rich Repeat family protein or Receptor kinase TRKe
Brachypodium	super0.6137	AV835525	Putative disease resistance protein
Brachypodium	super0.6138	TC179969	NB-ARC domain containing protein expressed Or Putative disease resistance protein
Brachypodium	super0.6139	AV835525	Putative disease resistance protein
Brachypodium	super0.6140	AV835525	Putative disease resistance protein
Brachypodium	super0.6141	TC154712	Putative receptor-like protein kinase;
Rice	LOC_Os03g63140	TC174458	nitrate-induced NOI protein, expressed
Rice	LOC_Os03g63150	TC193530	powdery mildew resistance protein PM3b, putative, expressed
Rice	LOC_Os03g63200	TC193530	powdery mildew resistance protein PM3b, putative, expressed
Rice	LOC_Os03g63220	TC182036	powdery mildew resistance protein PM3A, putative
Rice	LOC_Os03g63240	TC153988	disease resistance protein, putative, expressed
Rice	LOC_Os03g63420	TC162403	OsGrx_S14 - glutaredoxin subgroup II, expressed
Rice	LOC_Os03g63470	TC181664	nucleotide binding protein, putative, expressed

Seventeen genes were common to barley, rice and *Brachypodium*. Eight of them were kept to develop new markers because of their high levels of sequence similarity between homologues (Table 7). Only one among them was sure to be involved in resistance: TC181664 (Table 7).
The new marker, ABC09095, was based on the sequence of TC186954, producing a SnRK1-interacting protein. Among the possible SnRK1 (sucrose-nonfermenting-1-related-protein kinase-1) interacting protein, there is the transcription factor of the ethylene responsive element binding protein also called ERF (Ethylene Response Factor) (Halford *et al.*, 2003), which could be involved on the resistance, since they play an important role in the regulation of gene expression under biotic stress, such as the *TaERF1* gene, identified in *Triticum aestivum* by Xu *et al.* (2007).

Table 7 : result of the synteny study, showing the genes for which homologues were detected in *Brachypodium*, barley and rice

Brachy gene	Barley EST	Rice gene	Annotation (rice)
super0.9091	TC180982	LOC_Os03g63640	Putative ribosomal protein
super0.9092	BQ664615	LOC_Os03g63290	alcohol dehydrogenases, putative, expressed
super0.9093	**TC183920**	LOC_Os03g63280	Putative regulatory protein
super0.6094	**TC190539**	LOC_Os03g63270	Regulatory protein putative expressed
super0.6097	TC167963	LOC_Os03g63110	Putative uncharacterized protein
super0.6107	TC173662	LOC_Os03g63360	integral membrane protein, putative, expressed
super0.6112	**TC157328**	LOC_Os03g63370	polyphosphoinositide binding protein Ssh2p, putative, expressed
super0.6113	TC182066	LOC_Os03g63380	expressed protein
super0.6114	**TC161519**	LOC_Os03g63390	chemocyanin precursor, putative, expressed
super0.6115	TC161686	LOC_Os03g63400	transcription factor BTF3, putative, expressed
super0.6119	**TC173786**	LOC_Os03g63410	elongation factor Tu, mitochondrial precursor, putative, expressed
super0.6121	**TC155912**	LOC_Os03g63430	26S proteasome non-ATPase regulatory subunit 12, putative, expressed
super0.6128	TC186954	LOC_Os03g63450	snRK1-interacting protein 1, putative, expressed **
super0.6129	**TC181664**	LOC_Os03g63470	nucleotide binding protein, putative, expressed *
super0.6130	TC181471	LOC_Os03g63480	ankyrin repeat domain-containing protein 2, putative, expressed
super0.6131	TC173833	LOC_Os03g63490	pantoate--beta-alanine ligase, putative, expressed
super0.6136	**TC155769**	LOC_Os03g63500	ATPase, putative, expressed

In bold = barley gene used to develop new markers; * involved in the resistance process; **: maybe involved in the resistance process

The gradient PCR indicates that most of the primer pairs designed to develop new markers were not specific enough, because they all amplified multiple fragments. However two primer pairs had clear and single PCR products; TC161519 (50°C < Tm < 58°C) and TC155912 (co-dominant SCAR at Tm=61°C).

Because of the lack of time, sequencing and mapping of those two markers could not be realised in the frame of this study.

3.3.2 Phenotyping of available homozygous recombinants in the *Rphq16* region

Twenty-eight recombinants in the region of *Rphq16* have been identified previously and progeny lines of those recombinants were selected for homozygozity of the recombined fragment. We evaluated the latency period of those homozygous recombinant lines in three replications. SusPtrit was the parent with the smallest standard deviation over the three replicates, so it was used to calculate the RLP50S. The correlation factor for RLP50S between two replicates was ranged from 0.8 and 0.9. Similarly, low (ranged from 0.2 and 3.9) standard deviations were observed between replicates for each genotype (Table 8). The average of RLP50S of the three replicates was used to order the lines according to their level of partial resistance (Table 8).

A significant difference between the RLP50S (average of the three replicates) of the susceptible and the resistant genotypes was found ($p<0.001$, ANOVA table in Appendix 6). This allowed us to measure a QTL effect of 8 hours (10 hours for Yeo Kuok San, 2007).

Yeo Kuok San (2007) located *Rphq16* between GBS0408 and GMS002. According to Table 8, all susceptible homozygous recombinants had the SusPtrit allele for GBS0408 and the Dom allele when the level of partial resistance was higher.

The homozygous recombinants D38.02 was an exception to this, since it had an intermediate value of RLP50S (closed to the RLP50S of the resistant genotypes) and the SusPtrit allele for GBSS0408. It also had a high standard deviation between replicates (3.6, Table 8)

This was not the same case for GMS002. Indeed the two susceptible homozygous recombinants D44.06 and D15.03 had the Dom allele for GMS002 and the resistant genotype D16.01 had the SusPtrit allele here (Table 8)

The two homozygous recombinants D30.01 and D01.01 seem to have been wrongly genotyped by Yeo Kuok San (2007) for MWG2249 regarding the genotype of its flanking markers GBS0576 and GBS0408 (Table 8).

Table 8 : phenotyping results (RLP50S) from the seedling disease test performed on the homozygous recombinants from Yeo Kuok San (2007). The genotypes and the order of the markers are based on Yeo Kuok San (2007) results.

Homozygous recombiants	RLP50S (Sus)	*Sd*	ABG391	scsnp00635	cMWG650	scsnp07825_3	ABG390	MWG2193	ABC622	scsnp03275	Dst-33	GBS0576	MWG2249	GBS0408	GMS002	GMS001	scssr03907	scsnp03683
D38.08	93,93	2,3				H	H	H	H	H								
D44.06	96,65	2,8																
D41.02	97,92	1,3		U														
D31.02	98,16	0,6	U															
D08.01	98,68	1,9										H	H					
D40.01	98,73	0,2																
D23.1	99,55	1,6	U	U														
BC2.5F1.6	99,82	0,4	U	U	U				U			U						
BC2.7F1.3_02	99,82	0,9													H	H	H	H
D30.01	99,83	3,1										H		U				
D01.01	100,02	1,3		U	U									U			U	
D35.02	100,38	2,4	U									H						
D15.03	100,84	2,5																
D04.06	100,85	1,1			U													
D17.02	100,99	0,7																
D42.01	101,03	2,8		U	U			U	U	U		U		U		U		U
D13.02	101,70	1,7											H	U	H	H	H	H
D38.02	101,80	3,6				C	H	H	H	H								
BC2.1F1.4_02	102,03	1,7											C	U	H	H	H	H
D36.04	102,41	2,1	H	U	H	H	H	H	H	H	H	H	C					
D05.01	103,05	3,2																
D16.01	103,21	3,9																
D45.02	103,32	3,5	H	U	H	H	H	H	H			U						
D33.01	104,29	2,6	U	U	H	H	H	H	H									
D13.03	105,45	2,0											C	U	H	H	H	H
D11.03	105,50	0,9				H	H	H	H	H	H	H	H	H	H	H	H	H
D39.01	105,55	2,5																
D32.08	106,15	3,9	U	U	H	H	H	U	U	U	U	U						U
D18.02	106,67	1,5																

Black bars= SusPtrit introgression ; Grey bars= Dom introgression; Shaded bars = heterozygote; U = unknown; Red lines = putative location of the QTL *Rphq16*; Red colour = susceptible genotypes;; Yellow colour = resistant genotypes; Sd.:Standard deviation

3.3.3 Phenotyping and genotyping of the strategic recombinant families

The offspring of the 12 strategic recombinant families (360 plants in total, 30 per families) were genotyped with flanking markers of *Rphq16* and their genotypes were combined with the measure of their latency period to pointed out the one segregating (or not) for the QTL for partial resistance. L94 was used to calculate the RLP50S

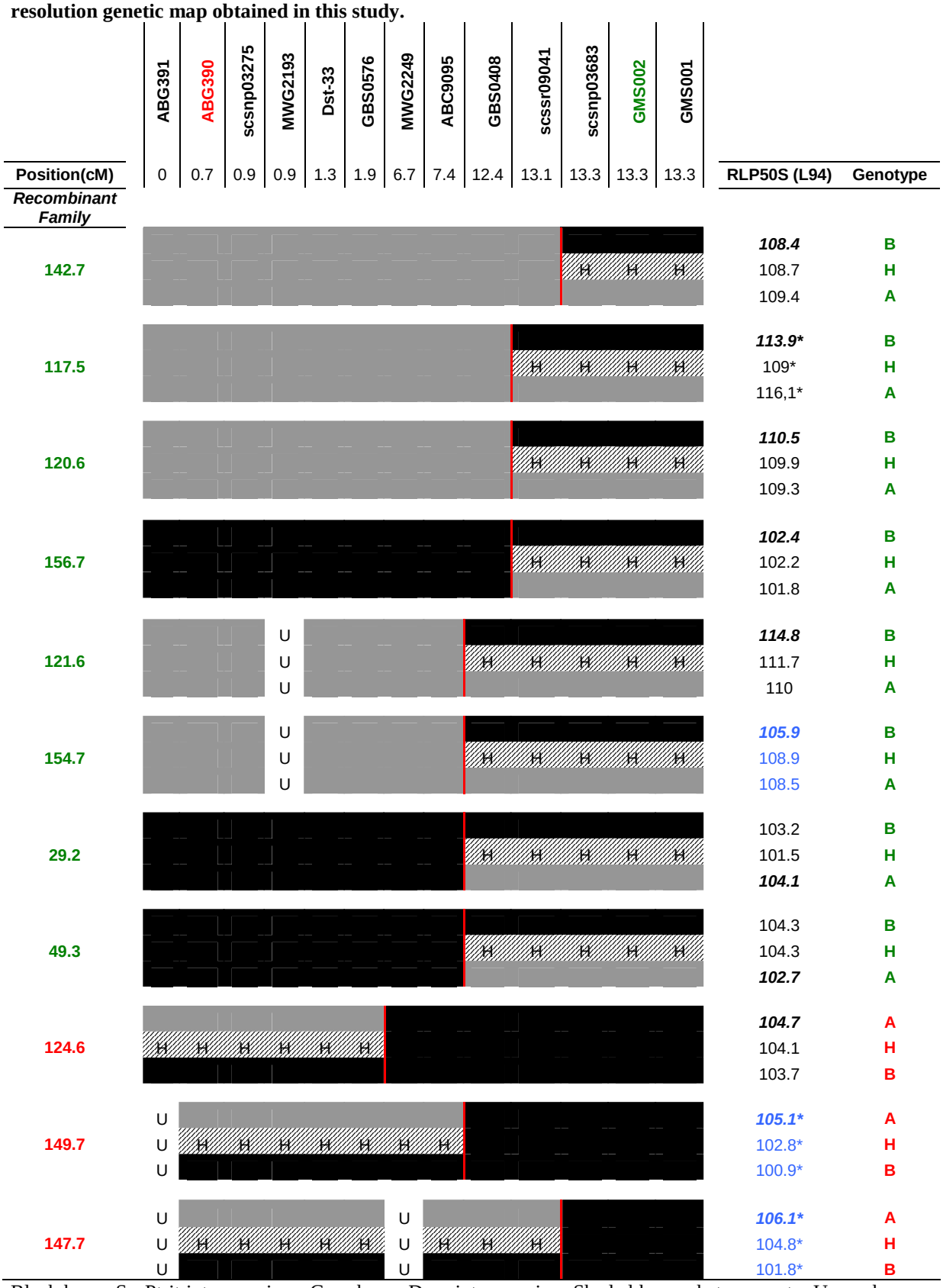
The family 124.6 was a control family with no resistant allele of the QTL for sure, and having the recombination point far from the putative location of the QTL. All individuals from this family did not segregate for the QTL, with an RLP50S of 104 on average (Table 9). Same observation for 130.6, having also an homozygous susceptible allele of *Rphq16*, giving an average RLP50S of 103 (not displayed in Table 9 since the recombination between flanking markers was not confirmed).

The two families 142.7 and 147.7 agreed on each other since 147.7 was segregating for the QTL and not 142.7 which had the opposite genetic profile; i.e.: 2 families have the opposite genetic profile when they have the same recombination site but one is homozygous (in *Rphq16* region) before the recombination site and the other after.

All the families having a recombination site between GBS0408 and scssr09041 (being homozygous before the recombination site) were not segregating for *Rphq16*. The family 117.5 was an exception to this since it was segregating but with an abnormal segregation pattern: the homozygous plants with the SusPtrit allele (for GMS002) had a higher RLP50S value than the heterozygote ones and were not significantly different from the homozygous with the Dom allele (Table 9).

Same observations were reported for the four families having the recombination site GBS0408 and ABC09095 (homozygous before the recombination site). On the contrary the 149.7, having the opposite genetic profile, agreed on this since it was segregating for *Rphq16*. The family 121.6 has a strange RLP50S for the homozygous plants with the SusPtrit allele (114.8), since it is higher than the homozygous plants with the Dom allele (110) but was not segregating for *Rphq16* (Table 9).

Table 9 : results of the genotyping and phenotyping (RLP50S) of the strategic recombinant families developed in this study. The order of the markers and the genetic distances are based on the new high resolution genetic map obtained in this study.



Black bars= SusPtrit introgression ; Grey bars= Dom introgression; Shaded bars = heterozygote; U = unknown; Red colour= plants genotyped with ABG390; Green colour= plants genotyped with GMS002; *=significant differences between genotypes; Blue colour = family segregating for the QTL; Red line= recombination site; A= Dom allele; B= SusPtrit allele; H = heterozygote allele; In bold and italic : homozygous lines ;

According to Figure 12, *Rphq16* seemed to be partially dominant since the homozygous plants, with the Dom allele and the heterozygous plants had a longer RLP50S than the homozygous plant, with the SusPtrit allele, but the heterozygous plants had a slightly lower RLP50S value than the homozygous plants, with the Dom allele.

Even if the differences between genotypes were not significant, the family 154.7 seemed to show the same segregation pattern as the segregating families (Figure 12).

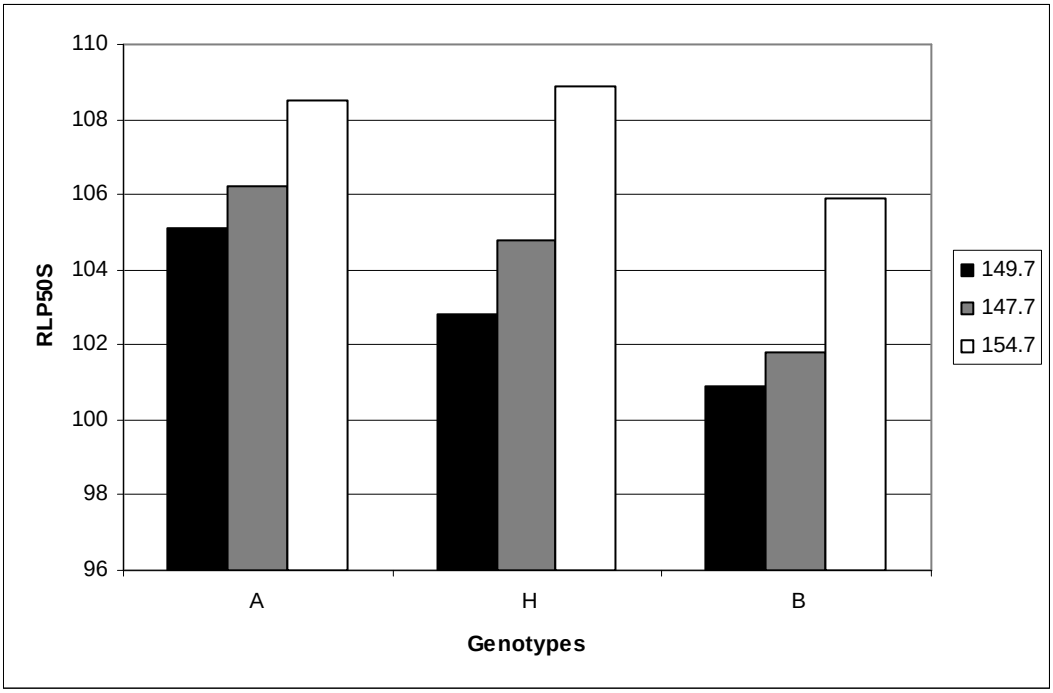


Figure 12 : RLP50S for the two families 149.7 and 147.7 segregating for *Rphq16*. and 154.7, nearly segregating for the QTL. A= homozygous with the Dom allele; B=homozygous with SusPtrit allele; H=heterozygous

3.4 Discussion

3.4.1 Marker development

The Work on marker development succeeded in the development of only one new marker. This marker, ABC09095, is co-dominant and located right in the middle of the genetic gap where only one dominant marker, MWG2249, was present.

The TDM from which is derived this new marker (contig9095, Potokina *et al.*, 2008) was mapped by eQTL on the edge of the region of interest at 171.5 cM and the marker developed turns out to map right in the middle of the region. So a solution to fill the genetic gap or to add markers in region closed to the QTL would be to develop markers derived from the contigs mapped downstream from contig9095 such as the one mentioned in Table 10.

Table 10 : some of the TDMs found by Potokina *et al.* (2008) mapping downstream from contig9095

Contig name	Position (cM)
contig11814	173,5
contig14958	173,5
contig06258	173,5
contig06261	173,5
contig15422	174,0
contig24342	174,0
contig08225	174,0

The contig11814 is quite interesting since it is conserved between barley, rice and *Brachypodium*.

The synteny work, even not completed, establishes a foundation basis to continue working on it by indicating which sequences are conserved through barley, rice and *Brachypodium*. Yeo Kuok San put forward a disruption in the synteny between rice and barley, but thanks to the new contigs available, the region seems to show similarities with the rice chromosome 3 and the *Brachypodium* contig super_0 (Figure 11). However Rostoks *et al.* (2005) brought forward that chromosome 4H of barley is also showing similarities with chromosome 3 from rice. So it could be possible that the markers derived from synteny for the *Rphq16* region on does not map chromosome 5H as expected but on chromosome 4H.

The light scanner markers were not as successful as expected. In the end, the light scanner should not be used to develop new markers (i.e. it does not avoid to make a gradient on the new marker and to sequence it to locate the SNPs) but rather to do large scale screening with for instance the amplicon, which had shown some relevant results. .

3.4.2 Fine mapping of *Rphq16*

The first good point is the confirmation of the fine genetic map of *Rphq16* established by Yeo Kuok San (2007) with a similar marker order and very few differences (see part 3.3.1, Figure 10).

According to the phenotyping of the homozygous recombinants (in *Rphq16* region) from Yeo Kuok San (2007), *Rphq16* seemed to segregate with the marker GBS0408. The homozygous recombinant D38.08 was again not ageing with the substitution map (Yeo Kuok San, 2007). This could mean that this line had a more resistant background, i.e. even if the resistant allele *Rphq16* is not present, the presence of a multitude of other small QTLs for partial resistance interacting with each other and conferring an intermediate level of partial resistance.

The family 124.6 indicated that *Rphq16* was not upstream from MWG2249 (no segregation). The two families 142.7 (not segregating for *Rphq16*) and 147.7 (segregating for *Rphq16*) agreed on the fact that the QTL is not present between scssr09041 and the cluster of markers formed by GMS002, GMS001 and scsnp03683. So by looking at their profiles, *Rphq16* should be upstream from GMS002 (Table 10).

As the four families, 29.2, 49.3, 121.6, 154.7, were not segregating for the QTL but 149.7 (opposite genetic profile) did so, it would seem that the QTL is upstream from GBS0408 according to the recombination site and the profile of these five families. This was confirmed by the absence segregation among the three families (117.5, 120.6, 156.7) having a recombination site between GBS0408 and scssr09041 (homozygous before the recombination site). The family 117.5 could not be seen as segregating for the QTL because of its irrelevant segregation pattern.

To summarize, Rphq16 should be between ABC09095 and MWG2249: in a genetic windows of 0.7 cM out of a region of 13.3 cM (Figure 13). The hypothesis is supported by Table 11 showing that all new homozygous line in *Rphq16* region (coming from the 12 strategic recombinant families) having the SusPtrit allele for MWG2249 and ABC09095 had a short RLP50S and the contrary was observed for the ones having the Dom allele for MWG2249 and ABC09095 (Table 11)

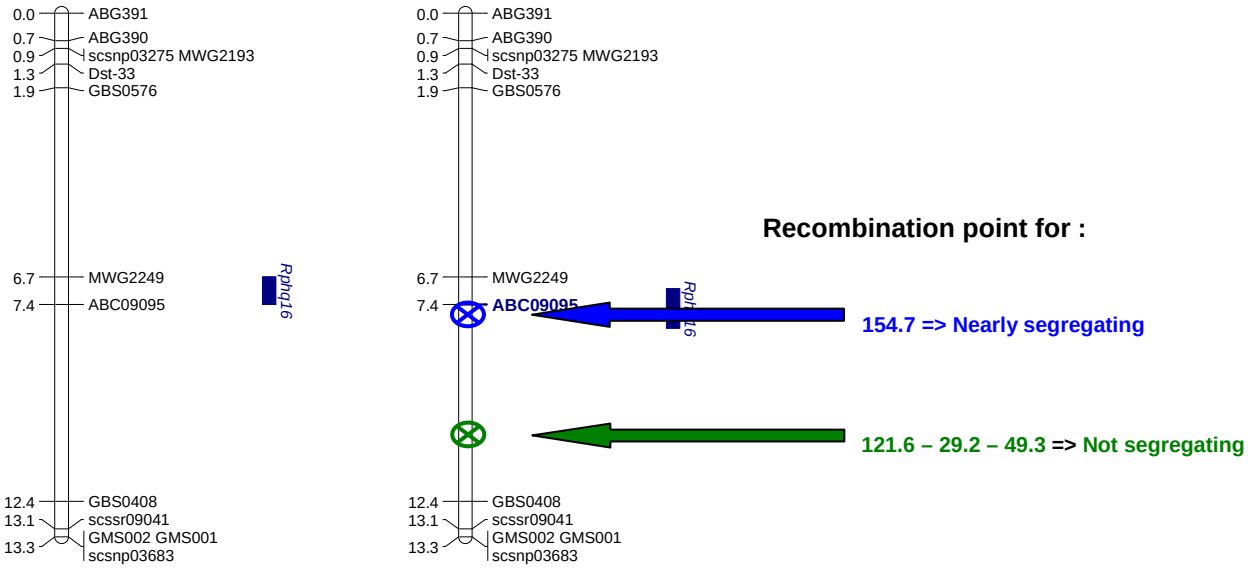
Table 11: RLP50S for the new homozygous recombinants (coming from the strategic recombinant families). The RLP50S was an average of RLP50S from all the homozygous recombinants in each family.

	ABG391	ABG390	scsnp03275	MWG2193	Dst-33	GBS0576	MWG2249	ABC9095	GBS0408	scssr09041	scsnp03683	GMS002	GMS001	
Posistion (cM)	0	0.7	0.9	0.9	1.3	1.9	6.7	7.4	12.4	13.1	13.3	13.3	13.3	**RLP50S (L94)**
Family														
156,7														101,8
49,3														102,7
29,2														104,1
124,6*														104,7
149,7	U													105,1
154,7				U										105,9
147,7	U						U							106,2
142,7														108,4
117,5														113,9
121,6				U										114,8
120,6														110.5

Black bars= SusPtrit introgression ; Grey bars= Dom introgression; Shaded bars = heterozygote; U = unknown; Red colour= plants genotyped with ABG390; Green colour= plants genotyped with GMS002; Blue lines = putative location of the QTL *Rphq16*; *=control line with the susceptible allele of *Rphq16*.

But one question remains: although they look similar in their genetic profile, why 154.7 seem to segregate for the QTL and not 29.2, 49.3 and 121.6? An explanation could be that *Rphq16* location might be extended a little bit downstream from ABC09095 (maybe co-segregating with it). . The families 154.7 would have a recombination on the QTL (closed to ABC09095) and the families 29.2, 49.3 and 121.6 have one closed to GBS0408 (not on the QTL) as showed on Figure 13.

Figure 13 : putative location of *Rphq16* and recombination point for some of the strategic recombinant families (green and blue) on the genetic fine map generated during this study. The distances between markers are in cM. Left: new potion of Rphq16; Right: new hypothesis of location to be confirmed



4. General discussion and future prospect

4.1 A complex QTL mapping for partial resistance against P. hordei in OWB at adult stage

The combination of data from Yeo Kuok San (2007) and the one from this study showed us two regions which could be QTL potential QTLs for partial resistance against *P. hordei*, because a QTL has been detected in those regions with more than one dataset.

One QTL for partial resistance was detected on chromosome 2H (Table 1, Figure 4), at a location where *Rphq18*, a QTL for partial resistance, was detected in OWB by Marcel *et al.* (2007) at seedling stage, and also where *Rphq6*, was identified in L94xVada population (Qi *et al.*, 1999). So *Rphq18* might be present also at adult stage, so stage independent. A second QTL for partial resistance was mapped on chromosome 5H, detected only in the third replicate, but with 3 different dataset (Table 1, Figure 4) coming from the pustules counting of the flag leaves and the F-1 leaves. This brings forward two things: the F-1 showed some relevance to assess the level of partial resistance and the level of partial resistance could homogeneous through the plant. But *Rphq16*, one of the main interesting QTL of this population (Marcel *et al.*, 2007) is definitely absent at adult stage.

The question of a pleiotropic effect of QTLs for plant development (such as heading date or plant height) on the level of partial resistance to explain the first QTL on chromosome 2H was put forward since several QTLs for plant height and heading date have been mapped at this location such as in in L94xVada population (Qi *et al.*, 1999) and Steptoe x Morex (Yeo Kuok San, 2007 with data from GrainGenes). In other population, the *Pdp-H1* gene, a major gene for earliness, has been mapped there (Emebiri *et al.*, 2005; Cuesta Marcos *et al.*, 2008a&b; Sameri *et al.*, 2006). But this hypothesis turned out to be wrong since no QTL for earliness (Table 2, Figure 4, Figure 6) were detected at this locus in this study.
Even if all the plants have been inoculated at the same development stage to avoid an effect of this stage on the RLP50A (Palveliet, 1979), a pleiotropic of the inoculation group on the level of partial resistance was observed and two putative QTLs for partial resistance turned to be due to a side-effect of the inoculation groups.
To confirm the QTLs for heading date found in this study, it could be possible to set up an experiment such as what has been done by Yeo Kuok San (2007) with Steptoe x Morex

population: build a randomized block design (field or greenhouse trial), infect the plants at the same time, measure the AUDPC (Area Under Disease Progress Curve) to assess the level of partial resistance and also measure as accurately as possible all kind of physiological trait related to the plant stage (plant height, day to heading or other agronomic trait mentioned by Sameri *et al.,* 2006). A greenhouse should be more appropriated to be sure that nothing else that the genetic of the plant is influencing the growth of the plants.

The main feeling rose by looking at the different QTLs for partial resistance mapped for OWB at adult stage in this study, was that a multitude of minor QTLs were playing a role in the level of partial resistance. A large part of them are maybe too difficult to detect because of their little effect and their effectiveness only under certain circumstances. This feeling was confirmed by the LOD profile (obtained with MapQTL5) showing no clear peaks for the interval mapping or even for the restricted multiple QTL mapping of some linkage group, but a multitude of small peaks. Even if the peaks are under but close from the threshold, they could be very minor QTL.

That's why a field trial for the OWBs would not be relevant for partial resistance assessment since the partial resistance seems to be handled by minor QTLs, so their effect could be even more masked, because in a field trial not only the genetic is influencing the level of resistance (not like in the greenhouse).

4.2 New insights in the fine mapping of Rphq16

The fine map realized in this study brought forward a new putative location for *Rphq16*. It was mapped in new position in the high resolution genetic map (different from where Yeo Kuok San (2007) mapped it): in a genetic window of 0.7 cM (out of a genetic map of 13.3 cM) between MWG2249 and ABC09095 (Figure 14). Regarding the small size of this genetic window (0.7cM) and the low ratio (0.2 to 0.9 Mb/cM) between the physical map and the genetic map (Yeo Kuok San, 2007), the next step of the fine-mapping would be to move to "map-based cloning" approach to clone the gene. However the *Rphq16*-NILs are not ready yet and *Rphq16* is in a region with no marker around (Figure 14). So some complementary work could be done to first confirm the position of the QTL on the genetic map and to check the new hypothesis of the co-segregation of *Rphq16* with ABC09095 and secondly to add new markers to ease the "map-based cloning" approach.

A first experiment would consist in re-genotyping some of the recombinants in *Rphq16* region with the markers present in the region.

Because of some missing data in the genotyping of the 168 new recombinants obtained in this study with 14 markers present in the region, they could be re-genotyped with these 14 markers in order to confirm the high resolution map and especially the distance between ABC09095 and MWG2249. The DNA of those recombinants is still available and ready for use.

The three markers MWG2249, ABC09095 and GBS0408 could be run again on some of the homozygous recombinants from Yeo Kuok San (2007). Regarding the profile of those recombinants, D40.01 should be genotyped as well as D30.01 and D01.01. The genotypes of the other recombinants are known and sure for the three markers mentioned. Indeed if the SusPtrit is for MWG2249 and for GBS0408, ABC09095 should also have this allele and vice-versa with the Dom allele. D30.01 and D01.01 have strange genotypes in the region of the three markers. The genotype for ABC09095 could not be guessed for D40.01 since MWG2249 has the Dom allele and GBS0408 the SusPtrit allele. Regarding Table 8, this will allow us to see if the QTL can segregate with ABC09095 if the SusPtrit allele is present in D40.01. D42.01 could also be included because of the unknown and unclear data for the three markers.

The second point would be to perform an equivalent experiment of what has been done with the recombinant families but this time on the progenies of recombinants having a recombination site between MWG2249 and ABC09095 to confirm of *Rphq16* could be (or not) close from ABC09095, maybe co-segregating with it. Two types of families should be selected: one being homozygous before the recombination point and one after. Then the homozygous recombinants from this extra recombinant families could be phenotyped with the new homozygous recombinants (from the 12 strategic recombinant families) in an experiment similar to the one realized in this with Yeo Kuok San's homozygous recombinants in order to be able to compare them to confirm QTL position on the new substitution map.

A big emphasis could be accorded to the marker saturation of the area between GBS0408 and MWG2249 to ease the map-based cloning. So it justifies the importance of the TDMs mapped downstream from contig9095 by Potokina *et al.* (2008) as listed in Table 10. The new insight about synteny with rice and *Brachypodium* should be completed because it is also another source of markers and only eight barley TC were tried, more can be tested as listed in table. The last relevant results with the light scanner also opened new possibilities for markers and to increase the speed of the screening, such as the amplicon.

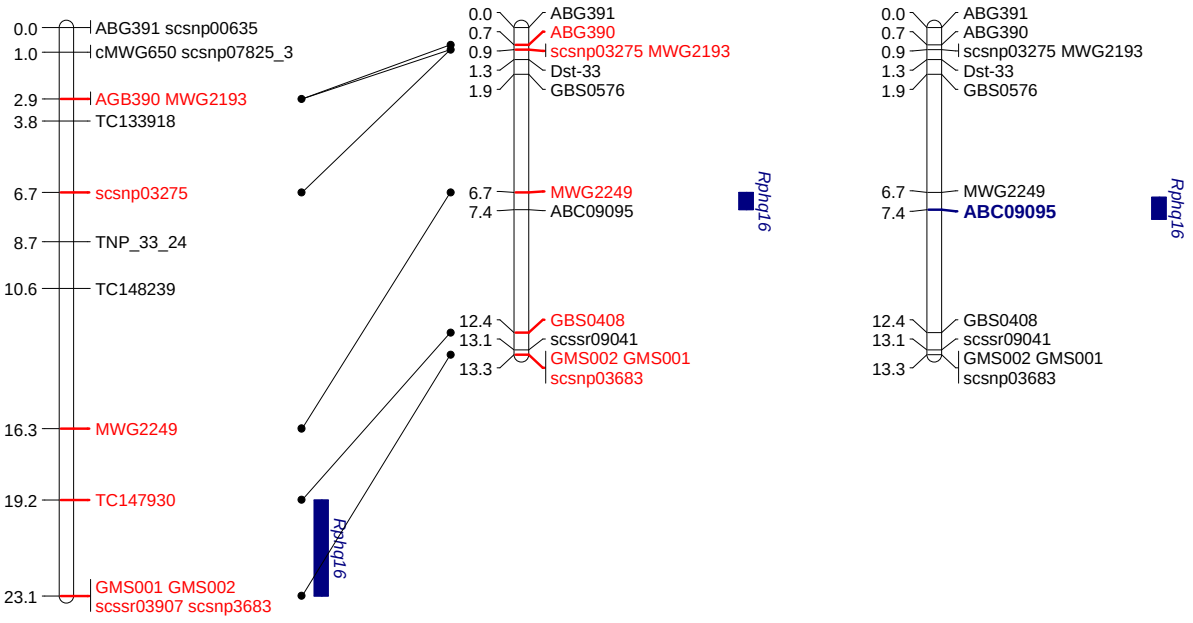


Figure 14 : evolution of the fine mapping of *Rphq16*. From left to right: fine map from Yeo Kuok San (2007); new fine map and location of *Rphq16*; new hypothesis of *Rphq16* location to be confirmed

To complement this molecular work, the investigation of the resistance mechanism of *Rphq16* could be investigated. Lorriaux (2007) studied the size of *P. hordei* colonies and the types of colonies (non-penetrant, early aborted, established) on seedlings from the two lines OWB30 (having only one QTL for partial resistance *Rphq18*) and OWB84 (having 5 QTLs for partial resistance: *Rphq12*, *Rphq16*, *Rphq17*, *Rphq18*, *Rphq19*). She put forward that compared to OWB30, OWB84 had colonies of a smaller size, more early aborted colonies and a longer RLP50S. But were these differences due to the effect of *Rphq16*, since there were more than one QTL for partial resistance of difference between OWB30 and OWB84. That's why it could be interesting to perform a similar study on our homozygous recombinants in *Rphq16* region, since if there are differences, they could be mainly due to the presence/absence of *Rphq16*.

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6. Appendices

6.1 Appendix 1: Analysis of variance to check the effect of the DBIG on the RLP50A

Analysis of variance

Source	d.f.	s.s.	m.s.	v.r.	F pr.
EXPE	2	4765.9	2382.9	10.65	< 0.001
DBIG	4	9673.6	2418.4	10.80	***< 0.001***
Residual	178	39841.5	223.8		
Total	184	54280.9	295.0		

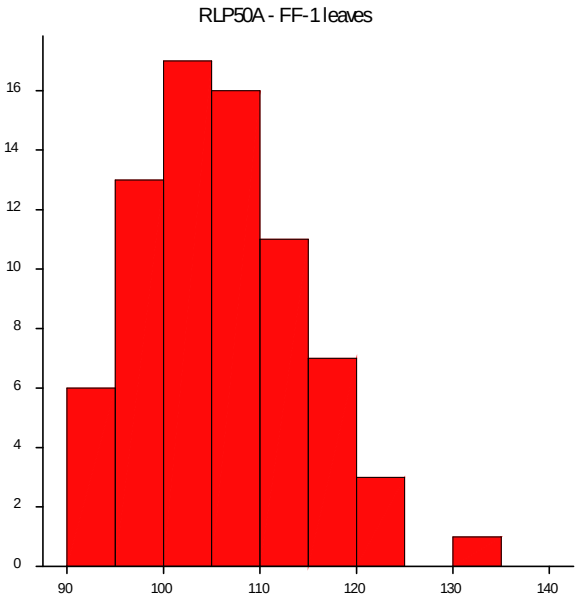
Predictions from regression model

Response variate: RLP50A The prediction is for the RPL50A

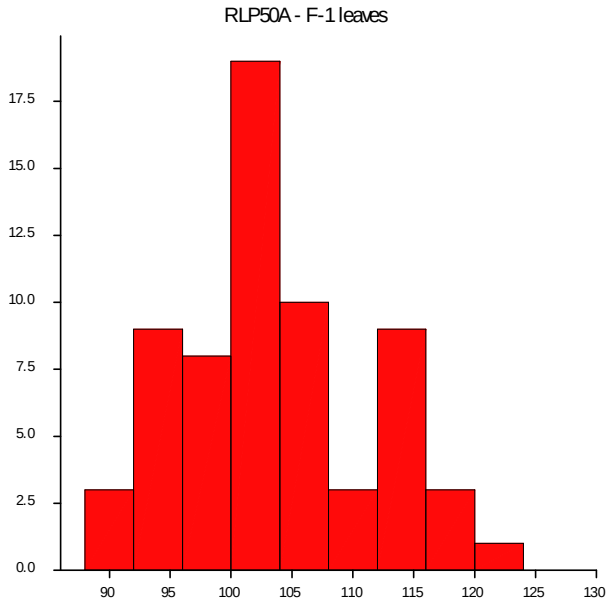
	Prediction
DBIG	
1	***119.8***
7	114.6
8	108.8
15	***100.4***
22	***120.9***

Minimum least significant difference	6.17
Average least significant difference	11.07
Maximum least significant difference	16.24

6.2 Appendix 2: frequency distribution of the RLP50A for the F-1 leaf and for the average of RLP50A for the flag and the F-1 leaf

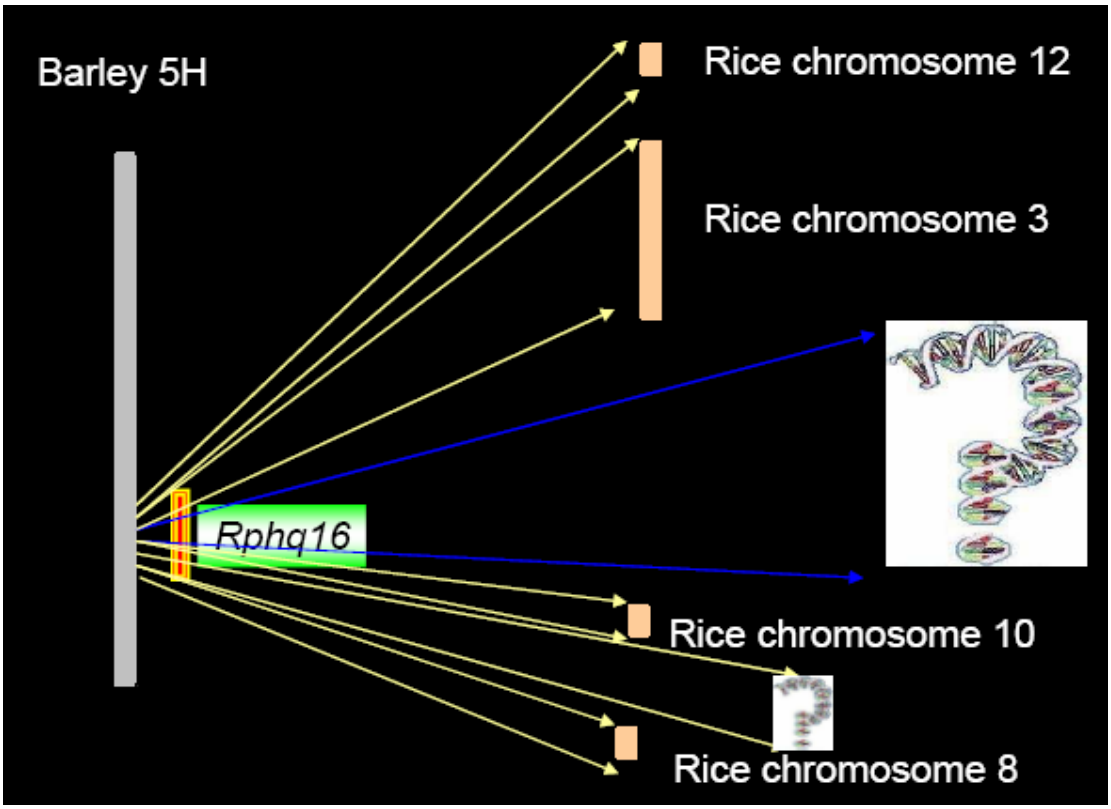


F-1 leaf

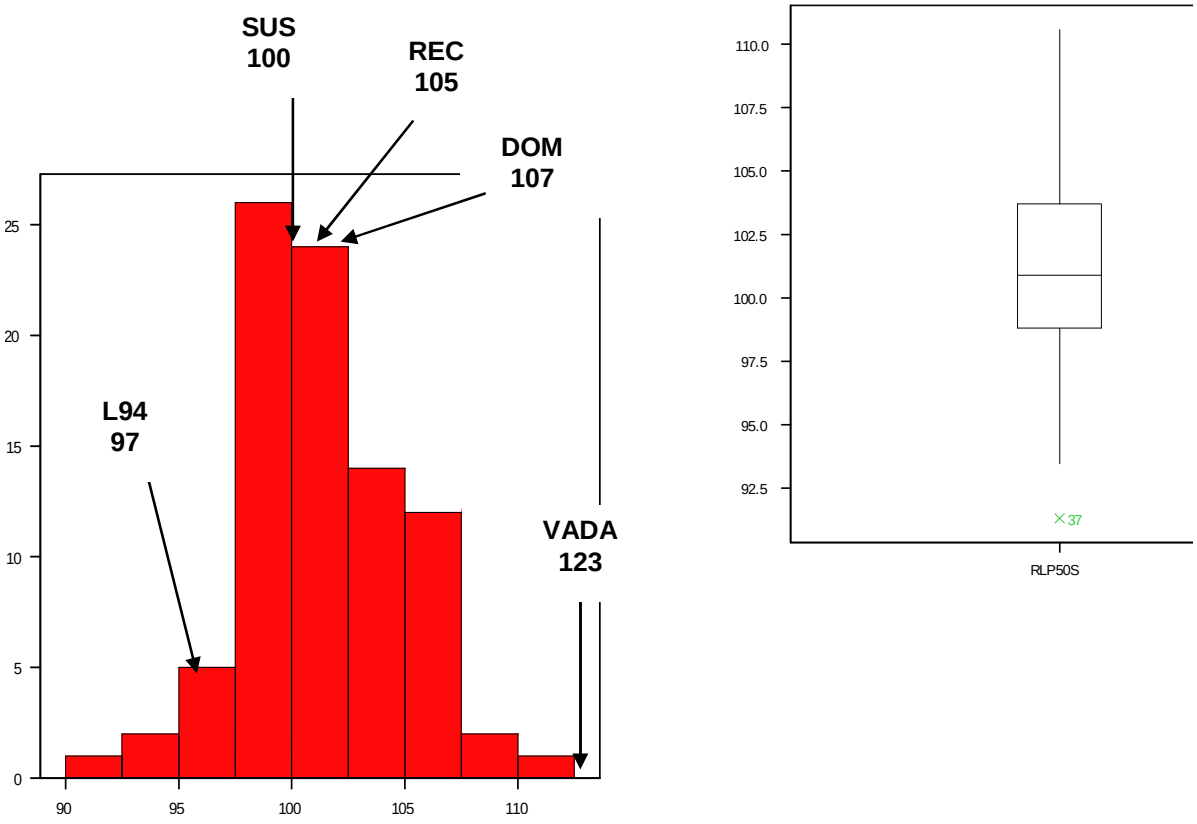


Flag and F-1 leaf

6.3 Appendix 3: synteny between rice and barley (Yeo Kuok San, 2007)



6.4 Appendix 4 : frequency distribution of phenotypes for the RLP50S of the available homozygous recombinants in Rphq16 region



6.5 Appendix 5 : Tuckey permutation test on RLP50S for the available homozygous recombinants in Rphq16 region

Identifier	Mean	
D38.08	93.9	|
D44.6	96.7	|
D41.2	97.9	|
D31.2	98.2	|
D8.1	98.7	|
D40.1	98.7	|
D23.1	99.6	|
DF 1.6	99.8	|
D1.3 02	99.8	|
D30.1	99.8	|
D1.1	100.0	|
D35.2	100.4	|
D15.3	100.8	|
D4.6	100.9	|
D17.2	101.0	|
D42.1	101.0	|
D13.2	101.7	
D38.2	101.8	
D1.4 02	102.0	
D36.04	102.4	
D5.1	103.0	
D16.1	103.2	
D45.02	103.3	
D33.01	104.3	
D13.03	105.4	
D11.03	105.5	
D39.1	105.6	
D32.08	106.2	
D18.2	106.7	

6.6 Appendix 6 : ANOVA table to check the difference of RLP50S between the resistant and susceptible available homozygous recombinants in Rphq16 region

Analysis of an unbalanced design using GenStat regression

Variate: RLP50S

Accumulated analysis of variance

Change	d.f.	s.s.	m.s.	v.r.	F pr.
Type	1	160.520	160.520	47.88	***<.001***
Residual	27	90.528	3.353		
Total	28	251.048	8.966		

Predictions from regression model

Response variate: RLP50S

	Prediction
Type	
R	103.9
S	99.2