University of Modena and Reggio Emilia

Ph.D. SCHOOL OF AGRI-FOOD SCIENCES, TECHNOLOGIES AND BIOTECHNOLOGIES

Fine mapping of two QTLs for resistance to the leaf rust *Puccinia brachypodii* in the model grass *Brachypodium distachyon* (L.)

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Abstract

The Brachypodium-*Puccinia brachypodii* pathosystems is a useful model for translational genomics of rust interactions with their cereal hosts.

Three quantitative trait loci (QTL) *Rpbq1*, *Rpbq2* and *Rpbq3*, for resistance to the *Ki* isolate of the leaf rust pathogen, were previously mapped, in a segregating population derived from the cross between the inbred lines Bd3-1 and Bd1-1 on Brachypodium chromosomes 2, 3 and 4, respectively. For validation of the three QTLs, F3 to F7 families derived from single F2 plants, and harbouring single QTLs at homozygous state, were selected by genotyping of flanking markers, and phenotyped inoculating with the *K*i isolate. *Rpbq2* and *Rpbq3* were confirmed and prioritized for further research.

The main aim of the present study was to fine map the two genomic regions associated to the leaf rust resistance in order to obtain a precise targeting of candidate genes towards the cloning of the two QTLs.

To reach this aim, two large segregating populations composed of 497 individuals for *Rpbq3* and 434 for *Rpbq2* were developed. Sequences of the QTL regions of the two parental lines were then aligned and searched for the discovery of gene-based single nucleotide polymorphisms (SNP) and insertion-deletions (InDel). SNPs and sequence tagged sites (STSs) markers have been designed in order to find recombinant individuals.

Eleven recombinant individuals for *Rpbq2* and ten for *Rpbq3* were found and multiplied by selfing to stabilize the recombinations at homozygosity; a second round of genotyping was thus necessary to select homozygous recombinant lines. To refine the position of the two QTLs, the recombinants were phenotyped with the *Ki* isolate of *Puccinia brachypodii*. Their progenies were phenotyped one year later to validate the experiment.

Exploiting the developed recombinants and data obtained from the screening, the *Rpbq3* interval was redefined, and the chromosome segment comprised between markers Bd4g09970 and Bd4g10230 was identified as the most linked with the resistance. The *Rpbq2* interval was reduced as well, to the segment comprised between markers bd3g16091 and bd3g16140 harbouring only three genes.

Subsequently, a functional protein domain analysis was performed for all gene residing at the two refined intervals for the two QTLs using NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and InterPro: protein sequence

analysis & classification (http://www.ebi.ac.uk/interpro).

Comparing the results obtained from the bioinformatics analysis and the fine mapping study, a first list of most promising candidate genes for the false brome rust resistance in *B. distachyon* including one gene for *Rpbq2* and two for *Rpbq3* was obtained. Identification of these candidate genes, already reported to be involved in plant-pathogen interaction may lead to the cloning of the two QTLs.

Moreover, an evaluation of isolate-specificity of the conferred resistance was performed by means of inoculations of the developed plant material with other four different isolates of *Puccinia brachypodii*: *De*, *Vij*, *Fl* and *Co*. As a complementary approach, a diversity panel of 50 different accessions of *Brachypodium distachyon* collected around the world and representative of Brachypodium natural variation was screened for response to *Puccinia brachypodii* isolate *Ki*. These 50 accessions are being re-sequenced with a project funded by United States Department Of Energy Joint Genome Institute (DOE-JGI). This study represents the first fine mapping approach applied to QTL cloning of the resistance genes to leaf rust pathogen *Puccinia brachypodii* in the model plant *Brachypodium distachyon*. The results obtained can be used in the future for translational genomics to the economically relevant cereals.

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Chapter 1

Brachypodium distachyon as a model plant

1.1 *Brachypodium distachyon*

Brachypodium distachyon (L.), also known as false brome, is a long day C3 grass from Middle East. Because of its ideal features, that include small stature (ranged from 15 to 20 cm in height), short generation time (8 – 12 weeks) and the ability to self-pollinate, it has emerged as a genetic model in recent years. (Brkljacic et al. 2011; Brutnell et al. 2015; Draper et al. 2001) The first genome draft of the Bd21 characterized by a high quality due to the very low percentage of repetitive DNA and the incorporation of a big amount of BAC ends was released to the community in 2009. The small genome size, 272 Mb, that is one of the smallest genome sizes of its family, contributed to the high quality of the assembly.

The first whole genome assembly v1.0 was released in 2010 (International Brachypodium Initiative, 2010), while recently, an improved new annotation V3.1 were released by the United States Department of Energy (DOE) Joint Genome Institute (JGI), that includes ~ 270 Mb of improved Brachypodium sequences with a total of 1.43 MB base pairs. Version 3.1 contains more than 6,000 genes that were not in the initial annotation (v 1.0). The aim of the JGI researchers was to place *B. distachyon* in the small group of organisms having the finished genome sequenced.

Another key feature making Brachypodium a model plant is its close phylogenetic relation with the more important cold temperate cereals. Brachypodium is the closest sequenced relative of grains such as wheat (Triticum aestivum), barley (Hordeum vulgare), rye (Secale cereale) and oat (Avena sativa) within the subfamily of the *Pooideae* (**Figure 1**) (International Brachypodium Initiative 2010). The high sequence similarity with wheat enables Brachypodium markers to hybridize most of the time with the wheat genome, while markers obtained from the rice (Oryza sativa), the former model plant used for temperate cereals, often used to fail (Griffiths et al. 2006). Smaller regions with a high degree of synteny were identified, and this highlights the great importance of Brachypodium genome as a bridge for positional cloning, however numerous rearrangements shown in comparison between genetic maps of wheat and Brachypodium make the efficiency of cloning depend on the segment under investigation (Berkman et al. 2011; Burt and Nicholson 2011; Choulet et al. 2010; Fleury et al. 2010; Huo et al. 2009; Krattinger et al. 2011; Liu et al. 2011; Qi et al. 2010; Qin et al. 2011; Somyong et al. 2011; Zhang et al. 2010; Zhang et al. 2011) (**Figure 2, Figure 3, Figure 4**).

In parallel with the first release of the Brachypodium genome assembly, other infrastructure and networks were built and are still growing in the Brachypodium community.

Nowadays a large number of protocols and genomic tools, such as genome sequences, genetic maps, transformation protocols, crossing protocols, mutagenesis protocols, expression data, T-DNA mutants, libraries made from expressed sequence tag (EST) and bacterial artificial chromosome (BAC) are ready to use for the Brachypodium Community. All these resources, accessible without relevant economical costs, are necessary to make Brachypodium an efficient model plant system for the cold temperate cereals (Brutnell et al. 2015).

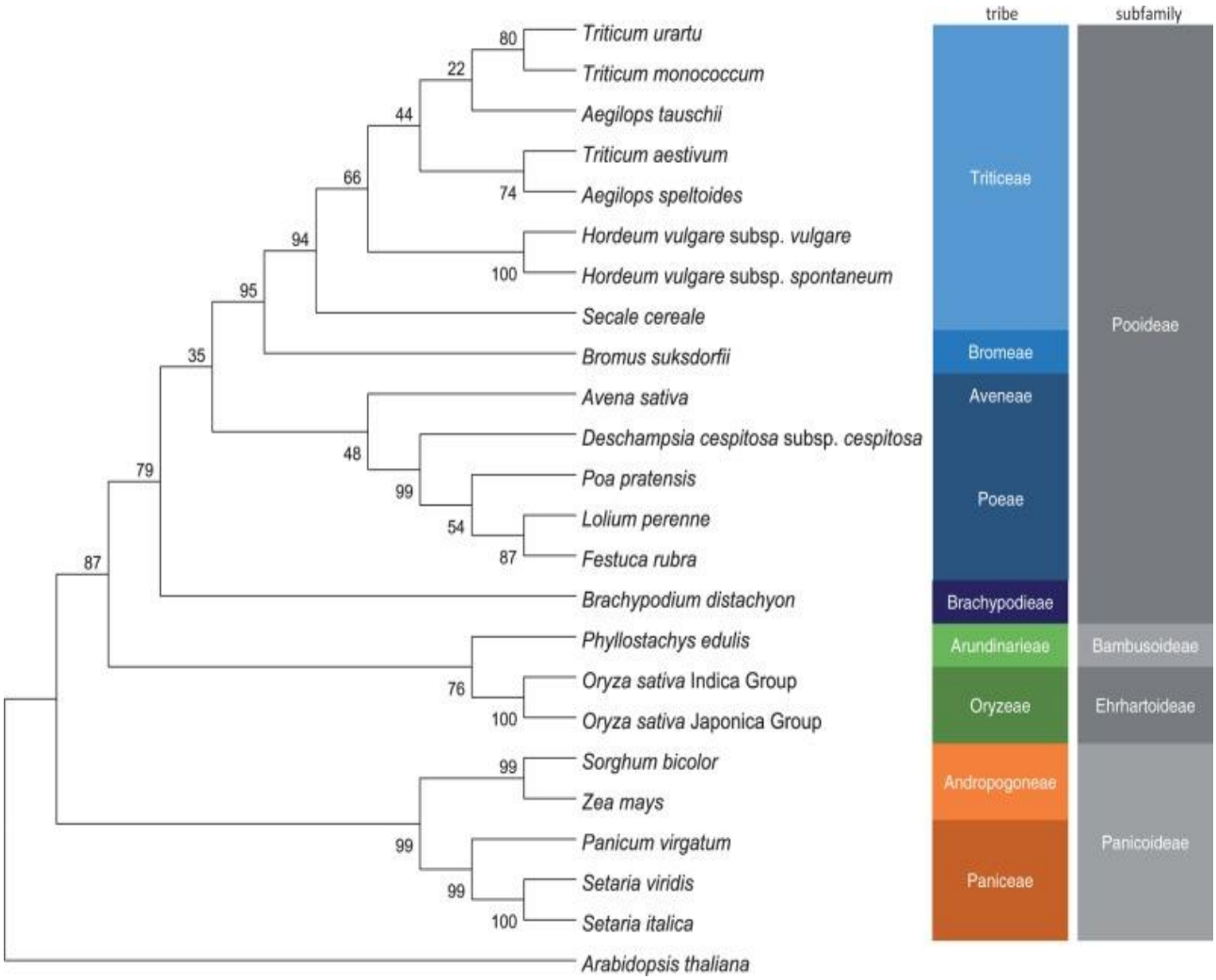

Figure 1. The phylogenetic relation between *Brachypodium distachyon* and the other grass species. The three was designed using the Neighbor–Joining method of the software MEGA v. 5.2 [http://www.megasoftware.net/ (Tamura et al. 2011)] exploiting the alignments of the gene *ndhF*. In every node of the three the bootstrap of 1000 replicates has been calculated. Figure credit: Mochida and Shinozaki, 2013.

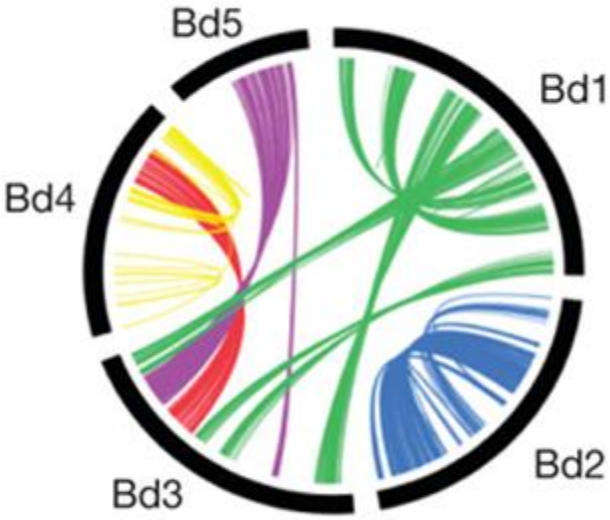

Figure 2. The six most important interchromosomal duplications are shown in this figure. The association underlined is made of 723 paralogous sequences that are coloured by different colour according to the link between the chromosomes. Figure credit: International Brachypodium Initiative, 2010 (modified).

Figure 3. Genome relations between Brachypodium (in the middle), rice (on the top) and sorghum (on the bottom). The region coloured in white is the centromeric region and the orthologous portions are coloured following this pattern: chromosome 1 blue; chromosome 2 yellow; chromosome 3 violet; chromosome 4 red; chromosome 5 green. The collinearity was made using 25,532 Brachypodium genes, 8,533 rice genes and 7,216 genes of sorghum. Figure credit: International Brachypodium Initiative, 2010 (modified).

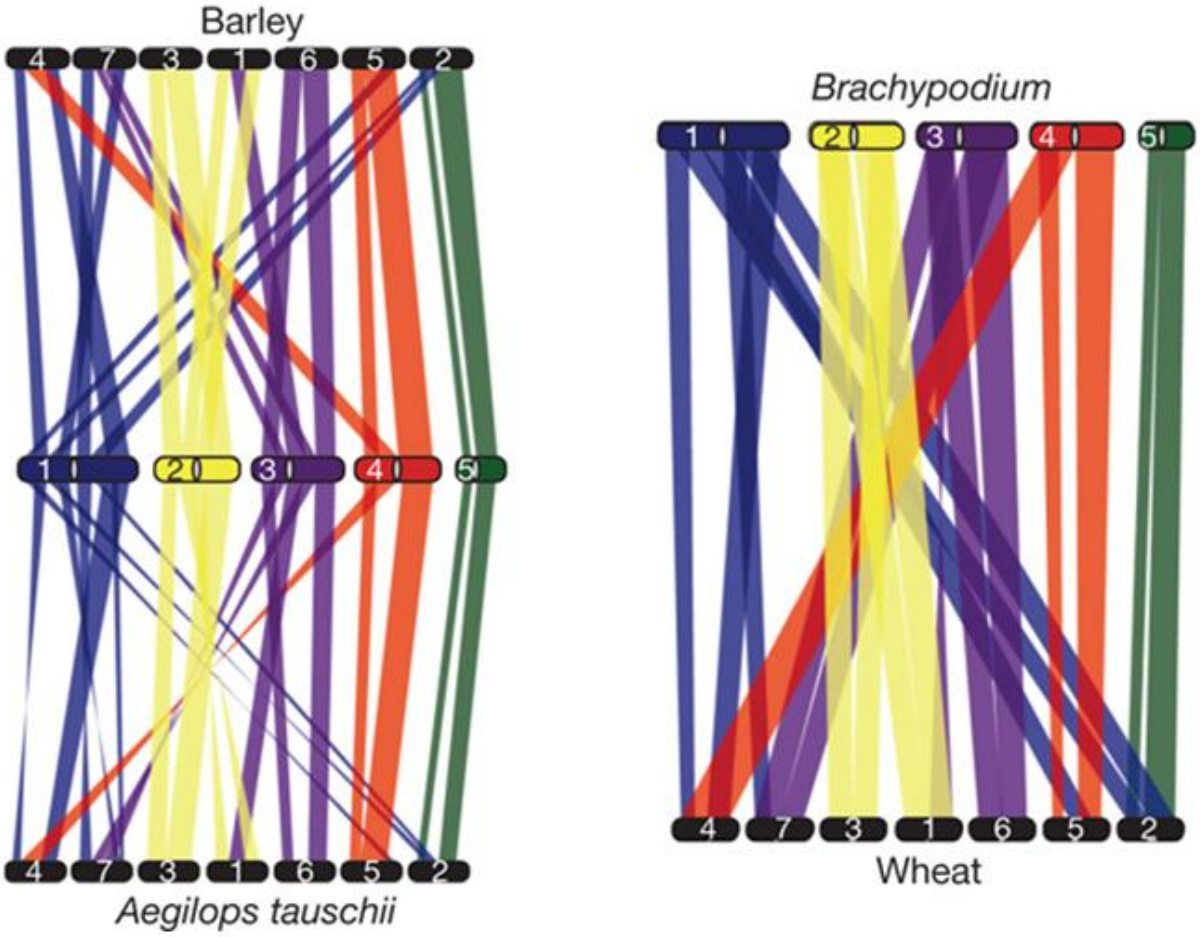

Figure 4. (Left) Chromosome orthologous relationships between Brachypodium (in the middle), barley (on the top) and Ae. tauschii (on the bottom). (Right) Comparison between hexaploid wheat and Brachypodium. The collinearity was made using 5,003 ESTs of bread wheat. Colours and centromeric regions are the same from figure 3. Figure credit: International Brachypodium Initiative, 2010 (modified).

1.2 Brachypodium community resources

1.2.1 Brachypodium genomics on-line resources

A numerous genomic tools have been developed for the false brome grass. All of these resources are a tassel that makes Brachypodium an exceptional model grass. A resume of Brachypodium online bio-informatic resources is listed in **Table 1**.

Table 1. Online resources for Brachypodium research. Table credit: Mochida and Shinozaki, 2013 (modified).

Resource Name	Description	URLs
Phytozome	Genome sequence Gene annotation ESTs RNA-seq data sets T-DNA tag lines FTS on the Bd21 genome Resequencing data sets	http://www.phytozome.net/
Brachypodium genome database–MIPS	Genome sequence Gene annotation Protein doman Comparative genome view	http://mips.helmholtz-muenchen.de/plant/brachypodium/
NCBI RefSeq	Gene annotation as RefSeq entry	http://www.ncbi.nlm.nih.gov/refseq/
Ensembl Plants	Genome sequence Gene annotation Wheat transcriptome alignments	http://plants.ensembl.org/Brachypodium_distachyon/Info/Index
Gramene	Gene annotation Comparative maps Pathways (BrachyCyc)	http://www.gramene.org/
RIKEN Brachypodium Full-length cDNA Database	Gene annotation Full-length cDNAs Triticeae transcriptome alignment	http://brachy.bmep.riken.jp/ver.1/index.pl
PlaNet	Gene expression profiles based on the GeneChip Co-expression analysis	http://aranet.mpimp-golm.mpg.de/
BRACHYTIL	TILLING lines	http://urgv.evry.inra.fr/UTILLdb
GramineaeTFDB	Transcription factors	http://gramineaetfdb.psc.riken.jp/
PlantTFDB	Transcription factors	
BrachyCyc	Pathways Gene Ontology	http://pathway.gramene.org/BRACHY/class-tree?object=Pathways
KEGG	Pathways Gene annotation	http://www.genome.jp/kegg/
Mapman	Mapping to MapMan Ontology	http://mapman.gabipd.org/
Plant Genome Duplication Database	Syntenic relationships	http://chibba.agtec.uga.edu/duplication/
PLAZA	Syntenic relationships Gene annotation Gene Ontology	http://bioinformatics.psb.ugent.be/plaza/
E-TALEN	Web service to design TALENs	http://www.e-talen.org/E-TALEN/

1.2.2 Germplasm collections

It is desirable for any model plant to have a collection of ecotypes showing a large variation at molecular level and in phenotype. Usually the variation is exploited for the development of segregating populations that are the basis for genetic map construction and also for positional cloning.

Historically, the first collection of Brachypodium inbred lines was the one assembled at the University of Aberystwyth, Wales (UK). The collection was listed on a website and included 38 ecotypes. This collection is no longer available on the Brachyomic website. The largest *Brachypodium distachyon* germplasm collection is the one held by the USDA National Plant Germplasm System (NPGS) (https://npgsweb.ars-grin.gov/gringlobal/taxonomydetail.aspx?7597). Nowadays, this collection has 256 different accessions and also merges two independent collections from Turkey. The first is composed by diploid accessions collected in different location across Turkey by the research group of the Namik Kemal University, lead by Hikmet Budak (Filiz et al. 2009) and named with an initial prefix "BdTR". The second collection was collected by researchers from the Sebanci University Metin Tuna with the help of John Vogel and his collaborators (Vogel et al. 2009) and the lines named with 3 letters coming from the place in which the seeds were collected (e.g., "ADI" stands for "Adiyaman" a region in Turkey). In total 53 locations in Turkey were exploited from the two scientists (Filiz et al. 2009; Vogel et al. 2009) in their surveys (**Figure 5**). Single seed descent inbreeding was used to develop 200 inbred lines representing all 53 locations of the Turkish germplasm collection and moreover, in the last years, more collections containing more than 2,000 accessions have recently been established. Most of them are the ones from Turkey (Filiz et al. 2009; Vogel et al. 2009), other are from France, Portugal, Spain (Catalan et al. 2012; Manzaneda et al. 2012; Mur et al. 2011), Italy, Georgia and Ukraine (A. Caicedo, unpublished data). Filiz (et al. 2009) and Vogel (et al. 2009) both did a molecular diversity analysis using respectively amplified fragment length polymorphism AFLP and 43 new SSR markers with the result showing that there was a terrific molecular diversity across the collections (**Figure. 6**).

From all these accessions 8 single seed descent derived inbred lines were developed from the USDA-NPGS accessions. These inbred lines are the so called "core inbred line set" (Bd1-1, Bd3-1; Bd2-3, Bd18-1, Bd21, Bd29-1; Bd30-1; Bd21-3) and from these lines several Recombinant Inbred Lines were developed thanks to two methods, freely available on line

from Garvin's lab (http://www.ars.usda.gov/Research/docs.htm?docid=18531) and Vogel's lab (http://jgi.doe.gov/our-science/science-programs/plant-genomics/brachypodium). The first recombinant inbred line comes from single seed descent advancement of the Bd3-1 x Bd21 was used to map a gene controlling natural variation for resistance to barley stripe mosaic virus (BSMV). In this study (Cui et al. 2012), the Bd3-1 x Bd21 RIL population was employed to map a BSMV resistance gene with SNP-markers previously used to develop a SNP-map in the F2 generation of the same population (Huo et al. 2011). More RILs have been developed from crosses between Bd21 x Bd1-1, Bd3-1 x Bd2-3, and Bd21 x Bd30-1 at Garvin's Lab (David Garvin unpublished data), and more are in development by other researchers. A Bd3-1 x Bd1-1 population was used to develop a linkage map, anchored to the genome sequence of Brachypodium, obtaining the first QTL analysis in the species by Barbieri (et al. 2012). The same population was used in this study to achieve the fine mapping of two QTLs for resistance to *Puccinia brachypodii* from researchers of the University of Modena and Reggio Emilia (**Chapter 3**).

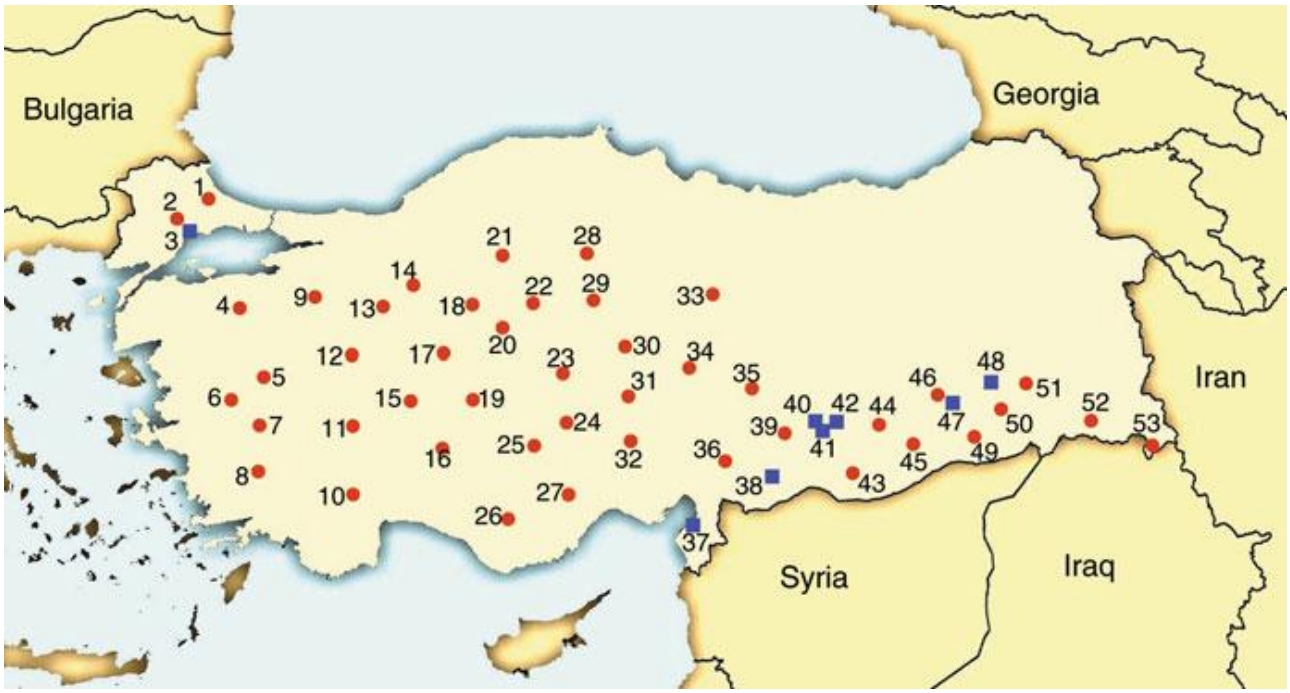

Figure 5. Fifty three places where seeds of Brachypodium were collected by Hikmet Budak (Filiz et al. 2009) in blue and Mehtin Tuna (Vogel et al. 2009) in red respectively. Figure credit: Vogel et al. 2009

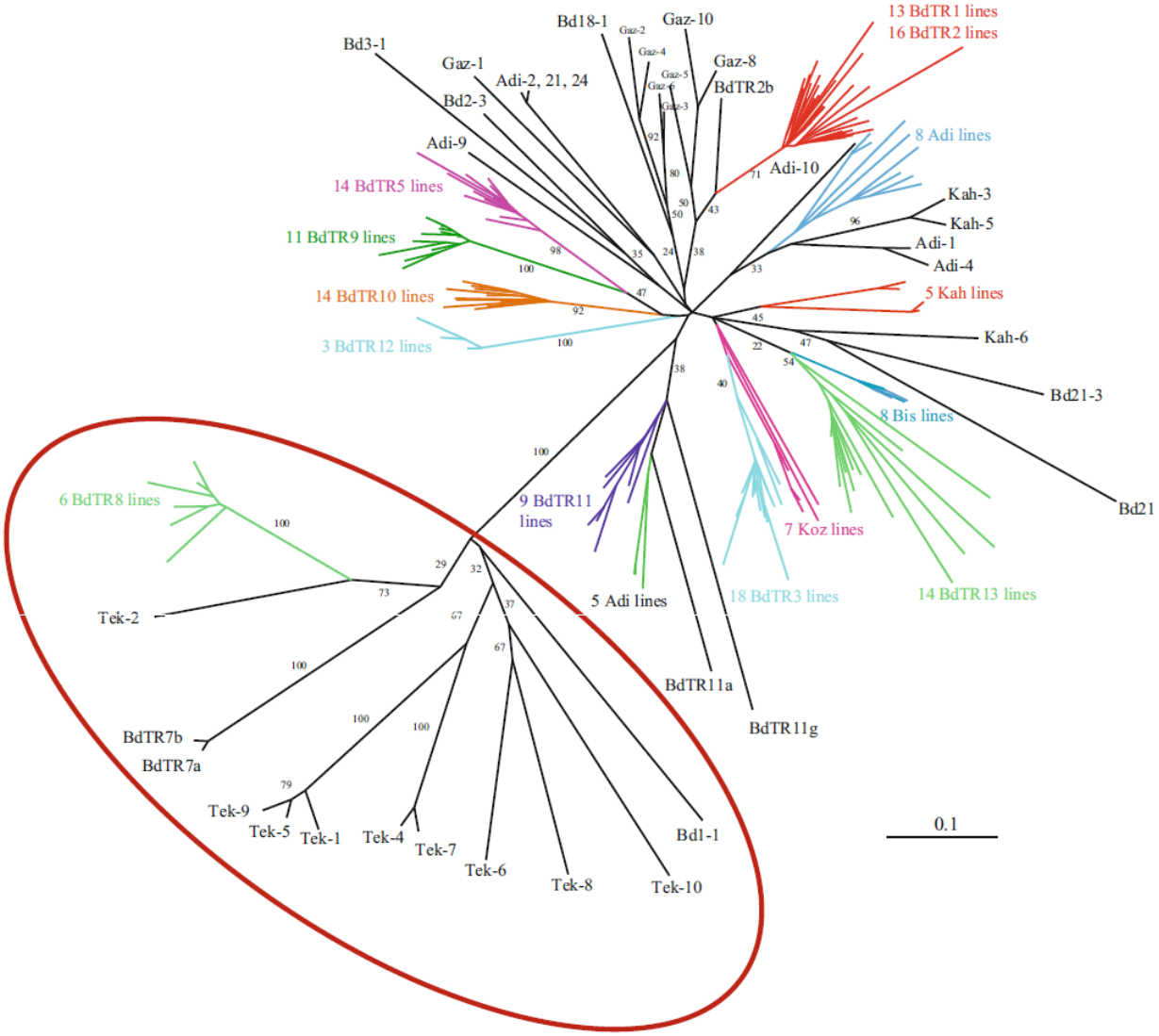

Figure 6. Forty three brand new SSR markers were used to genotype 187 accessions of *Brachypodium distachyon*. As a result a phylogenetic tree was made with thick branches with a bootstrap value higher than 20. The accessions clusterize in two big groups: the first one is on the top of the figure and have inside the Turkish and Iraqi accessions; the second one is composed of the individuals inside the red ellipse and they all have common features such as a long vernalization requirement, small grains and a lemma without hair. Figure credit: Vogel et al. 2009.

1.2.3 DNA libraries

DNA libraries, such as BAC and cDNA and expressions libraries are a powerful tool that can be exploited in research. The strength of Brachypodium as a model derives from the numerous libraries that have been created from 2010 until now. The main BAC libraries are

the three made from the reference inbred line Bd21, and one of which was also used during the sequencing of the reference genome (Huo et al. 2006, 2008; Febrer et al. 2010).A library of one of the core inbred lines, Bd3-1, has been recently obtained (http://www.genome.arizona.edu/orders; M. Bevan, unpublished data).

Two free access gateway-ready Brachypodium distachyon cDNA libraries ready to use were created and cloned into a two hybrid yeast vector (Y2H). This Gateway™ is going to speed up studies for multiple user defined purposes. The Y2H has a direct application for screening and discovery of interactions between proteins (Cao et al. 2011). Another cDNA's library of around 12700 clones as total has been made using mRNA from a various kind of Brachypodium's tissues and also treatments have been made and used to end up with EST sequencing (International Brachypodium Initiative 2010; Vogel et al. 2006). In 2013 39,000 full-length cDNA clones of Brachypodium were created and sequenced constituting an exceptional tool for annotation and expression studies (Mochida et al. 2013).

1.2.4 Transformation system, mutagenesis and genome editing

A model plant for being a successful one needs an efficient transformation system. During the years, Brachypodium has been proven to be inclined to transformation and tissue culture (Bablak et al. 1995; Draper et al. 2001). The community of Brachypodium can proudly say that after optimizations of the methodologies, the false brome grass is now the easiest and most efficiently transformed *Agrobacterium tumefaciens*-mediated grass (Pacurar et al. 2008; Vain et al. 2008; Vogel and Hill 2008). The line used for transformation is one of the core inbred line set, Bd21-3. The efficiency achieved in Brachypodium is 45% with the amount of 100 transgenic lines ready every week (Bragg et al. 2012). T-DNA collection was one of the reasons of the success of *Arabidopsis thaliana* as a model species and it will be also for Brachypodium distachyon because a project founded by the DOE has created more than 23,000 T-DNA Brachypodium lines (**Figure. 7**). Another T-DNA collection obtained at John Innes Centre (Norwich, England) and not longer publicly available was composed of more than 5000 T-DNA mutants of which 1000 were sequenced.

The genome editing is seen as the next generation system for modification of target genes (Mikami 2014). Transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA

endonucleases are now two new powerful class of tools that are redefining the frontiers of biological research (Gaj et al. 2013; Hsu et al. 2014; Lozano-Juste and Cutler 2014; Bortesi and Fischer 2015). Both TALENs (Shan et al. 2013) and CRISPR/Cas9 (Brutnell et al. 2015) systems are successful in Brachypodium, and this can be the start of a new revolution in plant functional genomics due to the high potential of these two techniques in high-throughput application to study and modify the functionality of target genes.

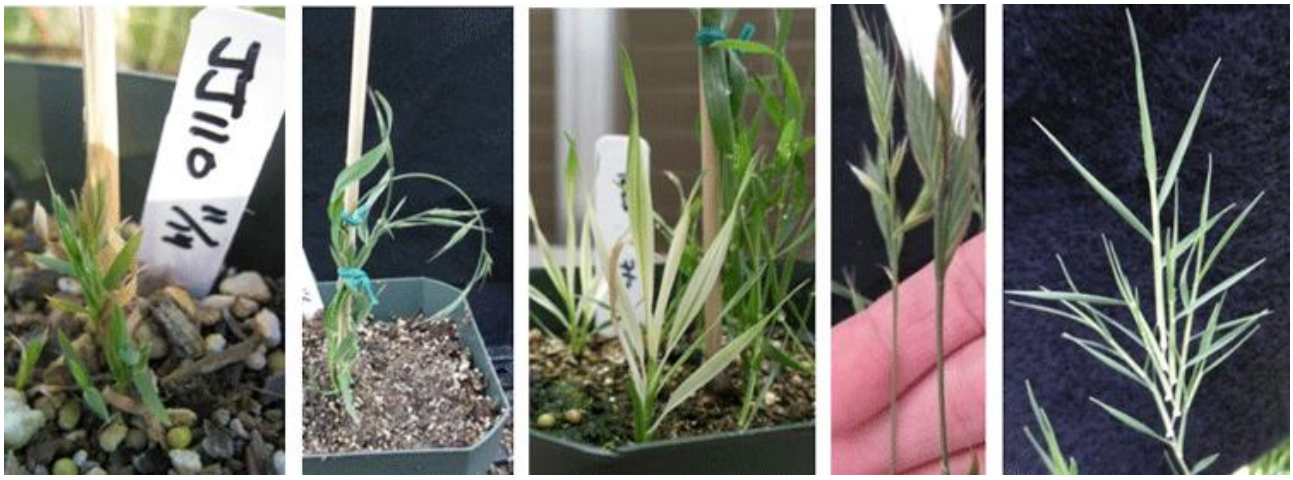

Figure 7. *Brachypodium distachyon* families segregating T-DNA insertions. Mutant phenotypes from left to the right: dwarf, curly, pale, spikes, branchy. Picture credit J. Bragg.

1.2.5 Mutagenesis

The Bd21-3 inbred line was used also as a resource for the creation of chemically induced mutant collections. Targeting Induced Local Lesions in Genomes (TILLING) methodology using ethyl methanesulfonate (EMS) (McCallum et al. 2000) and sodium azide was applied (Dalmais et al. 2013). The point mutations derived can be used both for the reverse genetic as for the forward genetic screens). The biggest TILLING collection, of over 10,000 Bd21-3 mutated accessions called BRACHYTIL, is available in the collection is and is available in the Unité de Recherche en Génomique Végétale (URGV) TILLING database (http://www-urgv.versailles.inra.fr/tilling) at INRA-Versailles. A second TILLING pipeline has been established in the Brutnell's laboratory at the Boyce Thompson Institute, affiliate of Cornell University in the US (Brutnell et al. 2015). The next step will be to implement TILLING for species *Brachypodium stacei* and *Brachypodium hybridum* which now have no T-DNA collections available. In 2015, WRRC project “Creating a genome-wide sequence-

indexed collection of grass mutants" started from a collaboration between the INRA-IJPB and the USDA-ARS with the aim of sequencing both the EMS and sodium azide Bd21-3 mutated populations. The result will be a useful tool for forward and reverse genetics (source: http://jgi.doe.gov/our-science/science-programs/plant-genomics/brachypodium/).

1.2.6 Expression analysis

The BradiAR1b520742 microarray originated from a collaboration between the Affymetrix Inc, and Mockler's laboratory at Oregon State University. The microarray was used both as a tiling and expression array and had the result to create an expression atlas that was freely accessible online in the former Brachypodium official website "brachypodium.org". A useful coexpression network has been established and is a part of a bigger project of the PlaNet (http://aranet.mpimp-golm.mpg.de) (Mutwil et al. 2010) a platform of web-tools dedicated to visualization and analysis of plant co-function networks which involves not only Brachypodium, but also other important plant models like Arabidopsis and also plant that are economically relevant such as wheat, soybean and barley. Recently microarrays has been replaced by RNA-seq methodology has taken the place of the microarrays that before where largely used for gene expression studies (Huan et al. 2013; Kakei et al. 2015).

1.3 *Brachypodium distachyon*: Exploiting the potential of a model monocot host for many pathogens to improve cereal crop resistance.

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Abstract:

With its small genome, tractable transformation, and the availability of a range of forward and reverse genetic tools, Brachypodium is now a well-established model species for the grass family Poaceae. A number of research groups have developed Brachypodium pathosystems employing a diverse array of pathogens to aid in the analysis of diseases of cereal, turf, and bioenergy grass species. The Brachypodium genome sequence has aided in the mapping of key disease resistance traits in larger genome Triticeae crops, and has served as a syntenic genome sequence to survey for variation in defence gene families. As a biological model Brachypodium has served in defining key events in susceptibility and resistance to diseases. This review outlines how Brachypodium is being exploited for plant pathology research and outlines future opportunities to employ Brachypodium to advance disease resistance in crops. Ultimately, Brachypodium research aimed at defining mechanisms of nonhost resistance, and obtaining a deeper understanding of determinants of susceptibility to pathogens, will reveal novel strategies for enhancing disease resistance in grass crops, in a manner that is complementary to traditional breeding methods.

Pathogen profiles, where disease-causing organisms are extensively reviewed, are an important feature of this journal, and they have proven to be enormously enlightening to pathologists at every career stage. In contrast, perhaps excepting Arabidopsis, model host plant species have not been fully considered. For obvious phylogenetic reasons, Arabidopsis is not a suitable model for cereal crop diseases. However, the model grass *Brachypodium distachyon* (hereafter referred to as Brachypodium, unless dealing with a range of *Brachypodium* species), with its small genome and, equally importantly, its close phylogenetic relationship to many of our most important cereal grain crops that form the basis of human diets, is positioned to fulfill such a role (Draper et al. 2001; Mur et al. 2011; Brkljacic et al. 2011). Here we consider how the range of pathosystems involving Brachypodium are being exploited to advance or understanding of plant-pathogen interactions and how this model can be further exploited to improve cereal crop resistance to many diverse diseases.

Brachypodium pathosystems *a la carte*

Arabidopsis thaliana is the premier model plant species, and its interactions with viruses, fungi, bacteria and oomycetes have been extensively characterized (Mauch-Mani & Slusarenko, 1993; Quirino & Bent, 2003; Hou et al. 2009). During the course of its emergence as a model grass over the past decade, many interactions between Brachypodium and a diverse range of pathogens of significance to grass crops have now been identified and described (**Table 2**). Central to these interactions is the defining of pathosystems encompassing biotrophic and necrotrophic interactions, including cereal foliar, inflorescence, and root pathogens.

Chronologically, the response of Brachypodium to challenge with *Magnaporthe grisea* - the causal agent of the economically devastating disease rice blast - was the first to be characterized (**Figure 8A**). The selection of pathogen was fortunate as this pathosystem readily revealed differential responses between genotypes, including susceptibility which was equivalent to that seen on rice (Routledge et al. 2004; Parker et al. 2008). Furthermore, a range of degrees of resistance was observed, including monogenic resistance in Brachypodium accession ABR5 (Routledge et al. 2004). The subsequent history of research of this pathosystem highlights the value of Brachypodium. The substantial similarity between

responses of rice and Brachypodium to *M. grisea* was demonstrated through metabolomic analyses (Parker et al. 2008). Other work indicated that jasmonates were associated with resistance to *M. grisea* in *B. distachyon* (Mur et al. 2004; Allwood et al. 2006), a finding that preceded a similar result in rice (Mei et al. 2006). This body of research on a single pathogen demonstrated that responses to a pathogen in Brachypodium can be readily compared to those of their primary cereal hosts to provide novel insights, as well as confirm the potential of Brachypodium to serve as a surrogate species to accelerate research in less tractable crop relatives. An extension of Brachypodium and its use in exploring plant interactions with *M. grisea* is furnished by the work of Yang *et al.* (2013), who used algorithms to identify rapidly evolving putative rice blast nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domain (NBS–LRR) resistance genes in Brachypodium as well as maize (*Zea mays*) and sorghum (*Sorghum bicolor*). The Brachypodium NBS-LRR genes with homology to the *Rp1/Pi37* gene family was found to be functional against *M. grisea* in transgenic rice as were some 25% of the other genes isolated from other species. The demonstration of the presence of rice blast *R* genes in distant relatives of rice led the authors to suggest a concept of "constrained divergence", whereby rapidly evolving pathogens are constrained to follow only a few evolutionary paths, and ancestral resistance genes in presumed non-host species can remain active against such pathogens (Yang et al. 2013).

Comparative analysis of response to infection between Brachypodium and grass hosts for other pathogens extends to other very diverse classes of interactions. An example of this is the interaction involving members of the fungal genus *Fusarium*, which cause Fusarium head blight (FHB). Despite decades of intensive research seeking to improve FHB resistance in wheat and barley, the disease remains a global problem due to the complex genetic nature of resistance in these crops, which is only partial and is overcome during epidemic years. Further, the molecular interactions between *Fusarium spp.* that cause FHB and these crops are complex and so remains poorly understood. Thus, a model species for exploring FHB resistance has great value for identifying new approaches to improving resistance. Peraldi et al. (2011) first demonstrated that both *F. graminearum* and *F. culmorum* can infect Brachypodium spikes and give rise to necrotic lesions, a hallmark symptom of FHB seen in infected cereal crop spikes (**Figure 8B**). Recent studies have used *F. graminearum* mutants (Blümke et al. 2014) and global transcriptomic and metabolomic analyses (Pasquet et al. 2014) to demonstrate comparable defence responses to the fungus in Brachypodium as observed in cereal crops. These studies expand upon earlier research on the role of

detoxification of trichothecene mycotoxins such as deoxynivalenol (DON) as one mechanism of resistance to *F. graminearum*. DON can be detoxified by UDP-glycosyltransferases (UGT) (Poppenberger et al. 2003) and UGTs able to convert DON into the inactive form DON-3-O-glucoside have been found in Brachypodium (Schweiger et al. 2013), including a presumed ortholog of a barley UGT that both converts DON to its glucosylated form (Schweiger et al. 2010) and which, when expressed in Arabidopsis, increases DON resistance (Shin et al. 2012). Brachypodium has now been used to help reveal new mycotoxin processing pathways as well (Kovalsky Paris et al. 2014). Further, in an intriguing finding, low-dose DON application to Brachypodium was observed to prime resistance in to *F. graminearum* infection. The reduced susceptibility to FHB was associated with enhanced cell wall changes and hyperactivation of pathogenesis-related gene expression (Blümke et al. 2014). Taken together, these results indicate that Brachypodium will continue to be a potent model for further dissection of the FHB pathosystem in cereals.

The list of necrotrophic fungi that are able to infect *B. distachyon* has been extended by studies of Sandoya & Buanafina (2014) and Falter & Voigt (2014) (**Table 2**). Falter & Voigt (2014) evaluated opportunities to establish Brachypodium-based pathosystems for the energy grasses. Full susceptibility was observed in Brachypodium to leaf pathogens from sugarcane (*Fusarium sacchari, Stagnospora tainanensis*) as well as maize (*Cochliobolus heterostrophus*). The opportunity to use Brachypodium to inform biological and genomic studies of potential energy grasses such as *Miscanthus x giganteus* and switchgrass (*Panicum virgatum*) has been noted (Bevan et al. 2010), and the value of Brachypodium for such research is highlighted by the fact that the U.S. Department of Energy considers Brachypodium a flagship species for its biofuel research programs. As the land area planted to energy grasses increases dramatically to accommodate cellulosic ethanol production, it will be imperative to guard them against potential diseases that may reduce not only yield but also stand persistence. Characterizing disease resistance mechanisms in these species may be particularly challenging and time-consuming because of both their complex genomes and genome structure and breeding systems. Thus, using Brachypodium as a surrogate for research on energy grass diseases may more rapidly lead to discoveries relevant to improving disease resistance in the crop species, resulting in useful and efficient translational breeding strategies.

Brachypodium has also emerged as an excellent model host for some viral diseases of grasses (Mandadi & Scholthof, 2013). Panicum mosaic virus (PMV) is the causal agent of the

lawn grass disease St. Augustine Decline. Brachypodium has proven to be an alternate host for PMV and its satellite virus (SPMV) and as such has been used to demonstrate the transcriptomic effects of viral infection (Mandadi & Scholthof, 2012). This study revealed wide-ranging shifts in a range of metabolic pathways and an up-regulation of signalling and defence processes regulated by salicylic acid but potential suppression of comparable processes mediated by jasmonic acid and ethylene. Interestingly, in some instances SPMV attenuated the amplitude of defence gene induction compared to the response to PMV alone.

Brachypodium is also host for another virus, Barley stripe mosaic virus (BSMV). BSMV is an RNA virus than can infect many species of the Poaceae, and particularly in barley has demonstrated the potential to cause significant yield losses with well researched strains including North Dakota (ND18), Norwich (NW), CV42, CV17, China, Russian and Argentina mild (Gustafson et al. 1982). A recent study reported that natural variation for resistance to ND18 exists in Brachypodium with genotype Bd21 (which represents the reference genome sequence) exhibiting susceptibility and Bd3-1 resistance (Cui et al. 2012). Using a recombinant inbred line (RIL) population developed from a cross between these two lines, BSMV resistance in Bd3-1 was determined to be due to a single locus termed *Bsr1* (*Barley stripe mosaic virus resistance 1*). Remarkably, it was possible to fine map *Bsr1* to a 23 kb interval in this population, which numbered only 165 RILs. The interval contained 5 putative genes, with the most promising candidate gene being a nucleotide binding site-leucine rich repeat (NBS-LRR) gene (Cui et al. 2012). Interestingly, BSMV Norwich (NW) strain is able to circumvent Bd3-1 resistance and genome-reassortment experiments using BSMV ND18 and NW strains revealed that the major NW virulence resides in the BSMV NW triple gene block 1 (TGB1NW) movement protein. Site-specific mutations at positions 390 and 392 of the two TGB1NW amino acids have proved to be critical residues affecting Bd3-1 *Bsr1* resistance (Lee et al. 2012). Such gene discovery in Brachypodium may be particularly useful for genetic linkage-based testing to determine whether *Bsr1* is orthologous to any known barley BSMV resistance genes, thus circumventing the major effort of map-based cloning of such genes in barley.

In contrast to *M. grisea* and *Fusarium* spp., Brachypodium interactions with cereal-adapted rust fungi which consistently give full susceptibility are still being sought. Rust diseases–for example, the wheat stem rust pathogen *Puccinia graminis* f. sp. *Tritici*, are receiving significant scientific attention due to the socio-economic threat that new virulent races emerging on the African continent pose to global cereal production (Kolmer, 2005).

Barbieri et al. (2011) found only a single race of *P. striiformis* f. sp. *hordei* (barley stripe rust, 42 tested), and no *Puccinia striiform*is f. sp. *tritici* (wheat stripe rust, 33 strains tested) where some susceptibility was demonstrated on inoculated Brachypodium. Ayliffe et al. (2013) subsequently screened 140 Brachypodium genotypes with *Puccinia graminis* f. sp. *tritici*, *P. triticina*, and *P. striiformis* and by far the most common responses were rapid callose deposition and autofluorescence consistent with the elicitation of basal resistance. Some genotypes did exhibit large lesions and some pustule formation was noted, but full susceptibility was not observed. This was in contrast to the excellent pathosystems developed for Brachypodium using rusts adapted to the genus *Brachypodium*. For instance, when infecting Brachypodium with rust *Puccinia brachypodii* collected from *B. sylvaticum* responses ranged from resistant to fully susceptible (**Figure 8C**) (Barbieri et al. 2011; 2012). When Figueroa et al. (2013) inoculated Brachypodium lines with *P. graminis formae speciales*, including *P. graminis* f. sp. *lolii* (Lolium stem rust) and timothy stem rust (*P. graminis f. sp. phlei-pratensis*) both were able to infect Brachypodium and complete their life-cycles to produce sporulating uredinia, although forming amongst necrotic patches of leaf tissue indicative of a latent host resistance response. Significantly, they observed phenotypic variation for the degree of resistance, which opens up possibilities to explore the genetic and molecular determinants of resistance to non-adapted rust pathogens, which may lead to identifying critical components of nonhost resistance that can be exploited for improvement of stem rust resistance in cereal crops under threat from the new races emerging from Africa.

With these observations in mind, examining the list of pathogens that can infect Brachypodium (**Table 2**; Mandadi & Scholthof, 2013), some general themes emerge. Most apparent is the fact that Brachypodium is a poor host for biotrophic, cereal-adapted fungal pathogens. While some degree of virulence may be displayed by cereal-adapted rusts, a delayed necrotic response is the outcome of interactions with the most susceptible Brachypodium genotypes (Barbieri et al. 2011; Draper et al. 2001; Figueroa et al. 2013). In this context, it is notable that no compatible interactions with the biotrophic powdery mildews have been described. While we have observed some mildew haustorial formation, these abort approximately 24 h post inoculation and fail to feed ectopic hyphal development, and no conidiophore formation is observed (Mur, unpublished observations). A second theme that emerges from the aforementioned studies is that it appears that compatible interactions between Brachypodium and both turf and forage grass-adapted pathogens as well as necrotrophic pathogens are more readily established. Such data could suggest that the

domestication of the major cereal species was matched by co-evolutionary specialisation of major biotrophic pathogens, such that they are no longer able to readily infect related grass species. Such a hypothesis was also proposed by Ayliffe et al. (2013) who noted that Brachypodium was generally more susceptible to Pooid-adapted (for example, *P. graminis* f. sp. *phalaridis*) compared to cereal-adapted rusts.

Ongoing and emerging opportunities for Brachypodium pathosystem based studies.

The emergence of Brachypodium as a model host for plant-pathogen interactions must be viewed against the backdrop of global food security. The current and future strategies for Brachypodium pathosystem-centred research for must mainly aim to accelerate the development of disease-resistant cereal crops. Currently, these are mostly focused on exploiting the genome resources for Brachypodium. The Brachypodium genome along with those of rice and sorghum has helped in mapping gene order in barley (IBGSC, 2012), wheat (IWGSC, 2014) and *Lolium* (Spannagl et al. 2013; Pfeifer et al. 2013; Hernandez et al. 2012). Further, in developing annotated cereal crop genomes, the well-defined Brachypodium transcriptome can be used to identify full length open reading frames (ORF) in other grass crops (Mochida et al. 2013), as well as suggest potential ORF function based on analysis of gene expression under different conditions (Mochida & Shinozaki, 2013). Thus, the Brachypodium genome sequence itself provides a powerful structural and functional reference for the analysis of more complex crop genomes. Obviously, Brachypodium also lends itself to functional genomic analysis of defence-associated genes as well. Thus, between 126 and 178 NBS-LRR resistance genes have been identified in the Bd21 genome (TIBI, 2010; Tan & Wu, 2012). Clearly, this same approach can be extended to any other class of defence genes; for example, receptor-like proteins (RLPs) cell surface receptors (Wang et al. 2008); pattern-recognition receptors (Han & Jung, 2013) or WRKY (TIBI, 2010; Bakshi & Oelmuller, 2014). Such computationally identified and characterized genes could be tested in heterologous hosts for functionality against a panel of pathogens, and this means that Brachypodium may serve as a reservoir of novel defence-related genes that could be deployed into elite cereal crop varieties.

For the plant pathologist, it is also clear that such information can directly aid breeding research. For instance, breeders frequently search for novel sources of disease resistance in a broad range of germplasm, for example landraces or wild crop relatives (Varshney et al. 2009). One challenge that is often encountered is linkage drag, in which linked deleterious genes are introgressed with the desired gene. Genetically separating the deleterious genes can be a daunting task, but the Brachypodium genome sequence can assist on this front. For instance, gene *Pch1* confers resistance against the important necrotrophic pathogen *Pseudocercosporella herpotrichoides* and was introgressed from *Aegilops ventricosa* to chromosome 7D of bread wheat (Burt & Nicholson, 2011). The introgressed genomic fragment was found to include genes which confer a yield penalty. Thus, the Brachypodium genome sequence was mined to develop conserved orthologous sequence (COS) markers to more finely resolve the location of *Pch1* within the introgressed region. These markers will permit more stringent marker-assisted selection for *Pch1* genetically uncoupled from the genes affecting yield. A similar example was the utilisation of Brachypodium-Poaceae co-linearity to develop flanking markers to the *P. striiformis* f. sp. *tritici* resistance gene *Yr26*, which should ultimately lead to its identification and isolation (Zhang et al. 2013). In yet another study that leveraged the genome sequence of Brachypodium, resistance genes and QTLs in barley landraces were extensively mapped using markers developed based on genome syntenic relationships between members of the Poaceae, including Brachypodium (Silvar et al. 2012, 2013). Based on this approach, QTLs for resistance to the fungus *Blumeria graminis* f. sp. *hordei*, assigned to barley chromosome arms 7HS, 7HL and 6HL were found to be associated with gene clusters including large numbers of NBS-LRR genes or protein kinase genes (Silvar et al. 2013). This leveraging of the high quality Brachypodium genome sequence and the well-characterized syntenic relationships to wheat and barley (TIBI, 2010) highlights the broad applicability to crop improvement programs at many levels, including to marker-assisted breeding and disease resistance gene discovery.

The power of exploring the Brachypodium genome directly to reveal novel and important insights into disease resistance was demonstrated in a study by Lucas et al. (2012). These authors screened the Bd21 genome for defence-associated genes and revealed such as Brachypodium orthologues of the wheat leaf rust *R* gene- *Lr10* (Bd2g39537) and the *Triticum dicoccoides* stripe rust resistance gene *Yr36*. Most interestingly, this study also investigated how microRNAs (miRNAs) could regulate *R* genes - in this case following infection with *Fusarium culmorum.* In this context, the NBS-LRR family gene, Bd1g34430 was of particular

interest, and was polymorphic among Brachypodium lines. Crucially, in lines where miRNA target sites were deleted, this correlated with increased Bd1g34430 gene expression during infection (Lucas et al. 2012). Interest in epigenetics in plants is rapidly expanding, as recognition that DNA sequence variation is not the only determinant of phenotypic variation has emerged (Law and Jacobsen, 2010). Small RNA-based post-translational modification are one type of epigenetic variation and besides the Lucas et al. (2012) study, Brachypodium has be used to show the roles of small RNAs in flowering (Wu et al. 2013) and drought (Bertolini et al. 2013). In an a exciting development, Wang et al. (2015) imposed a range of abiotic stress and noted the induction of novel, endogenous small interfering RNAs (stress-induced, UTR-derived siRNAs, or sutr-siRNAs) that originate from the 3' UTRs of a subset of coding genes. These could be acting by affecting mRNA transcript splicing or influencing interactions with *cis*-elements. However, he potential of Brachypodium for epigenetic studies is not limited to examining the role of small RNAs. Important initial epigenetic studies into epigenetic mechanisms based on DNA and histone methylation and histone acetylation have been published (Borowska et al. 2011; Wolny et al. 2014). As epigenetic effects includes being implicated in a range of plant-pathogen interactions (Latzel et al. 2012; Gijzen et al. 2014) we can expect these to feature in future studies of Brachypodium pathosystems.

In addition to the power of the annotated Brachypodium genome sequence for enhancing crop improvement through multiple angles as described above, the second and perhaps most potent use of Brachypodium is as a functional genomic tool to determine gene function, as has been advocated from the earliest Brachypodium publications (Draper et al. 2001; Hasterok et al. 2004). The availability of highly efficient *Agrobacterium*-mediated transformation and transgenic plant regeneration methods, and the emergence of additional genome resources such as T-DNA tagged mutant lines (Thole et al. 2011; Bragg et al. 2012) and TILLING populations (Dalmais et al. 2013) provide broad opportunities to do so. In a recent example, Goddard and co-workers (2014) used a T-DNA tagged Brachypodium line to demonstrate the conservation of gene function in disease resistance between Brachypodium and barley. Further, mutation of the gene encoding the brassinosteroid receptor BRI1 enhanced disease resistance to a wide range of necrotrophic and hemi-biotrophic fungal pathogens in Brachypodium, as it does in barley (Ali et al. 2014). These and the previously mentioned studies on FHB and rice blast resistance demonstrate that Brachypodium appears to frequently mimic the defence responses of its crop relatives, with orthologous genes involved. Given this, and the ease by which Brachypodium can be transformed compared to

its crop relatives such as wheat and barley, the potential of Brachypodium to rapidly investigate the roles of genes of interest to disease resistance before attempting comparable studies in the cereal hosts themselves is evident. Additional generic functional genomic tools can be integrated into such efforts; for instance that gene silencing based on the *Barley stripe mosaic virus* has been demonstrated in Brachypodium (Pacak et al. 2010). Virus-induced gene silencing (VIGS) was demonstrated for targeted genes in both roots and leaves. This approach offers the possibility of suppressing any gene at will, and as such represents an additional powerful functional genomics tool. In a complementary approach, the ease of Brachypodium transformation has proven useful in means to assess gene function or modify key aspects of the response, particularly since high throughput methods for its use have been developed (Yuan et al. 2011). Newer generation functional genomic tools such as TALENs and CRISPRs (Mahfouz et al. 2014) also provide methods to develop stable mutations in specific target genes, and when combined with the ease of Brachypodium transformation and regeneration, add even more power to assessing potential key genes associated with pathogen defence in a highly efficient manner compared to crop species themselves.

Given these resources and opportunities it may be questioned where Brachypodium could be best placed to make a distinctive contribution to plant-pathogen research. One area must certainly be in the area of non-host resistance based on which durable field resistance could be derived. Major gene resistance can be notoriously ephemeral under field conditions, so durable resistance is a major target of breeding programs (Ayliffe et al. 2008; Dangl, 2013). In this context, the non-host resistance of Brachypodium to a range of pathogens represents an opportunity to begin dissecting the molecular basis of non-host resistance, with a long term goal of introducing the genes involved into cereal crops to build a new layer of defence that will be long-lasting and function in a manner that complements native resistance. Although commonly thought to be regulated by complex genetics, Ayliffe et al. (2013) suggests that, at least in some instances, the genetic basis of variation in non-host resistance can be simple. Here, the ability of Brachypodium accession BdTr13K to form small lesions segregated as a single locus in crosses with Bd21. Progress toward the isolation of a single gene conferring variation in stem rust resistance is proceeding (Garvin, 2013). Indeed, it may be that the rare Brachypodium genotypes which exhibit significant susceptibility to a pathogen have incurred mutations in genes essential for non-host resistance. This may reveal the genetic basis of unusual relationships. For example, Ayliffe et al. (2013) found that in

some of the accessions studied, basal resistance to *P. striiformis* f. sp. *tritici* was inversely correlated with the relative degree of resistance to *P. graminis* f. sp. *tritici.*

A natural follow-on to such work, and, in view of the progress achieved in genome sequencing of other Brachypodium species, it should be possible to use Brachypodium-specialized pathogens such as *Puccinia brachypodii* to investigate the biological and molecular bases of host range. Barbieri et al. (2011) established *P. brachypodii* pathogenicity across the genus *Brachypodium*, especially on Brachypodium and on the perennial species *B. sylvaticum*. The results of their study suggested that *P. brachypodii* has co-evolved with the genus Brachypodium and seems not to have lost the ability to infect different species within it. These findings appear to contrast with the diversification of leaf rusts e.g. in the barley genus *Hordeum*. The closely related leaf rust fungi *P. hordei*, *P. hordei-murini*, and *P. hordei-bulbosi* are specialised and only pathogenic on their proper species (*H. vulgare/spontaneum*, *H. murinum*, *H. bulbosum*, respectively*)* (Barbieri et al. 2011). Moreover, the different levels of pathogenicity of *P. brachypodii vs*. Brachypodium and *B. sylvaticum* could be used to study differences in the co-adaptation of rust pathogens and hosts in annual *vs*. perennial species, where virulence could be "buffered" to survive through successive seasons.

The susceptibility of Brachypodium to many pathogens also provides a distinctive topic for plant pathology research. Our understanding of the role that effectors play in subverting plant defence and mobilizing host resources to the pathogens has been a major theme of the last decade (Chaudhari et al. 2014) even with necrotrophic pathogens (Wang et al. 2014). Although these effectors are delivered by different pathogens in different types of host interaction, some host components, such as plant hormone signaling cascades, may be commonly targeted (Kazan & Lyons, 2014). Therefore, the susceptibility to pathogens listed in **Table 2** could allow common susceptibility factors to be revealed. Such susceptibility factors could include WRKY-class transcription factors (Tripathi et al. 2012). Alleles of these targets that do not efficiently interact with the effectors may prove to be novel sources of decreased susceptibility. Examining mutagenized or targeted gene knockouts for altered susceptibility is well within reach with the current ensemble of functional genomic resources of Brachypodium.

It is hoped that this review has provided ample evidence of the power and potential of exploiting genome, epigenomic and functional genomic resources of Brachypodium to explore disease resistance, and how these findings have and will continue to be translated to crop improvement from many diverse angles. However, Brachypodium is also an ideal

platform for systems biology (Windram et al. 2014). Systems biology seeks to develop mathematical models to describe a system, but depends on accurate and comprehensive datasets. Within the context of plant pathology, transcriptomic systems models have been derived for Arabidopsis challenged with *Pseudomonas syringae* (Sato et al. 2010) and *Botrytis cinerea* (Windram et al. 2012). Given the current state of knowledge and infrastructure Brachypodium appears to be ripe for a systems biology approach. With the multiple pathosystems developed for Brachypodium, particularly interesting integrated multi-pathogen network models could be built for disease susceptibility and resistance, as a foundation for the Poaceae (Windram et al. 2014). A similar strategy has recently been demonstrated with interactions between potyviruses, the largest family of plant RNA viruses, and their host plants (Elena & Rodrigo, 2012). Here physical interactions between viral and plant proteins were used to derive computational models for susceptibility. Whilst developing such models for fungal pathogens would be difficult, the bioinformatics tools to target gene expression regulons have emerged; for example, WIGAMS, which describes co-regulated genes in time series expression studies (Polanski et al. 2014). It is conceivable that susceptibility models derived from transcriptomic studies could reveal both common and disease-specific regulons. These models could become powerful tools for predicting complex gene/allele combinations to be deployed for optimal effect in crop breeding programmes. Large-scale comparative transcriptome studies have already been undertaken in Brachypodium responses to abiotic stress (Priest et al. 2014), and it is highly likely that comparable research will be undertaken for responses to various pathogens. Such analyses would likely reveal novel insights into the transcriptional architecture of disease resistance, including new genes that may play previously unrecognized key roles in resistance, and which could be evaluated using the broad set of functional genomics tools in Brachypodium.

The vast majority of the research highlighted in this review has emerged from just over the last 5 years since the Brachypodium genome sequence was published (TIBI, 2010). We expect that rapid ongoing technological advances in omics research will continue to facilitate a large and impressive array of investigations of Brachypodium pathosystems, resulting in the identification and implementation of novel approaches to enhance disease resistance in the major cereal crops within the family Poaceae.

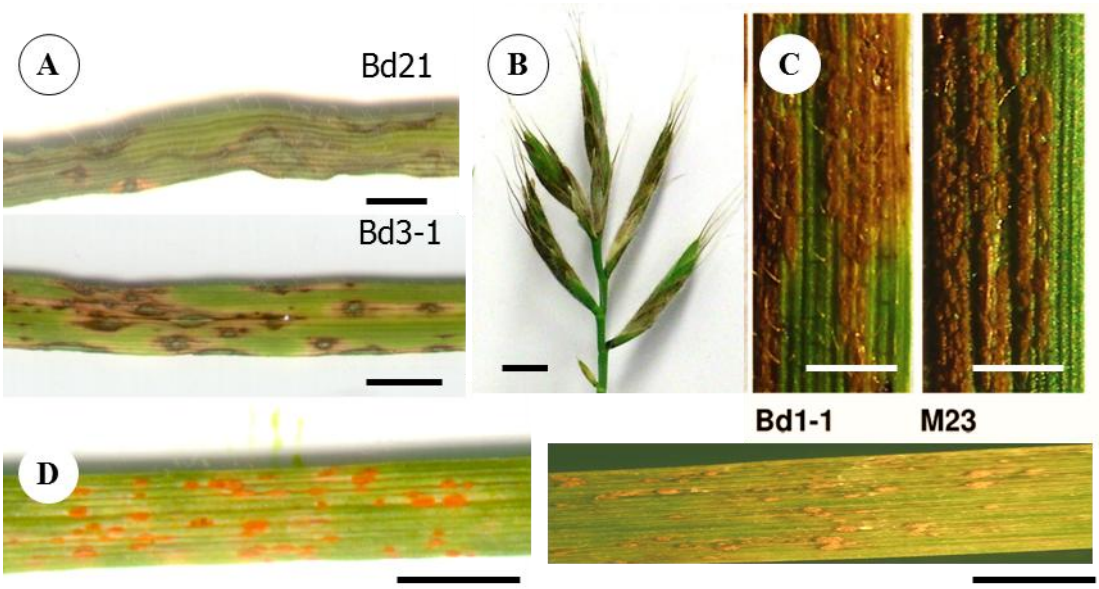

Figure 8. Disease symptoms in *B. distachyon* following inoculation with **(A)** *Magnaporthe grisea* Guy-11 **(B)** *Fusarium graminearum* **(C)** *Puccinia brachypodii* and **(D)** *Puccinia coronata*. Bar = 0.5 cm

Table 2: Model host-pathogen interactions involving *Brachypodium distachyon*.

Pathogen	Causal Disease	Reference
Magnaporthe grisea	Rice blast	(Routledge et al. 2004)
Fusarium graminearum	Fusarium head blight	(Peraldi et al. 2011)
Fusarium culmorum	Fusarium head blight	(Peraldi et al. 2011)
Oculimacula spp.	Eyespot	(Peraldi et al. 2014)
Ramularia collo-cygni	Ramularia leaf spot	(Peraldi et al. 2014)
Puccinia brachypodii	False brome rust	(Barbieri et al. 2012)
Bipolaris sorokiniana	Spot blotch and Common root rot	(Pogorelko et al. 2013)
Colletotrichum cereale	Anthracnose	(Sandoya and Bunafina, 2014)
Gaeumannomyces graminis var. *avenae*	Take-all	(Sandoya and Buanafina, 2014)
Magnaporthe poae	Summer patch	(Sandoya and Buanafina, 2014)
Ophiosphaerella agrostis	Dead spot	(Sandoya and Buanafina, 2014)
Ophiosphaerella korrae	Necrotic ring spot	(Sandoya and Buanafina, 2014)
Pythium aphanidermatum	Blight	(Sandoya and Buanafina, 2014)
Rhizoctonia solani	Sheath bBlight	(Sandoya and Buanafina, 2014)
Scleroctinia homeocarpa	Dollar spot	(Sandoya and Buanafina, 2014)
Ustilago bromivora	Smut	(Barbieri et al. 2012)
Cochliobolus heterostrophus	Corn leaf blight	(Falter and Voigt, 2014)

Fusarium sacchari	Sugarcane wilt	(Falter and Voigt, 2014)
Pithomyces chartarum	Leaf spot	(Falter and Voigt, 2014)
Pyrenophora teres	Net blotch of barley	(Falter and Voigt, 2014)
Stagonospora macropycnidia	Leaf spot of forage grasses	(Falter and Voigt, 2014)
Stagonospora nodorum	blotch (SNB)	(Falter and Voigt, 2014)
Stagonospora paspali	Leaf stripe disease	(Falter and Voigt, 2014)
Stagonospora tainanensis	Leaf blight of sugarcane	(Falter and Voigt, 2014)
Barley Stripe Mosaic Virus		(Cui et al. 2012)
Panicum Mosaic Virus	St. Augustine decline.	(Mandadi et al. 2012)
Partial susceptibility demonstrated in the following interactions		
Puccinia graminis f.sp*. Tritici*	Wheat Stem rust	(Figueroa et al. 2013)
Puccinia graminis f.sp*. Loli*		(Figueroa et al. 2013)
Puccinia graminis f.sp*. phlei-pratensis*	Timothy grass stemrRust	(Figueroa et al. 2013)

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Chapter 2

Aims of the work

2.1 General aims

The starting point of this work is reported in Barbieri et al. 2012. Three genomic regions for quantitative resistance to *P. brachypodii* were identified in a Bd3-1 × Bd1-1 cross population. Barbieri detected three QTLs on chromosomes Bd2, Bd3, and Bd4 respectively (**Figure 9**). According to Barbieri, Bd3-1 always contributed the resistance alleles for *Rpbq1* and *Rpbq2* and Bd1-1 the resistance allele for *Rpbq3*.

Rpbq2 and *Rpbq3* were validated by Vozabova et al. (unpublished data), indeed it was not possible to validate the *Rpbq1* QTL due to the lack of phenotypic data.

The overall aim of this investigation is to clarify the bases of quantitative resistances to the leaf rust pathogen *Puccinia brachypodii* in the model grass *Brachypodium distachyon* in order to exploit the biotic resistance in economically relevant temperate cereals using Brachypodium as a bridge for translational genomics.

2.2 Specific aims and outline of the thesis

The specific aims of the present study are the fine mapping of the two genomic regions associated to the leaf rust resistance in order to obtain a precise targeting of candidate genes towards the cloning of the two QTLs. A second aim of this thesis is to unravel and validate the Brachypodium-*P. Brachypodii* pathosystem.

In **Chapter 3** the fine mapping populations development is described and the phenotyping with the subsequent genotyping are presented in parallel with an observation of the behaviour of the inbred line Bd3-1 and Bd1-1 after the inoculation with the isolate of *Puccinia brachypodii Ki*, the isolate used for the fine mapping. For this experiment seeds of the core inbred lines Bd3-1 and Bd1-1 were obtained from 5 different germplasm collection: Unimore; Wageningen University; United States Department of Energy – Genome Joint Institute (DOE-GJI); United States Department of Agriculture - National Plant Germplasm System (USDA-NPGS) and University of Minnesota.

In **Chapter 4** the comprehensive evaluation of the Brachypodium-*P. Brachypodii* pathosystem is done by carrying out two parallel experiments. The first is the evaluation of the isolate-specificity of the resistance genes by performing inoculations on the developed fine mapping populations with other 4 different isolates of *Puccinia brachypodii.*

The second trial is an observation of host response to *Puccinia brachypodii* isolate *Kistápé* in fifty representative *Brachypodium distachyon* accessions collected around the world, to evaluate the plant-pathogen interaction within the species.

All phenotyping trials present on this work were done at Wageningen University under the supervising of Professor Rients E. Niks, associate professor of Plant Breeding.

In the final chapter (**Chapter 5**) is presented the general discussion together with the future perspective of the *Brachypodium distachyon* as a model plant for temperate cereals.

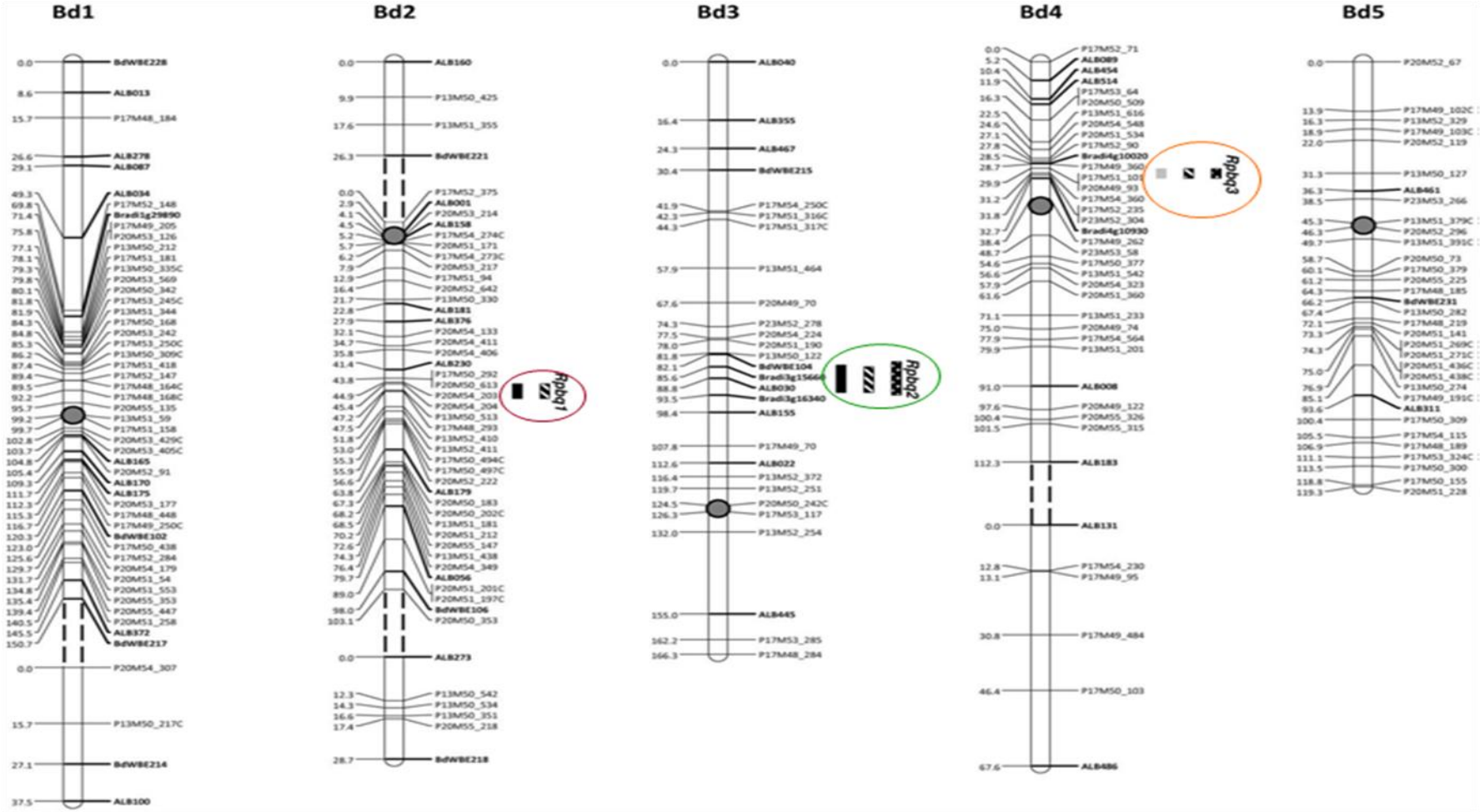


Figure 9. The Bd3-1 × Bd1-1 genetic linkage map. Short arms are located at the top. The circles in grey indicate the position of centromeres (Qi et al. 2010). On the left side map distances between markers (in Kosambi cM); marker and QTLs names are on the right. SSR and SNP anchor markers are in bold. QTL boxes are indicated as follows: F2A in solid gray; F2S in solid black; F3S1 in hatched; F3S2 in cross-hatched. On the right side of the boxes the QTL name is given. The QTL support intervals (DLOD 1.0) correspond to the length of a box.

Chapter 3

Fine Mapping of *Rpbq2* and *Rpbq3* QTLs conferring the resistance to the pathogen *Puccinia brachypodii*

3.1 Development of the fine mapping population

3.1.1 Materials and methods

3.1.1.1 Preparation of segregating materials and genotyping

One hundred and ten pseudo-RILs derived by single seed descent from an original cross between *Brachypodium distachyon* lines Bd3-1 (spring) x Bd1-1 (winter) were sown in the greenhouse of the Crop Production Group of the University of Modena and Reggio Emilia, Italy, in order to identify segregating plants. Bd3-1 and Bd1-1 originate from Iraq and Turkey respectively. Genomic DNA was extracted from young leaf tissue of plantlets harvested 20 days after sowing, using a CTAB-based protocol of Steward and Via (1993), adjusted for 96-well format. Polymerase chain reactions (PCR) of flanking marker haplotypes was performed for selection of Bd3-1 x Bd1-1 RILs, with the target QTL in a heterozygous state, while the other two QTLs were selected to be homozygous for the susceptible allele (**Table 1**).

Rpbq1 flanking markers genotyping

Two Brachypodium SSR markers (ALB230 and ALB179), shown to be polymorphic between Bd3-1 and Bd1-1 (Vogel et al. 2009), were used as flanking markers for *Rpbq1*. The nomenclature of SSR markers follows Vogel et al. (2009). Each forward primer r was tailed with an M13 sequence (5′-CACGACGTTGTAAAACGAC- 3′) labelled with a FAM or VIC fluorophore to allow labelling of the amplification products.PCR were carried out in a final volume 10 μL on an Applied Biosystems 2720 thermal cycler, as described in Vogel et al. (2009). Reactions were carried out in 1X DreamTaq® Green Buffer (Thermo Scientific), 1.5 mM of MgCl2 (Thermo Scientific), 0.25 mM of dNTPs, 0.5 U of DreamTaq® DNA polymerase (Thermo Scientific), 15 ng template genomic DNA, 2 μM marker specific reverse primer, 0.4 μM marker-specific M13- tailed forward primer and 1 μM of FAM-labelledor VIC-labelled M13 primer. The amplification was performed following a touchdown PCR protocol: first a denaturing step at 94 °C for 2 min, then a second denaturation at 94 °C for 30 s, an annealing temperature of 57 °C for 30 s and an elongation at 72 °C for 1 min, cycled 10

times and each time the annealing temperature decreases by 0.5 °C, 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, cycled 30 times with a final extension of 72 °C for 30 min. For the separation of the fragment and its analysis, μL of dye-labelled PCR products was added to a mix containing 0.4 μL of GeneScan 500 LIZ size standard (Applied Biosystems; standard for fragments with size between 35 and 500 bp) and 12μL of formamide. After denaturation (3′ at 95°C), a capillary electrophoresis was performed using the genetic analyzer ABI PRISM 310 (Applied Biosystems) and for the determination of the size of each fragment the software GeneMapper 4.0 was used.

Rpbq2 flanking markers genotyping

Two Brachypodium gene-derived markers, one dCAP (Bd3g15660) and one CAP (Bd3g16340) with the locus nomenclature adopted from Phytozome *Brachypodium distachyon* v3.1 and shown to be polymorphic between Bd3-1 and Bd1-1 (Barbieri et al. 2012) were used as flanking markers for *Rpbq2*.Amplification was performed using Applied Biosystems 2720 thermal cycler in a 15 μL final volume, containing 25 ng of genomic DNA, 1× Green GoTaq reaction buffer (Promega), 1.5mM of MgCl2, 0.5mM of each dNTP, 0.4 μM of each primer, and 0.5 U of GoTaq DNA polymerase (Promega). After an initial denaturing step at 94 °C for 2 min, the touchdown PCR profile was as follows: 94 °C for 30 s, 62 °C for 20 s, 72 °C for 30 s, cycled 10 times and each time the annealing temperature decreased by 0.5 °C, 94 °C for 30 s, 57 °C for 20 s, 72 °C for 30 s, cycled 25 times with a final extension of 72 °C for 10 min. PCR products were then separated on a standard 1.5% agarose gel. After PCR amplification, 5 μL of PCR products were digested following the manufacturer protocol (Thermo Scientific). The restriction enzymes used were DdeI (HpyF3I) for dCAP Bd3g15660, while BspMI (BveI) was used for the CAP marker Bd3g10340 (all the enzymes are product of Thermo Scientific); the visualization of the digestion products were visualized on standard 2% agarose gel.

Rpbq3 flanking markers genotyping

Two Brachypodium gene-derived markers CAP, (Bd4g10017 and Bd4g10710) with the locus nomenclature adopted from Phytozome *Brachypodium distachyon* v3.1 (http://phytozome.jgi.doe.gov/pz/portal.html) shown to be polymorphic between Bd3-1 and Bd1-1 (Bd4g10017 is from Barbieri et al. 2012 while Bd4g10710 is a brand new marker) and were used as flanking markers for *Rpbq3*. Amplification was performed in a 15 μL final volume, on an Applied Biosystems 2720 thermal cycler, containing 25 ng of genomic DNA, 1× Green GoTaq reaction buffer (Promega), 1.5 mM of MgCl2, 0.5 mM of each dNTP, 0.4 μM of each primer, and 0.5 U of GoTaq DNA polymerase (Promega). After an initial denaturing step at 94 °C for 2 min, the touchdown PCR profile was as follows: 94 °C for 30 s, 62 °C for 20 s, 72 °C for 30 s, cycled 10 times and each time the annealing temperature decreases by 0.5 °C, 94 °C for 30 s, 57 °C for 20 s, 72 °C for 30 s, cycled 25 times with a final extension of 72 °C for 10 min. Separation of the PCR products was performed on a standard 1.5% agarose gel. The Bd3-1 and Bd1-1 sequences were analyzed with the software Web Cutter 2.0 (http://rna.lundberg.gu.se/cutter2/) to find restriction site to be cut by an endonuclease in order to exploit size polymorphisms generated with this approach. After PCR amplification, 5 μL of PCR products were digested following the manufacturer protocol (Thermo Scientific),. The restriction enzymes used were BstuI (Bsh1236I) for Bd4g10017, while RsaI was used for the Bd4g10710 marker (all enzymes are products of Thermo Scientific); the digestion products were visualized on standard 2% agarose gel.

Table 1. Confirmation of the heterozygous RILs by flanking markers scoring. The two population harbour QTL *Rpbq1* in homozygous state, while for *Rpbq2* population plants harbouring susceptible alleles for *Rpbq3* were selected and vice versa for *Rpbq3* population

							Rpbq3										*Rpbq2*								
		Marker	Locus	Marker Type	enzyme	Class.	M007	M007	M007	M007	M007	M092	M092	M092	M092	M092	M092	M021	M021	M021	M061	M061	M061	M061	M061	M061
	1	ALB230		SSR-FAM	-	(a,b,h)	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
Rpbq1	2	ALB179		SSR-VIC	-	(a,b,h)	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
	3	Bd3g15660	Bradi3g15660	dCAPs	HpyF3I (DdeI)	(a,b,h)	b	b	b	b	b	b	b	b	b	b	b	h	h	h	h	h	h	h	h	h
Rpbq2	4	Bd3g16340	Bradi3g16340	CAPs	BveI (BspMI)	(a,b,h)	b	b	b	b	b	b	b	b	b	b	b	h	h	h	h	h	h	h	h	h
	5	Bd4g10020	Bradi4g10020	CAPs	Bsh1236I(BspMI)	(a,b,h)	h	h	h	h	h	h	h	h	h	h	h	a	a	a	a	a	a	a	a	a
Rpbq3	6	Bd4g10710	Bradi4g10710	CAPs	RsaI	(a,b,h)	h	h	h	h	h	h	h	h	h	h	h	a	a	a	a	a	a	a	a	a

3.1.1.2 Segregating populations development

3.1.1.2.1 Plant material

Two new large segregating RIL populations have been developed in the present research for each QTL separately to fine map *Rpbq2* and *Rpbq3*. These marker-selected heterozygous RILs have been selfed to obtain large segregating populations for each QTL. For *Rpbq3* and *Rpbq2* a first set of three and four F3 families were selected after genotyping, respectively. For *Rpbq3* two F3 families, M007 and M092, while two for *Rpbq2*, M021 and M061, were chosen for further analysis (**Table 2**). Twenty plants from each selected family/line were sown in a greenhouse, the growth conditions were 20h light/4h dark photoperiod, 24 °C during the day and 20 °C at night with cool-white fluorescent lighting at a level of 0,85 PAR μmol/m-2 s-1. Then genomic DNA has been extracted from fresh leaf tissue of each plant using a CTAB protocol (Steward and Via 1993) and was used to confirm that in each individual only the target QTL was heterozygous for the flanking markers. After genotyping, the plants were grown in appropriate conditions to set seed as described by Vogel et al. (2006), who reported that the winter inbred line Bd1-1 requires 6 weeks on 4 °C in order to fulfill vernalization requirement and induce flowering. Two new large populations of 2100 seeds for *Rpbq3* population and 920 for *Rpbq2* were obtained from selected plants. Groups of fifty of these seeds were sown in a Petri dish with a tissue paper containing 3 mL of bi-distillated water and covered in aluminium for 3-4 days and placed in the greenhouse in the same environmental conditions as described before. Germinated plants, 497 individuals for *Rpbq3* and 434 for *Rpbq2* (**Table3**) were genotyped for finding the heterozygous recombinants and the selected recombinant were grown following Vogel's indication to set seeds (Vogel et al 2006a).Genomic DNA samples from every individual of the two segregating populations were collected using the DNA extraction kit Wizard® Magnetic 96 DNA Plant System (Promega). For a practical reason plants has been renamed following a new nomenclature: L, followed by the chromosome number harbouring of the QTL of interest and then the progressive number of the plant.

Table 2. *Rpbq2* and *Rpbq3* families selected for further studies.

Rpbq3		***Rpbq2***	
M007	**M092**	**M021**	**M061**
5	24	40	56
8	27	47	57
10	32	49	58
16	35		60
19	37		62
	38		68

Table 3. Number of individuals for fine mapping segregating populations. In brackets the new nomenclature

Rpbq3		***Rpbq2***	
M007-8 (L401)	1	M021-40 (L301)	74
M007-19 (L402)	115	M021-49 (L302)	124
M092-24 (L403)	59	M061-58 (L303)	26
M092-27 (L404)	37	M061-62 (L304)	113
M092-32 (L405)	40	M061-68 (L305)	97
M092-37 (L406)	161		
M092-38 (L407)	84		
TOTAL	**497**	**TOTAL**	**434**

3.1.1.2.2 Molecular markers design

The whole genome sequences of the parental lines Bd3-1 and Bd1-1 were requested from John P. Vogel and Sean P. Gordon, United States Department Of Energy Joint Genome Institute, California. The regions of the two QTLs were screened to identify the sequence of every putative gene involved in the resistance using as reference the Brachypodium JBrowse v.1.0 (www.brachypodium.org - website and Jbrowse version not available anymore). As a result 75 sequences for *Rpbq2* and 78 for *Rpbq3* for both lines Bd3-1 and Bd1-1 were found.

Two regions of 560 kb for *Rpbq2* and 1026 kb for *Rpbq3* corresponding to 10 cM for *Rpbq2* and 4 cM for *Rpbq3* were screened to identify SNPs and InDels between the two parental lines.

An alignment of Bd3-1 and Bd1-1 sequences was done using the multiple sequence alignment program ClustalW2 (www.ebi.ac.uk/tools/msa/clustalw2 - tool nowadays replaced with ClustalOmega http://www.ebi.ac.uk/Tools/msa/clustalo/). Genes with physical distance around 100 kb from each other and containing SNPs and INDELs between Bd3-1 and Bd1-1were chosen. Ten SNPs (Single Nucleotide Polymorphism) based and 2STSs (Sequence Tagged Site) based molecular markers for *Rpbq2* and 12 SNPs based and 4 STSs based molecular markers for *Rpbq3* designed using the program Oligo Explorer (http://www.genelink.com/tools/gl-oe.asp) were designed and are listed in **Table 4** and **Table 5**.

Table 4. List of *Rpbq2* molecular marker used to obtain the fine mapping population. The primer type, the primer name, the genetic position of the gene for which the primer was designed, the name of the gene and the annotation of the protein family coded are listed.

Rpbq2					
		Gene Genomic Position (bp)			
Primer type	**Primer Name**			**Gene**	**Annotation**
SNP	Bd3g15660	13945922	13950387	Bradi3g15660	Leucine Rich Repeat, Protein kinase domain
SNP	Bd3g15661	13945922	13950387	Bradi3g15660	Leucine Rich Repeat, Protein kinase domain
SNP	Bd3g15770	14002885	14005150	Bradi3g15770	no functional annotations for this gene
STS	Bd3g15860	14095653	14096819	Bradi3g15860	Glutaredoxin
SNP	Bd3g16010	14203912	14205781	Bradi3g16010	Domain of unknown function (DUF1817)
SNP	Bd3g16020	14215701	14218152	Bradi3g16020	Plant protein of unknown function
SNP	Bd3g16140	14301088	14304259	Bradi3g16140	Protein of unknown function, DUF573
SNP	Bd3g16141	14301088	14304259	Bradi3g16140	Protein of unknown function, DUF573

SNP	Bd3g16192	14339149	14341683	Bradi3g16192	no functional annotations
STS	Bd3g16270	14426485	14428344	Bradi3g16270	DnaJ C terminal region; DnaJ C domain
SNP	Bd3g16340	14510617	14513786	Bradi3g16340	Myb-like DNA-binding domain
SNP	Bd3g16341	14510617	14513786	Bradi3g16340	Myb-like DNA-binding domain

Table 5. List of *Rpbq3* molecular marker used to obtain the fine mapping population. The primer type, the primer name, the genetic position of the gene for which the primer was designed, the name of the gene and the annotation of the protein family coded are listed.

		Rpbq3			
		Gene genomic Position			
Primer type	**Primer Name**	**(bp)**		**Gene**	**Annotation**
SNP	Bd4g10017	9588079	9591745	Bradi4g10017	NB-ARC domain
SNP	Bd4g10018	9588079	9591745	Bradi4g10017	NB-ARC domain
CAP	Bd4g10020	9588079	9591745	Bradi4g10017	NB-ARC domain
STS	Bd4g10105	9702968	9704882	Bradi4g10105	F-box domain
SNP	Bd4g10220	9839854	9842915	Bradi4g10220	NB-ARC domain
SNP	Bd4g10221	9839854	9842915	Bradi4g10220	NB-ARC domain
SNP	Bd4g10345	9940324	9941555	Bradi4g10345	F-box associated, F-box domain
SNP	Bd4g10350	9945977	9948813	Bradi4g10350	no functional annotations for this gene
STS	Bd4g10410	10013560	10014033	Bradi4g10410	no functional annotations for this gene
SNP	Bd4g10460	10156436	10157873	Bradi4g10460	GDSL-like Lipase/Acylhydrolase
SNP	Bd4g10461	10156436	10157873	Bradi4g10461	GDSL-like Lipase/Acylhydrolase
STS	Bd4g10520	10205999	10208752	Bradi4g10520	Protein kinase domain
SNP	Bd4g10640	10296567	10297395	Bradi4g10640	no functional annotations for this gene
STS	Bd4g10690	10406353	10407543	Bradi4g10690	no functional annotations for this gene
SNP	Bd4g10720	10439922	10442308	Bradi4g10720	no functional annotations for this gene
SNP	Bd4g10721	10439922	10442308	Bradi4g10720	no functional annotations for this gene
SNP	Bd4g10920	10609231	10611341	Bradi4g10920	Translationally controlled tumour protein
SNP	Bd4g10930	10612338	10614527	Bradi4g10930	WD domain, G-beta repeat
SNP	Bd4g10931	10612338	10614527	Bradi4g10930	WD domain, G-beta repeat

The STSs protocol

Amplification was performed on an Applied Biosystems 2720 thermal cycler in a 25 µL final volume. Four following different amplification profiles (**Table 6)** where used:

Profile 1) 2 ng of genomic DNA as template, 1X DreamTaq® Buffer including25mM of $MgCl_2$ (Thermo Scientific), 0.8 mM of the dNTP mix, 0.4 µM of each primer, and 1U of DreamTaq® DNA polymerase (Thermo Scientific). The amplification was performed with an

initial denaturing step at 95°C for 5 min, the denaturation at 95°C for 1 min, annealing at 55°C for 30 s, elongation at 72°C for 45 s, cycled 35 times, with a final extension of 72°C for 7 min.

Profile 2) 25 ng of genomic DNA as template, 1X DreamTaq® Buffer (Thermo Scientific) 1.5 mM of $MgCl_2$ (Thermo Scientific), 0.8 mM of the dNTP mix, 0.4 μM of each primer, and 1U of DreamTaq® DNA polymerase (Thermo Scientific) initial denaturing step at 95°C for 5 min, the denaturation at 95°C for 1 min, annealing at 57°C for 30 s, elongation at 72°C for 45 s, cycled 35 times, with a final extension of 72°C for 7 min.

Profile 3) 25 ng of genomic DNA as template, 1X DreamTaq® Buffer (Thermo Scientific) 1 mM of $MgCl_2$ (Thermo Scientific), 0.8 mM of the dNTP mix, 0.4 μM of each primer, and 1U of DreamTaq® DNA polymerase (Thermo Scientific) initial denaturing step at 95°C for 5 min, the denaturation at 95°C for 1 min, annealing at 59°C for 30 s, elongation at 72°C for 45 s, cycled 35 times, with a final extension of 72°C for 7 min.

Profile 4) Is a Touchdown PCR starting with25 ng of genomic DNA as template, 1X DreamTaq® Buffer (Thermo Scientific) 1 mM of $MgCl_2$ (Thermo Scientific), 0.8 mM of the dNTP mix, 0.4 μM of each primer, and 1U of DreamTaq® DNA polymerase (Thermo Scientific). The touchdown PCR profile is performed with an initial denaturing step at 94 °C for 2 min, 94 °C for 30 s, 66°C for 20 s, 72 °C for 30 s, cycled 10 times and each time the annealing temperature decreases by 0.5 °C, 94 °C for 30 s, 61 °C for 20 s, 72 °C for 30 s, cycled 25 times with a final extension of 72 °C for 10 min.

Amplicon size was estimated on a standard 2% agarose gel.

Table 6. List of the STS molecular markers used to obtain the fine mapping populations. The QTL name, the primer name, the name of the gene locus and the amplification information with the PCR profile are listed.

QTL	MARKER NAME	LOCUS NAME	PRIMER FW	PRIMER RV	Amplicon size (bp) Bd3-1	Amplicon size (bp) Bd1-1	Delta Amplicon	PCR Profile
Rpqb2	Bd3g15860	Bradi3g15860	TTCCAGGAGCAGCAGAG	AGTAGATCACCACTTTCCCC	136	148	12	3
Rpqb2	Bd3g16270	Bradi3g16270	ATGCAGACTAATTCAGATGTTC	AAAAAATTCAATGTCACACTG	133	153	20	1
Rpqb3	Bd4g10105	Bradi4g10105	GAAGAGCGTCTTGGTTGGTAAC	AGGACTTGGAAGTGCTCACAGA	133	147	14	2
Rpqb3	Bd4g10410	Bradi4g10410	GGCAAGTGTGTGACCATAC	CAACTGTTTGGACATCTTCC	165	186	21	1
Rpqb3	Bd4g10520	Bradi4g10520	TGAGAGGGAAATTAGTTGC	GGGTGTTGTTTCTCTTCTG	228	273	45	1
Rpqb3	Bd4g10690	Bradi4g10690	TGCTACAACGGTACTGAACGAC	GAACCCGAAGGAGTAGCCA	362	506	144	4

The SNPs protocol

SNPs detection was performe dusing the low/mid-multiplex SNP genotyping Fluidigm EP1 system™ .SNP type assays are allele-specific PCR assays that use three molecular markers to distinguish between two alleles; the allele specific primer 1 and 2 (ASP1 and ASP2 - forwardprimers) are labelled with FAM and HEX fluorochrome, while the locus specific primer (LSP - reverse primer) has no fluorochrome. The system is designed to genotype 192 samples using 24 assays in a single run; once in the well plate, the components are pressurized into the chip by an IFC Controller. All the components are then automatically combined into 4,608 parallel reactions.

As far as the preparation of the assays is regarded, sequences of at least 60 bp before and after the SNPs were chosen and sent to Fluidigm for the design of the custom SNP array. ASP1, ASP2 and LSP were designed by Fluidigm and used in the present study (**Table 7**).

a mixture containing 7.5 μM of SNPtype Assay ASP1 (Fluidigm),7.5 μM of SNPtype Assay ASP2 (Fluidigm), 20 μM of SNPtype Assay LSP (fluidigm) was added to 2 μL of 2x Assay Loading reagent (Fluidigm) in order to prepare the 10x Assay mix which was inserted in each of the 24 inlets present in the chip. For the sample mixture a total of60 ng of gDNA from each sample was added in an amplifcation mixture made of 2.25 μL of Biotium 2x Master Mix (Biotium), 0.225 μL of SNPtype 20X Sample Loading Reagent (Fluidigm), 0.075 μL of SNPtype Reagent (Fluidigm), 0.027 μL ROX (Invitrogen), 0.048 PCR certified water, and loaded into192.24 genotyping IFC (Integrated Fluidic circuit) and placed in IFC-Controller RX. Next step was the FC1™ Cycler, to thermal cycle IFCs. The thermal cycling condition were the following: after a hot start at 95 °C for 5 min, a touchdown PCR was performed with 95 °C for 15 s, 64 °C for 45 s, 72 °C for 15 s, cycled 4 times and each time the annealing temperature decreases by 1 °C, 95 °C for 15 s, 60 °C for 45 s, 72 °C for 15 s, cycled 34 times with a final cooling of 25 °C for 10 s.

The IFC is read by the EP1™ reader, an endpoint detection system and processed by an Analysis Software that gives a Cluster Plot and Call Map as a result.

Table 7. List of the SNP molecular markers used to obtain the fine mapping populations. The SNP name, the allele's polymorphism, the ASP1 allele, the ASP2 allele, the ASP1 marker sequence, ASP2 marker sequence, the LSP marker sequence are listed.

SNP_NAME	ALLELE	ASP1	ASP2	ASP1_SEQ	ASP2_SEQ	LSP_SEQ
Bradi3g15660	CT	C	T	AGGTACATCTTCTTCCATTATTTAGCGG	AAGGTACATCTTCTTCCATTATTTAGCGA	CCATGGCTTAAAGTATAAACGTGTATCAACA
Bradi3g15661	GT	G	T	ATGGGAGGCCTTTTTTGCTTG	ATAATGGGAGGCCTTTTTTGCTTT	GCTTACTATGAAATAACCAGTAAAAACAGGCAT
Bradi3g15770	AG	A	G	TCCTGTTAAACTGCGTGGACA	CCTGTTAAACTGCGTGGACG	CAAAGGACTTCCTCGACGGC
Bradi3g16010	TC	T	C	GTGCCAGCATTGCAGCA	TGCCAGCATTGCAGCG	CCCGTTGTGCTTGACTGGAG
Bradi3g16020	GC	G	C	GGAAACTCATGTCCTCCACGG	GAAACTCATGTCCTCCACGC	GGTTCACGCCCTTGAGCC
Bradi3g16140	GA	G	A	TTGTAGCCAGCAACAGCATTATTC	CTTGTAGCCAGCAACAGCATTATTT	CTGGGGACCCCGGTCA
Bradi3g16141	CT	C	T	TGCATAAGTTACATAGACACCACGG	ATGCATAAGTTACATAGACACCACGA	GGTAGCAGCGGTATCTAGGCA
Bradi3g16192	AG	A	G	TGTTTCCTTTGACTTCAAGACCCTAT	TGTTTCCTTTGACTTCAAGACCCTAC	TCTTAGAAAGTGAACACGATTTAGAACCTAATGAA
Bradi3g16340	AG	A	G	TCTGACAAGTGCTTTTCCATGGA	CTGACAAGTGCTTTTCCATGGG	CGTTCTCGAATCCGTCAGCC
Bradi3g16341	CT	C	T	GTTTAGTTAGCTCATTTGATCACCTGC	AGTTTAGTTAGCTCATTTGATCACCTGT	CCGATTTCAAACTTTCTCTAAGTCAAGCA
Bradi4g10017	CT	C	T	AGGTACATCTTCTTCCATTATTTAGCGG	AAGGTACATCTTCTTCCATTATTTAGCG	CCATGGCTTAAAGTATAAACGTGTATCAACA
Bradi4g10018	GT	G	T	ATGGGAGGCCTTTTTTGCTTG	ATAATGGGAGGCCTTTTTTGCTTT	GCTTACTATGAAATAACCAGTAAAAACAGGCAT
Bradi4g10220	GA	G	A	CCTGGAGAAGGGGCTTTCC	CCTGGAGAAGGGGCTTTCT	AGCTGCACCGGCTATGGA
Bradi4g10221	AC	A	C	CCAATTGTAAACCGCGGATTCTT	CCAATTGTAAACCGCGGATTCTG	CTTCCTTAATGACGCCCAGCA
Bradi4g10345	GA	G	A	GGCTCAGCAACCACGG	GGGCTCAGCAACCACGA	TCGCAGGACGCGTGGA
Bradi4g10350	CA	C	A	CAAGGTGTCCATCCTGCG	GCAAGGTGTCCATCCTGCT	CGGGACCAACCTGCCAG
Bradi4g10460	TG	T	G	TCCCGAGGTGGTGCTCT	CCCGAGGTGGTGCTCG	CCAAGGAGGAGGCACCGTA
Bradi4g10461	TC	T	C	AGTTGTAAACATGTGTGTACCAGGA	GTTGTAAACATGTGTGTACCAGGG	CACCGCCTTCTCCAAAATCGT
Bradi4g10640	TA	T	A	GAAATGCCGCAGCGCA	GAAATGCCGCAGCGCT	GCTGCGCCCATCTGAGAG
Bradi4g10720	GA	G	A	GGTTGCAAAGGTTAACCGGG	TGGTTGCAAAGGTTAACCGGA	CCTAGATATACAACAAAATATAGCAGTAGTAACA
Bradi4g10721	TC	T	C	CGCAAGAAAAGGCTCATCATCCTA	GCAAGAAAAGGCTCATCATCCTG	TGCACCGTTGCTAGATTATGTTTTTTGT
Bradi4g10920	AG	A	G	TCCTTGATTACAGAACGTGCACA	CCTTGATTACAGAACGTGCACG	CCACCCACTGCAAGCATTCA
Bradi4g10930	TC	T	C	CAGAGAAGACTCAGGTGTCAACAA	CAGAGAAGACTCAGGTGTCAACAG	CACTTGATTCTGTTTCTGTATGCAGTGAA
Bradi4g10931	TA	T	A	GTGCAATGGTAGTTTAAGTTTTGTCTGA	TGCAATGGTAGTTTAAGTTTTGTCTGAA	GAGGGCCACATTTTACACGCA

3.1.1.2.3 The linkage map construction

Chi-square tests ($\chi 2$) were performed on each SNP and STS markers in order to check the expected Mendelian segregation ratio 1:2:1 of each marker. The linkage groups were identified and the maps for *Rpbq2* and *Rpbq3*were designed using JoinMap 4.0 software (Van Ooijen 2006). A minimum LOD score of 4.0. ML (Maximum Likelihood) was used to determine the linkage groups and the order of the locus inside each linkage group. The Kosambi mapping function was used for the obtainment of the map distances starting from the recombination frequencies (**Table 8**, **Table 9**).

Table 8. The number of recombinants and the genetic distance for each marker interval within *Rpbq2*are reported.

	Rpbq2			
Marker interval	**N° recombinants Observed**	**N° of Genotypes**	**N° of Gametes**	**Genetic Distance cM**
Bd3g15661 - Bd3g15770	9	411	822	1,09
Bd3g15770 - Bd3g15860	10	411	822	1,22
Bd3g15860 - Bd3g16010	9	411	822	1,09
Bd3g16010 - Bd3g16020	0	411	822	0
Bd3g16020 - Bd3g16140	10	411	822	1,22
Bd3g16140 - Bd3g16141	0	411	822	0
Bd3g16141 - Bd3g16192	0	411	822	0
Bd3g16192 - Bd3g16270	3	411	822	0,36
Bd3g16270 - Bd3g16340	16	411	822	1,95

Table 9. The number of recombinants and the genetic distance for each marker interval within *Rpbq3* are reported.

	Rpbq3			
Marker interval	**N° recombinants Observed**	**N° of Genotypes**	**N° of Gametes**	**Genetic Distance cM**
Bd4g10017 - Bd4g10105	8	421	842	0,95
Bd4g10105 - Bd4g10345	23	421	842	2,73
Bd4g10345 - Bd4g10350	0	421	842	0
Bd4g10350 - Bd4g10410	2	421	842	0,24
Bd4g10410 - Bd4g10520	0	421	842	0
Bd4g10520 - Bd4g10640	15	421	842	2
Bd4g10640 - Bd4g10690	1	421	842	0,12
Bd4g10690 - Bd4g10720	0	421	842	0
Bd4g10720 - Bd4g10721	0	421	842	0
Bd4g10721 - Bd4g10920	0	421	842	0
Bd4g10920 - Bd4g10930	0	421	842	0
Bd4g10930 - Bd4g10931	0	421	842	0

3.1.2 Results

3.1.2.1 Fine mapping populations

A set of one hundred and ten pseudo-RILs derived from an existing mapping population (Barbieri et al, 2012), obtained by a single seed descent breeding method cross between a spring *Brachypodium distachyon* inbred lines Bd3-1 per a winter inbred Bd1-1 was used. The populations were genotyped using QTLs flanking markers to find plants with the target QTL in a heterozygous state, while the other two QTLs were in homozygous state for the susceptible allele.

The five known molecular markers used as flanking markers were all polymorphic between the two parental inbred lines Bd3-1 and Bd1-1. The brand new molecular marker, CAP Bd4g10710 was polymorphic between the two parental lines as well.

As a first result, F3 plants: M012, M021, M072 and M061, were chosen to become the progenitors of the segregating population for QTL *Rpbq2*; while for three F3 plants: M007, M092 and M023 were chosen for QTL *Rpbq3*. After an accurate analysis of the genotypic data and the status of the plant in the greenhouse compartment, plant M012 and M072 for *Rpbq2* and M023 for *Rpbq3* were discarded due to inconsistent in genotyping data.

The segregating population for *Rpbq2*, obtained from the seeds of the selected families was composed of 920 seeds, while the *Rpbq3* population numbered 2100 seeds. The final score of the seedling, germinated for Rpbq2 population, was 434 with a germinating ratio of 47.17%, while for the *Rpbq3* population the number of seedling germinated was 497, with a lower germinated ratio compared to the *Rpbq2* population of 23.67%.

3.1.2.2 Molecular markers pattern

A set of 12 molecular markers, 10 SNP-based and 2 STS-based, was used to amplify gDNA extracted from the 434 *Brachypodium distachyon* plants composing the *Rpbq2* segregant population. Most of the markers used had a clear pattern of the polymorphisms identified between the two inbred core lines used as parental.

As far as *Rpbq3* is regarded, a set of 16 molecular markers, 12 SNP-based and 4 STS-based was used to find heterozygous recombinant plants among the 497 individual of the fine

mapping population. Four SNP-based marker were found to be not polymorphic between the inbred lines Bd3-1 and Bd1-1; two of them were designed on gene Bradi4g10017, which is the upper flanking marker of the QTL, so they were replaced with the CAP molecular marker Bd4g10017 (Barbieri et al. 2012)described above in the *Rpbq3* flanking markers genotyping paragraph.

An example of an InDel exploited in the designing of STS markers and the related amplification product is shown in **Figure 1** and **Figure 2**.

```
3-1         TCCCGCGTGCAGGAGTTCCAGGAGCAGCAGAGGGCCGCGGCGCAGCGGCGGAGGAAGCAG 600
1-1         TCCCGCGTGCAGGAGTTCCAGGAGCAGCAGAGGGCCGCGGCGCAGCGGCGGAGGAAGCAG 600
            ************************************************************

3-1         CAAGAAGAGGAAGAAGAAGCCATGGCCATT------------GCGGAAGAAGAAGAATTC 648
1-1         CAAGAAGAGGAAGAAGAAGCCATGGCCATTGCCATGGCCATGGCGGAAGAAGAAGAATTC 660
            ****************************            ******************

3-1         CCGCCGCGCGAATCCCCAACGGGGGGGGAAAGTGGTGATCTACTTCACGAGCATCCGCGGC 708
1-1         CCGCCGCGCGAATCCCCAACGGGGGGGGAAAGTGGTGATCTACTTCACGAGCATCCGCGGC 720
            ************************************************************
```

Figure 1. The sequence of the Bd3g15860 marker used for the amplification of the *Rpbq2* population harbouring a deletion of 12 nucleotides in Bd3-1

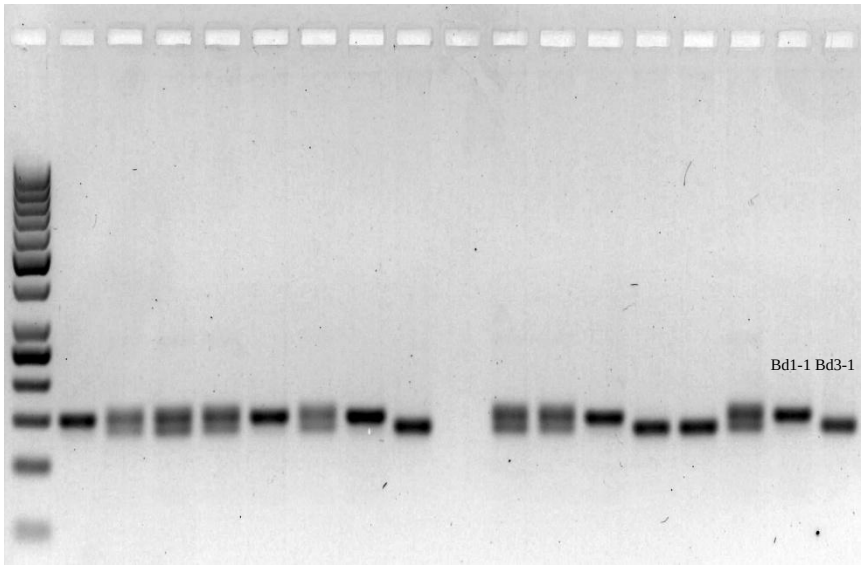


Figure 2. Amplification of the marker Bd3g15860. The upper band corresponds to Bd1-1 genotype, the lower band to Bd3-1 genotype and the double strand is the heterozygous.

An example of a SNP marker used for the discrimination of genotypes and the Fluidigm Ep1 System output are shown in **Figure 3** and **Figure 4,** respectively.

```
Bd3-1          CGAACACCTTAGGCGCAGGTCGCCGCCGTATCTCGCTCCACCCCCCGGTGCCTTTGGGGT 295
Bd1-1          CGAACACCTTAGGCGCAGGTCGCCGCCGTATCTCGCTCCACCCCCCGGTGCCTTTGGGGT 300
               ************************************************************

Bd3-1          GCACCGCTCCACCGCTGCGCCCATCTGAGAGGAAACGGCAAACGCCACCCCTCGCTGCGC 355
Bd1-1          GCACCGCTCCACCGCTGCGCCCATCTGAGAGGAAACGGCAAACGCCACCCCTCGCAGCGC 360
               ************************************************** *********

Bd3-1          TGCGGCATTTCTCCGAACATCTTTGTGGGTGCGGGCGCTCGTCTCGGGTTGGGCGCACCG 415
Bd1-1          TGCGGCATTTCTCCGAACATCTTTGTGGGTGCGGGCGCTCGTCTCGGGTTGGGCGCACCG 420
               ************************************************************
```

Figure 3. The sequence of the Bd3g16340 marker used for the amplification of the *Rpbq2* population. In red a thymin for Bd3-1 and an adenine for Bd1-1.

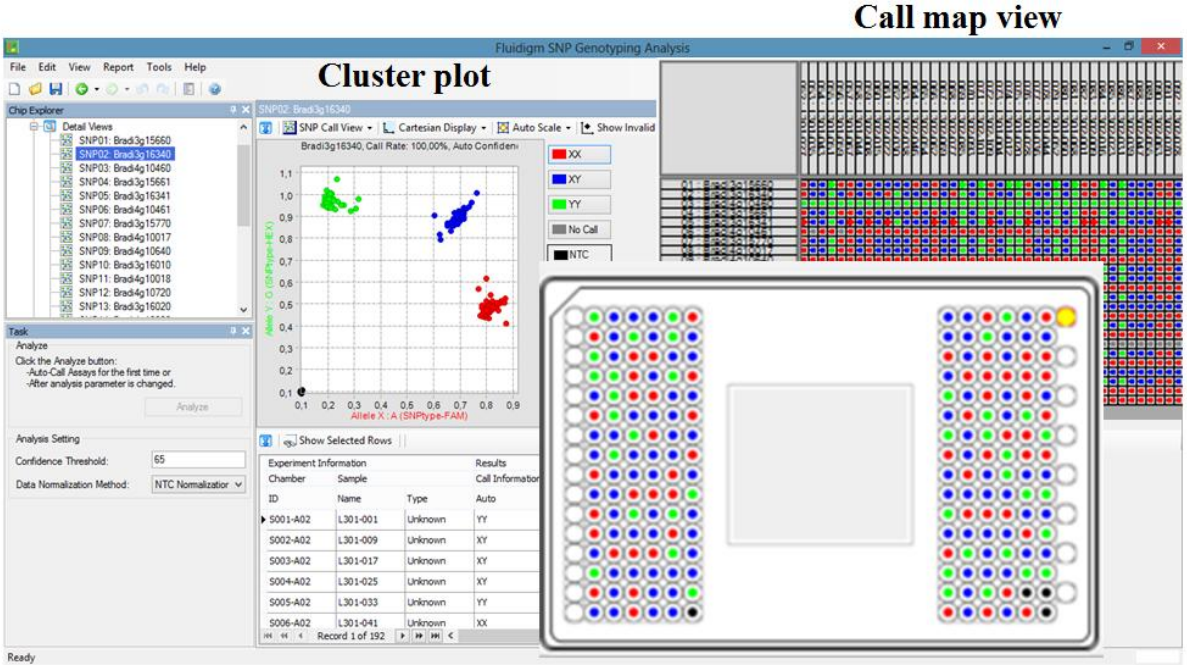


Figure 4. Output of the Fluidigm EP1 system: in the cluster plot all the amplification data are summarized. In blue the heterozygous, in red and in green the homozygous are clustered. In the call map view, data are ordered by the assay name and sample. In the chip disposition in yellow the assay and in black the NTCs samples are highlighted.

3.1.2.3 Genetic Maps

Rpbq2 map of recombinants was made after the exclusion of 23 from the 434 starting individuals for lacking of genotype data. The genetic segregation analyzed with the JoinMap 4.0 software (Van Ooijen 2006) in the *Rpbq2* population was done using 411 genotypes. Two markers Bd3g15660 and Bd3g16341 were discarded from the map analysis by the program due to lack of data. In order to obtain the genetic distance, Kosambi mapping function was used to convert the recombination frequencies into genetic distance Kosambi. All the markers were placed in the same linkage group, confirming the order of markers of the assembly of Brachypodium (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Bdistachyon). The map of recombinant for QTL *Rpbq2,* obtained using the maximum likelihood method, is shown in **Figure 5**.

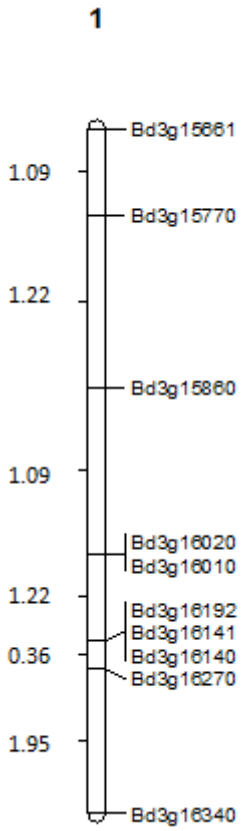


Figure 5. *Rpbq2* linkage map: all the markers are in the same linkage group and in the same order as on the Jbrowse of Brachypodium (https://phytozome.jgi.doe.gov/jbrowse/index.)

Ten markers were mapped spanning a total genetic length of 6.93 Kosambi cM. As no recombination events were observed between molecular markers Bd3g16010 and Bd3g16020 they were mapped at the same locus that correspond however to physical distance of 9920 bp as shown by genome sequence. Another lack of recombination events between markers Bd3g16140, Bd3g16141 and Bd3g16192 was pointed out. The first two markers, Bd3g16140 and Bd3g16141 were designed on the same gene Bradi3g16140, so another confirmation of the good quality of the map was given by this result. However the physical distance between Bd3g16140/Bd3g16141 and Bd3g16192 was34890 bp. The average distance between markers was1.155 Kosambi cM of (with the associated pair of markers not taken into account); the average was reduced to 0.693 cM of Kosambi taking account of the associated markers. The smallest gap between two markers was 0.36 cM between the group of associated markers composed of Bd3g16140, Bd3g16141 and Bd3g16192 and Bd3g16270; whereas the biggest gap was1.95 cM between Bd3g16270 and Bd3g16340. All the intervals were less than 2 cM and the 33.33% of the intervals were less than 1 cM.

As far as *Rpbq3* is regarded, the genetic map was made using 421 genotypes out of the 497 analyzed; 76 individuals were discarded due to lack of genotypic data. No pair of molecular markers was discarded from the JoinMap 4.0 software during the data analysis and as for *Rpbq2* data, all the pairs of marker were placed in the same linkage group and all the genetic frequencies were converted using the Kosambi mapping function in Kosambi cM.

The map of recombinant for QTL *Rpbq3* for resistance to *Puccinia brachypodii*, obtained using the maximum likelihood method, is shown in **Figure 6**.

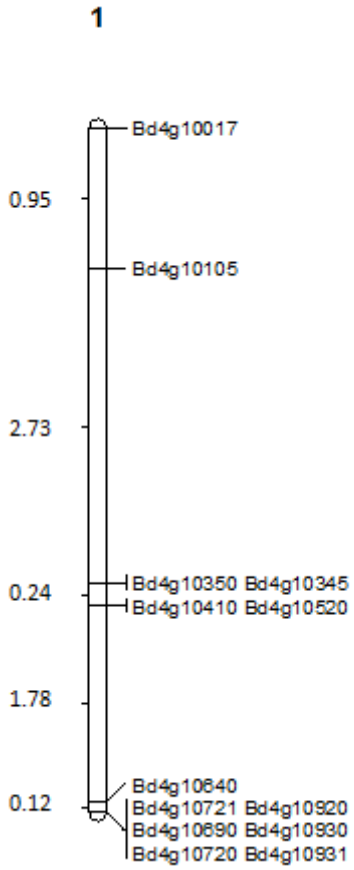


Figure 6. *Rpbq3* linkage map: all the markers are in the same linkage group and in the same order as on the Jbrowse of Brachypodium (https://phytozome.jgi.doe.gov/jbrowse/index.)

Rpbq3 linkage map was made elaborating data from 13 pairs of markers and its total genetic length was 5.82 Kosambi cM. Three lack of recombination events were found in the designing of *Rpbq3* map. The first was composed of markers Bd4g10345 and Bd4g10350 spanning a physical distance of4422 bp; while no recombination events were observed between molecular markers Bd4g10410 and Bd4g10520 and they were mapped at the same locus that correspond however to physical distance of191966 bp. Last group was composed of6 markers: Bd4g10690; Bd4g10720; Bd4g10721; Bd4g10920; Bd4g10930; Bd4g10931.Out of this 6 pairs, two couples were designed on the same genes (Bd4g10720/Bd4g10721 and Bd4g10930/Bd4g10931) and as for *Rpbq2* this was a proof of the high quality of the map. The physical distance between the two far ends of this association group was204795 bp. The average distance between pair of markers was1.164Kosambi cM, reduced to 0.447 Kosambi cM taking account of the marker without recombination events between each other. The

smallest gap between two markers was 0.12 cM between Bd4g10640 and the group of markers composed ofBd4g10690, Bd4g10720, Bd4g10721, Bd4g10920, Bd4g10930 and Bd4g10931; while the biggest gap observed was2.73 cM between Bd4g10105 and the group of marker pairs Bd4g10345 and Bd4g10350. There was only one interval with more than 2 cM and the 58.33% of the intervals were less than 0 Kosambi cM.

3.1.2.4 Novel Brachypodium distachyon fine mapping populations

Two novel populations were developed, one for each QTL respectively. The *Rpbq2* population was made of 11 families divided in 6 groups of recombination; while 10 families were developed for QTL *Rpbq3* and divided in 6 groups of recombination too. For every family the seeds were counted, a germination test was performed to understand the quality of the seeds and the germination rate was pointed out with the predicted number of seeds to sown in order to have at least 30 seedlings (**Table9**, **Table 10**).

Table 9. List of the *Rpbq2* family/lines developed. The primer name, the name of the line family/line with the haplotype, the number of seeds collected, the quality of the seeds harvested, the germination rate and the number of seeds to sown in order to obtain at least 30 seedling are listed.

	Rpbq2 families										
Marker	L301-059	L301-003	L305-059	L304-004	L302-025	L305-083	L301-001	L301-016	L305-067	L301-063	L301-062
Bd3g15661	a	a	h	b	h	h	h	h	a	h	h
Bd3g15770	a	a	h	b	h	h	h	h	a	h	h
Bd3g15860	a	a	h	b	h	h	h	h	a	h	h
Bd3g16010	h	h	a	h	h	h	h	h	a	h	h
Bd3g16020	h	h	a	h	h	h	h	h	a	h	h
Bd3g16140	h	h	a	h	a	a	b	b	a	h	h
Bd3g16141	h	h	a	h	a	a	b	b	a	h	h
Bd3g16192	h	h	a	h	a	a	b	b	a	h	h
Bd3g16270	h	h	a	h	a	a	b	b	h	b	a
Bd3g16340	h	h	a	a	a	a	b	h	h	b	a
N° of seeds	269	269	497	389	362	282	221	317	44	286	135
Quality of seeds	Ok	Very good	Very good	Ok	Very good	Very good	Very good	Very good	Ok	Very good	Ok
Germination rate (%)	100	100	50	66,7	50	16,7	100	100	50	66,7	100
N° of seeds to sow	40	40	80	65	80	180	40	40	80	65	40

Table 10. List of the *Rpbq3* family/lines developed. The primer name, the name of the line family/line with the haplotype, the number of seeds collected, the quality of the seeds harvested, the germination rate and the number of seeds to sown in order to obtain at least 30 seedling are listed.

	Rpbq3 families									
marker	L403-007	L407-021	L406-137	L402-079	L402-066	L404-002	L402-083	L402-069	L405-013	L406-041
Bd4g10017	h	h	h	b	a	a	h	h	b	h
Bd4g10105	h	h	h	b	a	a	h	h	b	h
Bd4g10345	a	a	b	b	a	a	h	h	b	h
Bd4g10350	a	a	b	b	a	a	h	h	b	h
Bd4g10410	a	a	b	h	a	a	h	h	b	h
Bd4g10520	a	a	b	h	a	a	h	h	b	h
Bd4g10640	a	a	b	h	h	h	b	a	h	h
Bd4g10690	a	a	b	h	h	h	b	a	h	b
Bd4g10720	a	a	b	h	h	h	b	a	h	b
Bd4g10721	a	a	b	h	h	h	b	a	h	b
Bd4g10920	a	a	b	h	h	h	b	a	h	b
Bd4g10930	a	a	b	h	h	h	b	a	h	b
Bd4g10931	a	a	b	h	h	h	b	a	h	b
N° of seeds	263	236	245	165	212	373	251	153	301	213
quality	Very good	Very good	Very good	Very good	Ok	Very good	Bad	Bad	Ok	Bad
Germination rate (%)	50	50	50	50	16,7	16,7	16,7	33,3	33,3	50
N° of seeds to sow	80	80	80	80	180	180	180	125	125	80

3.2 Bd3-1 vs. Bd1-1 inoculation tests

3.2.1 Materials and methods

3.2.1.1 Plant material

Brachypodium distachyon inbred lines Bd3-1 and Bd1-1 are known to show different symptoms after an inoculation with *Puccinia brachypodii* isolate *Kistápé*. The isolate was named "*Kistápé*" after the place in which it was collected in Hungary from *Brachypodium sylvaticum* (Barbieri et al. 2011a). Seeds of these two inbred lines from five different germplasm collections were used in order to better understand their response in seedling and adult stage inoculations. Seeds came from University of Modena and Reggio Emilia, Department of Life Science, Crop Production Group collection and were requested from: Rients E. Niks, Wageningen University, Plant Breeding Group; Margaret A. Gollnick, USDA-ARS Western Regional Plant Introduction Station & Seed Testing (WRPIS); David Garvin, University of Minnesota.

3.2.1.2 Pathogen multiplication

The same *Puccinia brachypodii* isolate used by Barbieri (et al. 2012)was used in the Bd3-1 vs. Bd1-1 inoculation experiments as well as in all the fine mapping trials described in following Paragraphs 3.3.1.4 and 3.3.2.3. Spores stored at -80 °C were multiplied to obtain fresh spores using the host *Brachypodium sylvaticum* (original host), and Bd21, Bd3-1 and Bd18-1 inbred lines of *Brachypodium distachyon*, which show high level of susceptibility to this rust species according to Barbieri et al, 2011(**Figure 7**, **Figure 8**). Plants were infected by spreading the inoculum (urediospores mixed with Lycopodium powder) on the leaves using a settling tower (Niks et al, 2011). The inoculated plants were incubated over night for 14 hours in a room at 100% relative humidity, to induce the germination of the spores, and then moved

to a specific greenhouse compartment. Ten-eleven days after infection, the spores were ready to be collected from the using a cyclone spore collector.

Figure 7. *B. distachyon* lines Bd 3-1, Bd21 and Bd18-1 and B. sylvaticum plants used for the rust multiplication

Figure 8. *B. sylvaticum* showing mature pustules of *P. brachypodii Ki*

3.2.1.3 Inoculation tests

The two inoculation tests were done at seedling stage, 21 days after sowing, and at adult stage, 35 days after sowing. The experiment was carried out in two independent replications. In every experiment 3 biological replicates from the inbred lines Bd3-1 and Bd1-1 from each of the 4different germplasm collection were transplanted, for a total of 24plants; last three spots were filled with the reference inbred line Bd21. Each replication was constituted by one tray (33.5 x 42 cm) containing 27 plants divided in 3 columns of 9 plants.

Twenty one days after sowing, one leaf per plant, were labelled, than fixed to the soil using pins with the adaxial sides faced up and all the new emerging leaves were cut to preserve an homogeneous distribution of the inoculum. The experiments were carried out with freshly collected spores, and on each inoculation box an average of 1.3 mg of *Puccinia brachypodii* isolate *Kistápé* spores were distributed. In addition, in order to homogenise the inoculum and have a uniform distribution of the spores over the leaves, the inoculation was performed in a settling tower and to the *Kistápé* spores, a ten-fold greater volume of Lycopodium spores was added (Niks et al 2011). A slide was added in every tray to check the quality and quantity of the spore with an optical microscope (**Figure 9)**. After an incubation overnight in a room having 100% of relative humidity, plants were moved to a specific greenhouse compartment with fixed temperature of 18°C. The observations began 10 days post inoculation, when the first pustules became mature and the number of pustules was counted everyday in a delimitated area of the leaf for a total of three days.

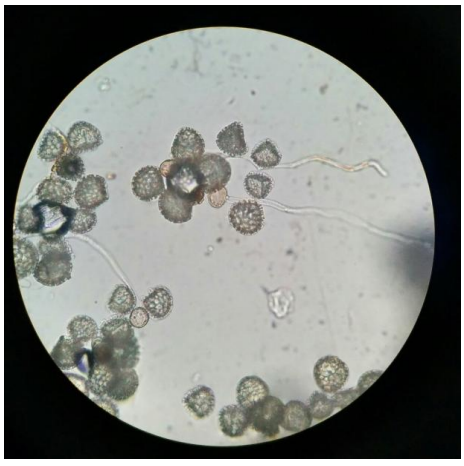


Figure 9. *P. brachypodii Ki* spores germination and germ tube growht are pointed out.

3.2.1.4Statistical analysis

All the data obtained were analyzed using Linear Mixed Model REML with GenStat 12.1 (VSN International Ltd. 2009, Hemel Hempstead, UK). The number of pustules was the Y-variate, the family/line was the Fixed Models, there were no Random Model due to the fact that the experiments were carried on using only one inoculation box. This method allowed to estimate missing values, make predicted means and calculate the Fisher Least Significant Difference at 5% (LSD). The correlations of the data obtained were performed using GenStat 12.1.

3.2.2 Results

The inbred line Bd3-1 and Bd1-1 infections

As expected *Brachypodium distachyon* inbred lines Bd3-1 and Bd1-1 leaves showed different symptoms as well as a different number of mature pustules after an inoculation with the isolate of *Puccinia brachypodii* isolate *Ki*. This trial was set in two replications with two different inoculation time points for each replication: the first at seedling stage (21 days after sowing) and the second at adult stage (35 days after sowing). The replications presented the same result for all replicates at both time points.

Line Bd1-1 has statistically significant average number of pustules lower than the Bd3-1. This data, analyzed using the Linear Mixed Model REML, were confirmed in all the days of observation and for both the growth stage in which the plants were inoculated. No statistical significant difference due to the origin of the line was observed. In most of the cases line Bd21 was the most susceptible. In **Tables** from **11** to **14** the association between the origin of the germplasm collection of each line with the average number of pustules and the Fisher LSD (P 0.05) is reported.

Table 11. Inoculation experiment A, seedling stage. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

EXPERIMENT A - SEEDLING STAGE INOCULATION						
	Number of pustules observed					
Line and Origin	Mean 1	LSD	Mean 2	LSD	Mean 3	LSD
Bd3-1 Unimore	20,33	c	45,33	b	78,67	b
Bd1-1 Unimore	4	a	13,33	a	18	a
Bd3-1 WUR	16,67	bc	47,33	b	75,67	b
Bd1-1 WUR	2,33	a	11,33	a	25,33	a
Bd3-1 WRPIS	14	b	39	b	78,33	b
Bd1-1 WRPIS	1,67	a	7,33	a	14,33	a
Bd3-1 UMN	15,33	bc	40,33	b	73	b
Bd1-1 UMN	1,33	a	9,67	a	14,67	a
Bd21	28	d	62,33	c	82	b

Table 12. Inoculation experiment A, adult stage. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

EXPERIMENT A - ADULT STAGE INOCULATION						
	Number of pustules observed					
Line and Origin	Mean 1	LSD	Mean 2	LSD	Mean 3	LSD
Bd3-1 Unimore	27,67	bc	79,67	c	90,67	b
Bd1-1 Unimore	1,67	a	12,33	a	12,66	a
Bd3-1 WUR	21	b	64,33	b	81,33	b
Bd1-1 WUR	3,67	a	9,33	a	14,33	a
Bd3-1 WRPIS	23	b	63,33	b	82,33	b
Bd1-1 WRPIS	1,33	a	7,33	a	7,66	a
Bd3-1 UMN	27,33	bc	73	bc	8,33	b
Bd1-1 UMN	2	a	6,67	a	7,66	a
Bd21	35	c	91	d	134	c

Table 13. Inoculation experiment B, seedling stage. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

EXPERIMENT B - SEEDLING STAGE INOCULATION						
	Number of pustules observed					
Line and Origin	Mean 1	LSD	Mean 2	LSD	Mean 3	LSD
Bd3-1 Unimore	8,66	b	29,67	b	55,67	bc
Bd1-1 Unimore	0,33	a	7	a	13	a
Bd3-1 WUR	11,66	bc	28,33	b	50,33	b
Bd1-1 WUR	2,66	a	8	a	11,33	a
Bd3-1 WRPIS	15,33	c	33,67	b	53,67	b
Bd1-1 WRPIS	2,66	a	6,33	a	11,67	a
Bd3-1 UMN	13	bc	28	b	48	b
Bd1-1 UMN	4	a	7	a	11,67	a
Bd21	22,33	d	45,33	c	67	c

Table 14. Inoculation experiment B, adult stage. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

REPLICATION B - ADULT STAGE INOCULATION						
	Number of pustules observed					
Line and Origin	Mean 1	LSD	Mean 2	LSD	Mean 3	LSD
Bd3-1 Unimore	19,67	b	60,67	b	94,33	b
Bd1-1 Unimore	1	a	7,67	a	20,33	a
Bd3-1 WUR	17,33	b	54	b	93,33	b
Bd1-1 WUR	1,33	a	9	a	16	a
Bd3-1 WRPIS	22	b	56,33	b	78,33	b
Bd1-1 WRPIS	3	a	8,33	a	17,33	a
Bd3-1 UMN	18	b	60,50	b	79,50	b
Bd1-1 UMN	2,67	a	9,67	a	15,67	a
Bd21	34	c	80,33	c	117,67	c

High correlation was found between the experiment and within each experiment (**Table 15**).

Table 15. Correlation calculated for data obtained in Bd3-1 vs. Bd1-1 inoculation experiments. P1-2-3 indicates the day of observation; A-S indicates adult or seedling stage; A-B indicates the experiment (P1AA stands for pustules scored in the first day of observation at adult stage of the experiment A).

Experiments	P1AA	P1AB	P1SA	P1SB	P2AA	P2AB	P2SA	P2SB	P3AA	P3AB	P3SA	P3SB
P1AA	-											
P1AB	0,89	-										
P1SA	0,92	0,87	-									
P1SB	0,82	0,86	0,83	-								
P2AA	0,97	0,92	0,91	0,82	-							
P2AB	0,94	0,96	0,89	0,83	0,96	-						
P2SA	0,92	0,88	0,94	0,80	0,93	0,90	-					
P2SB	0,89	0,88	0,90	0,94	0,90	0,87	0,86	-				
P3AA	0,95	0,96	0,92	0,86	0,97	0,97	0,94	0,91	-			
P3AB	0,92	0,95	0,92	0,83	0,96	0,98	0,94	0,89	0,97	-		
P3SA	0,92	0,85	0,87	0,78	0,94	0,89	0,93	0,88	0,91	0,91	-	
P3SB	0,93	0,90	0,94	0,88	0,94	0,93	0,91	0,95	0,94	0,95	0,90	-

Phenotypically there was a clear difference between a Bd1-1 inoculated leaf compared to a Bd3-1. Bd1-1 leaves presented a reaction with production of chlorotic haloes and necrotic flecks/stripes with the presence of a low number of mature pustules, while line Bd3-1 present a classical reaction of a host with an high amount of pustules on a green background (**Figure 10** and **Figure 11**).

Figure 10. Bd1-1 leaf inoculated at seedling stage (on the left). Bd3-1 leaf inoculated at seedling stage (on the right). The symptoms were recorded 10 days after infection with the pathogen *Puccinia brachypodii* isolate *Kistápé***.**

Figure 11. Bd1-1 leaf inoculated at seedling stage (on the left). Bd3-1 leaf inoculated at seedling stage (on the right). The symptoms were recorded 15 days after infection with the pathogen *Puccinia brachypodii* isolate *Kistápé.*

3.3 Phenotyping and genotyping

3.3.1 Materials and methods - first inoculation trials (2014)

3.3.1.1 Plant material

A population of 462 heterozygous recombinant plants, 42 individual for each of the 11 recombination families, for *Rpbq2* and 420 plants, 42 individual for each of the 10 recombination families, for *Rpbq3* were sown in plateau (7 x 12 pots with square shape and with side of 4 cm) in the greenhouse of the Laboratory of Plant Breeding of Wageningen University, The Netherlands, in autumn 2014 (**Figure12**). These families derived from a cross of *Brachypodium distachyon* lines Bd3-1 (spring) x Bd1-1 (winter) and come from the two large population described above. Genomic DNA samples from every individual of the two 11 heterozygous recombinant families for *Rpbq2* and 10 heterozygous recombinant families for *Rpbq3* were extracted using the DNA extraction kit Wizard® Magnetic 96 DNA Plant System (Promega).

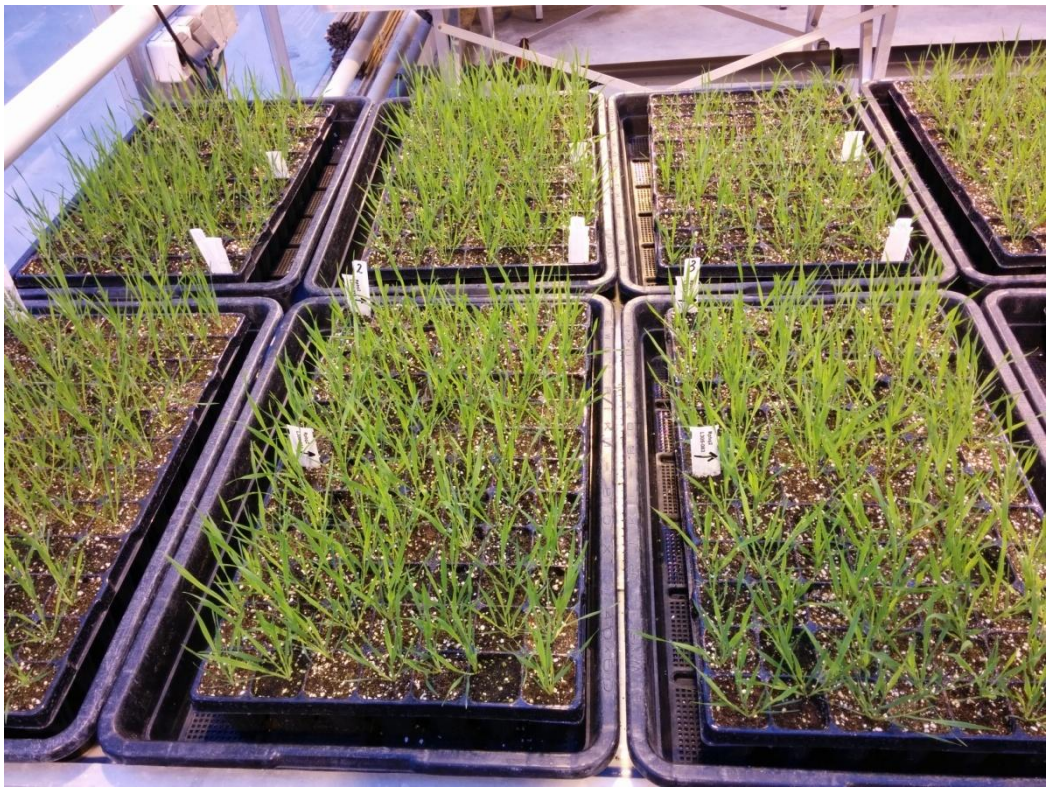


Figure 12. *Rpbq2* and *Rpbq3* heterozygous recombinant populations sown in the greenhouse of the Laboratory of Plant Breeding of Wageningen University, The Netherlands.

3.3.1.2 Pathogen multiplication

The same method described above in **Paragraph 3.2.1.2** was used for the isolate *Ki* multiplication in order to obtain fresh spores for the inoculation tests.

3.3.1.3 Genotyping of the heterozygous recombinants

In total, 643 genomic DNA were extracted, 326 and 317 from the heterozygous family of QTLs *Rpbq2* and *Rpbq3* respectively. For the genotyping the mid-throughput genotyping platform Fluidigm EP1 was used as described; three 192.24 genotyping IFC were loaded with 184 samples, 4 NTCs and 2 gDNA from the parental inbred line Bd3-1 and 2 gDNA of Bd1-1

each. After the genotyping, selected homozygous recombinants, with a segment of the QTL fixed with resistance alleles and the remaining interval fixes with the susceptible alleles, were transplanted in 8 trays for the inoculations.

3.3.1.4 Inoculation tests

The two inoculation tests were done at adult stage, 45 days after sowing. Each of the 8 trays (33.5 x 42 cm) contained 16 plants divided in 2 columns of 8 plants, exception for box number 4 of *Rbpq2* that contained 18 plants, 9 individuals per column (**Figure 13**). In every inoculation box 1 Bd3-1, 1 Bd1-1 inbred lines and at least 4 individuals with the same genotype of one of the parental line but with its own genetic background (from now on these plants will be called "homozygous") were placed as controls; the remaining spots were occupied by the plants to phenotype. In the four boxes for *Rpbq2,* 7 different haplotype recombinants were inoculated, (of them represented by only one plant), while for *Rpbq3* 6 different haplotypes were inoculated. For *Rpbq3* there were less homozygous recombinant plants compared to the number of *Rpbq2*, in fact for *Rpbq2* 34 recombinants, 24 homozygous were placed in 4 boxes; whereas for *Rpbq3* the number of recombinant tested was 31 with 25 homozygous. A total of 66 plants were inoculated for *Rpbq2*, while 64 were inoculated for *Rpbq3* trial. The inoculum density, incubation time and infection procedure were the same as described above in **Paragraph 3.2.1.3** with the difference that four leaves per plant, two couple of leaves per branch, forty five days after sowing were inoculated and the number of pustules was counted everyday in a delimitated area of the leaf for a total of two days.

Figure 13. Preparation of the inoculation trays. Inoculation trays before (left) and after (right) the inoculation procedure.

3.3.2 Materials and methods - Validation test (2015)

3.3.2.1 Plant material

A population of 147 homozygous recombinant plants for *Rpbq2*, 21 individual for each of the 7 haplotype and 126 plants, 21 individual for each of the 6 haplotype families of *Rpbq3*, 14 Bd3-1, 14 Bd1-1 and 14 homozygous for each parental line were sown in plateau (7 x 12 pots with square shape and with side of 4 cm) in the greenhouse of the Laboratory of Plant Breeding of Wageningen University, The Netherlands. This material was constituted by further generation of the plants genotyped and used in the previous inoculation test. Two weeks after sowing plants were transplanted in inoculation trays.

3.3.2.2 Pathogen multiplication

The pathogen isolate, infection procedure, inoculum density, incubation time and fresh spores collection were as described above in **Paragraph 3.2.1.2**with no differences.

3.3.2.3 Inoculation test

Four inoculation tests were performed, two at seedling stage (21 days after sowing), and two at adult stage (35 days after sowing). The 2 trays (33.5 x 42 cm) for *Rpbq2* disease tests contained 24 and 26 plants, while the 2 trays for *Rpbq3* contained 30 plants each (Figure g). In every inoculation box 2 Bd3-1, 2 Bd1-1 inbred lines and 3 individuals with the same genotype for each of the homozygous genotypes were placed as controls; the remaining 20 spots were used with the recombinant plants to phenotype. In the 4 boxes the same number of different haplotype as described before were inoculated. For *Rpbq2* 30 recombinants, 12 homozygous, 4 Bd3-1 and 4 Bd1-1 were transplanted in boxes; while for *Rpbq3* the number of recombinant tested was 40, 12 homozygous, 4 Bd3-1 and 4 Bd1-1. A total of 50 plants were inoculated for *Rpbq2*, while 60 were inoculated for *Rpbq3* trials. The inoculum density and incubation time were as described above in **Paragraph 3.2.1.3**; the infection procedure was slightly different with two distinct inoculations on one leaf infected for each plant. The first inoculation, at seedling stage, 21 days after sowing on the fifth leaves of the plants fixed to the soil using pins and with the adaxial side of the leaf faced up, the second, at adult stage, 35 days after sowing on the ninth or tenth leaf prepared to be inoculated (**Figure 14**). For the adult stage inoculation at first all the leaves that had shown rust symptoms were removed in order to re-inoculate the same plants in the same box used at seedling stage.

Figure 14. Preparation of the inoculation trays for seedling stage (left) and adult stage (right) inoculation .

3.3.2.4 Statistical analysis

The first two inoculation trials and all the four validation experimental data were analyzed using the Linear Mixed Model REML with GenStat 12.1 (VSN International Ltd. 2009, Hemel Hempstead, UK). The number of pustules was the Y-variate, the family/line were the Fixed Models and the Boxes were treated as the Random Model. The analysis of the experimental data using the REML method was performed for each day of observation (two days for every replication). This method allowed to estimate missing values, make predicted means and calculate the Fisher Least Significant Difference at 5% (LSD). Correlations between the first experiment and the subsequent validations were calculated using GenStat 12.1 too.

3.3.2.5 Genomic DNA extraction of plant material

The DNAs of two homozygous recombinant plants L302-025-01 and L301-001-11 carrying resistance alleles and of three homozygous susceptible recombinant plants L305-083-17, L305-083-20 and L305-083-22 to rust from QTL *Rpbq2*, together with the DNAs of seven homozygous recombinant plants: L403-007-01; L403-007-04; L403-007-05;L407-021-02; L407-021-03; L407-021-17 and L406-137-08 found to show resistance given by QTL *Rpbq3*, and DNA of L406-137-08 recombinant that showed high susceptibility, were extracted used the Wizard® Magnetic 96 DNA Plant System (Promega) kit. The DNAs of parental inbred lines Bd3-1 and Bd1-1 were extracted following the same procedure

3.3.2.6 Molecular marker design

For *Rpbq2*, in the interval between the flanking gene-based markers Bd3g16020 and Bd3g16140 five new CAPs molecular markers were designed using the alignment of sequences of Bd3-1 and Bd1-1 in order to find the location of the recombination point in the Brachypodium leaf rust pathogen resistant plants L302-025-01 and L301-001-11 and in the Brachypodium leaf rust pathogen susceptible plants L305-083-17, L305-083-20 and L305-083-22 (**Table 16**).

Molecular gene-based markers for QTL *Rpbq3* included five gene-based SSRs four of which were located respectively in four genes above the flanking marker Bd4g10017; one of them was located in the interval between the two flanking markers of this QTL such as Bd4g10017 and Bd4g10345. For further investigations seven new markers, 4 STS and 3 CAPs, were made on genes located in between the flanking markers (**Table 17**).

SNP, STS and SSRs identified were confirmed using the Jbrowse of Brachypodium V2.0 available on the website JGI Phytozome The Plant Genomic Resource. The nomenclature of these markers follows the one of this version of the Jbrowse (http://phytozome.jgi.doe.gov/pz/portal.html).

Table 16. List of *Rpbq2* molecular marker used to obtain the fine mapping. The primer type, the primer name, the genetic position of the gene for which the primer was designed, the name of the gene and the annotation of the protein family coded are listed.

Rpbq2					
Primer type	**Primer Name**	**Gene Genomic Position (bp)**		**Gene**	**Annotation**
CAP	Bd3g16040	14221835	14225383	Bradi3g16040	Sigma-70, region 4; Sigma-70, region 2; Sigma-70, region 3
CAP	Bd3g16060	14234125	14237588	Bradi3g16060	no functional annotations
CAP	Bd3g16091	14250658	14251473	Bradi3g16091	no functional annotations
CAP	Bd3g16110	14258351	14259939	Bradi3g16110	2OG-Fe(II) oxygenase superfamily
CAP	Bd3g16130	14290596	14294880	Bradi3g16130	ABC-2 type transporter; ABC transporter

Table 17. List of *Rpbq3* molecular marker used to obtain the fine mapping. The primer type, the primer name, the genetic position of the gene for which the primer was designed, the name of the gene and the annotation of the protein family coded are listed.

Rpbq3					
Primer type	**Primer Name**	**Gene Genomic Position (bp)**		**Gene**	**Annotation**
SSR	Bd4g09832	9314805	9315682	Bradi4g09832	no functional annotations
SSR	Bd4g09880	9401789	9402686	Bradi4g09880	no functional annotations
SSR	Bd4g09970	9540267	9549742	Bradi4g09970	SLIDE; SNF2 family N-terminal domain; HAND; Helicase conserved C-terminal domain
SSR	Bd4g09997	9618143	9623442	Bradi4g09997	Ribonucleases P/MRP protein subunit POP1; POPLD (NUC188) domain
STS	Bd4g10072	9720739	9722016	Bradi4g10072	no functional annotations
STS	Bd4g10158	9815711	9817518	Bradi4g10157	no functional annotations
CAP	Bd4g10230-E	9918754	9919614	Bradi4g10230	no functional annotations
STS	Bd4g10230-I	9918754	9919614	Bradi4g10230	no functional annotations
SSR	Bd4g10230-S	9918754	9919614	Bradi4g10230	no functional annotations
CAP	Bd4g10250	9938036	9944675	Bradi4g10250	Calponin homology (CH) domain; Kinesin (KAR3 subfamily)
STS	Bd4g10260	9944170	9948104	Bradi4g10260	F-box associated, F-box domain
CAP	Bd4g10327	9972516	9974242	Bradi4g10327	ubiquitin C

The CAP protocol

Amplification was performed on an Applied Biosystems 2720 thermal cycler in a 25 µL final volume containing: 2 ng of genomic DNA as template, 1X DreamTaq® Green Buffer (Thermo Scientific), 1.2 mM of $MgCl_2$ (Thermo Scientific), 0.8 mM of the dNTP mix, 0.4 µM of each primer, and 1U of DreamTaq® DNA polymerase (Thermo Scientific). Four different amplification profiles where used as follows:

Profile 1) initial denaturing step at 95°C for 5 min, the denaturation at 95°C for 1 min, annealing at 55°C for 30 s, elongation at 72°C for 45 s, cycled 35 times, with a final extension of 72°C for 7 min.

Profile 2) initial denaturing step at 95°C for 5 min, the denaturation at 95°C for 1 min, annealing at 60°C for 30 s, elongation at 72°C for 45 s, cycled 35 times, with a final extension of 72°C for 7 min.

Profile 3) initial denaturing step at 95°C for 5 min, the denaturation at 95°C for 1 min, annealing at 61°C for 30 s, elongation at 72°C for 45 s, cycled 30 times, with a final extension of 72°C for 7 min.

Profile 4) initial denaturing step at 95°C for 5 min, the denaturation at 95°C for 1 min, annealing at 57°C for 30 s, elongation at 72°C for 45 s, cycled 30 times, with a final extension of 72°C for 7 min.

After PCR amplification, 5 µL of PCR products were digested, following the manufacturer protocol (Thermo Scientific) and the visualization of the digestion products was done on standard 2% agarose gel. The restriction enzymes used were for QTL *Rpbq2*:

Bsh1236I (BstUI) for Bd3g16040; AluIfor Bd3g16060; BspMI (BveI) for Bd3g16091, HaeIII (BsuRI) for Bd3g16110 and XmiI (AccI) for Bd3g16130. Moreover for QTL *Rpbq3* were used BspmI for Bd4g10230; Tsp509I for Bd4g10250 and TaqI for Bd4g10327 (all the enzymes used are products of Thermo Scientific) (**Table 18**).

Table 18. List of CAP molecular markers used for *Rpbq2* and *Rpbq3*. The QTL name, the primer name, the name of the target gene locus, the sequences of the forward and reverse primers, the enzyme and the PCR profile used are listed.

QTL	MARKER	LOCUS	Sequence FW	Sequence RV	Enzyme	PCR profile
Rpqb2	Bd3g16040	Bradid3g16040	TCTGCATGAGAGGCTGATTG	CGTTGGCAACATTTTGATCC	Bsh1236I - BstUI	1
Rpqb2	Bd3g16060	Bradid3g16060	GGGGGAAATGGATAGATCAC	AGGAGAGGGAAGAAGAATGG	AluI	1
Rpqb2	Bd3g16091	Bradid3g16091	GTAGGGGAGCGTCAGCGAG	CAGTCGGCGACCCTAACCTC	BveI - BspmI	2
Rpqb2	Bd3g16110	Bradid3g16110	GGCAAAATCCAGTACAATGG	AAGATTGGCTGGTCATCAG	HaeIII - BsuRI	2
Rpqb2	Bd3g16130	Bradid3g16130	TGTACCACGACCACCAATAG	ATGACGGCTTCTTCTTGTTC	XmiI - AccI	1
Rpqb3	Bd4g10230-E	Bradid4g10230	GCGGTTCCCAAAGCAAATG	GCGACCAGTCGAGGAAGAGG	BspmI	3
Rpqb3	Bd4g10250	Bradid4g10240	TGCACTTCTGCAGTCTGTAC	CGAAGCAAAAGGAACTCAC	Tsp509I	1
Rpqb3	Bd4g10327	Bradid4g10326	GGTCCTCCCATTCTCAATG	AGTCCACCATCCTCCTTGT	TaqI	4

The SSR protocol

Five SSR were used only for the genotyping of the families that were carrying the resistance alleles of QTL *Rpbq3*. Each forward primer had an M13 tail (5′ - CACGACGTTGTAAAACGAC- 3′) to permit fluorescent labelling with either a FAM- or VIC-labelled M13 primer in the mixture for the amplification (**Table 19**). Polymerase chain reactions were carried out in a final volume of 10 μL on an Applied Biosystems 2720 thermal cycler. Reactions were carried out in 1X DreamTaq® Green Buffer (Thermo Scientific), 1.5 mM of $MgCl_2$ (Thermo Scientific), 0.25 mM of dNTPs, 1U of DreamTaq® DNA polymerase (Thermo Scientific), 15 ng template genomic DNA, 2 μM marker specific reverse primer, 0.4 μM marker-specific M13- tailed forward primer and 1 μM FAM-labelled M13 primer or VIC-labelled.

The amplification protocol followed a touchdown PCR method based on an initial denaturing step at 94 °C for 2 min, 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, cycled 10 times and each time the annealing temperature decreases by 0.5 °C, 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, cycled 30 times with a final extension of 72 °C for 30 min.

For the separation of the fragment and its analysis,2 μL of dye-labelled PCR products was added to a mix containing 0.4 μL of GeneScan 500 LIZ size standard (Applied Biosystems; standard for fragments with size between 35 and 500 bp) and 12 μL of formamide. After denaturation (3′ at 95°C), a capillary electrophoresis was performed using the genetic analyzer ABI PRISM 310 (Applied Biosystems) and for the determination of the size of each fragment the software GeneMapper 4.0 was used.

Table 19. List of SSR molecular markers used for *Rpbq3*. The primer name, the name of the target gene locus and the sequences of the forward and reverse primers are listed.

MARKER	LOCUS	Sequence FW	Sequence RV
Bd4g09830	Bradi4g09830	CACGACGTTGTAAAACGACACGCCTTGCTCTTGTTGTA	CGGATCCAGATTAGTTTGAG
Bd4g09880	Bradi4g09880	CACGACGTTGTAAAACGACATTCCAAAACCATAGAAACA	ATTCCAAAACCATAGAAACA
Bd4g09970	Bradi4g09970	CACGACGTTGTAAAACGACATCTACATCCGCACGAAATT	ACGAGGTGTTGTTTTTCCC
Bd4g09997	Bradi4g09997	CACGACGTTGTAAAACGACTCTAGGAAACTCCATTTGCC	CATGTAGCGGTGTGAAAGC
Bd4g10230	Bradi4g10230	CACGACGTTGTAAAACGACCGTGTTTCCTTTCCGCTTAC	AGCAGGGTCCTCGTCATTC

The STS protocol

STS were used only for QTL *Rpbq3.* Amplification was performed on an Applied Biosystems 2720 thermal cycler in a 25 μL final volume containing: 25 ng of genomic DNA as template, 1X DreamTaq® Green Buffer including 20 mM of $MgCl_2$ (Thermo Scientific), 0.8 mM of dNTPs, 0.4 μM of each primer, and 1U of DreamTaq® DNA polymerase (Thermo Scientific). The amplification profiles used were differed each other only for the temperature of annealing, indicated in table 6. The polymerase chain reaction started with a denaturing step at 95°C for 5 min, followed by a denaturation at 95°C for 1 min, an annealing at 55-61 °C (**Table 20**) for 30 s, elongation at 72°C for 45 s, cycled 30 times, with a final extension of 72°C for 7 min. In the end PCR products were separated on a 1.5% agarose standard gel

Table 20. List of STS molecular markers used for *Rpbq3*. The primer name, the name of the target gene locus, the sequences of the forward and reverse primers and the annealing temperatures are listed.

MARKER	LOCUS	Sequence FW	Sequence RV	Annealing temperature
Bd4g10072	Bradi4g10072	TCGTTGTACCAGACACATTC	GACGAGTACGTGAAGAAGTG	55°C
Bd4g10230-I	Bradi4g10230	TGCACTTCTGCAGTCTGTAC	CTCGGCTTCTTCCTCCAGG	61°C
Bd4g10260	Bradi4g10260	GTGGCGAGTCAAATGTAACG	CGGCAAGGGTACTACGATG	57°C
Bd4g10157	Bradi4g10157	CCGACTGTACCGAGCATTAG	CCTGGATACGGGTCAACTG	57°C

3.3.2.7 Bioinformatic search for candidate genes

The first step for searching the candidate genes for the resistance to *Puccinia brachypodii* in the QTLs *Rpbq2* and *Rpbq3* of the false brome grass was to align the sequences of the parental lines Bd3-1 and Bd1-1 for the 75 genes of the QTL *Rpbq2* and 78 of the QTL *Rpbq3* interval exploiting the Brachypodium Jbrowser of phytozome.net (version 1.0 - not available anymore) SNPs and InDels were identified. A functional protein analysis based on the translation of coding sequences into amino acid sequences using the ExPASy Proteomics Server Translate Tool (http://expasy.org/translate) was performed for every gene of the two QTLs in Bd3-1 and Bd1-1 using NCBI Conserved Domain Database (www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi) in order to identify the polymorphisms inside protein functional domains.

3.3.2.8 Analysis of synteny between Brachypodium distachyon and temperate cereals

For finding conserved regions between the model plant *Brachypodium distachyon* and some of the temperate cereals a syntenic analysis of the genes of the QTLs *Rpbq2* and *Rpbq3* has been done by confronting all the annotations for each gene of the two QTLs.

Syntenic genes in *Oryza sativa* (L.) and *Sorghum vulgare* (L.) were found using the protein homologs database (https://phytozome.jgi.doe.gov/pz/portal.html) with an all-against-all Smith-Waterman alignment available on phytozome.net. Syntenic genes were chosen on the basis of four parameters: Most Recent Shared Family (MRSF), score, percent similarity and annotation.

As far as *Hordeum vulgare* (L.) is regarded, a tblastx between the coding sequences of the QTLs genes and the MIPS's barley database (www.mips.helmholtz-muenchen.de.) was performed for a precise targeting of syntenic genes in barley. The parameters followed for the confirmation of syntenic genes were e-value and similarity. The genome zipper and the Comparative Genome Map Viewer tools (www.mips.helmholtz-muenchen.de.) were used to obtain additional data on *Brachypodium distachyon* and *Hordeum vulgare* (L.).

3.4 Results

3.4.1 Homozygous recombinant plants

To fine map the two QTL conferring resistance to the leaf rust pathogen *Puccinia brachypodii* from a population of 462 F4 recombinant plants for *Rpbq2* (11 families) and 420 for *Rpbq3* (10 families), 326 and 317 gDNA samples were extracted respectively. Two hundred and seventy five and two hundred and fifty samples were genotyped for *Rpbq2* and for *Rpbq3*, respectively to choose homozygous family lines.

From the eleven recombinant families for *Rpbq2*, a total of 34 F4 homozygous recombinant plants were inoculated. The individuals were divided as follows: 3 recombinant individuals for L305-067 family; 3 recombinants for L301-063; 1 recombinant for L301-001; 3 for L301-016; 2 for L301-059; 4 for L301-003; 3 for L305-059; 3 for L304-004; 3 for L302-025; 3 for L305-083 and 6 for L301-062.

From the ten recombinant families for *Rpbq3*, a total of 31 homozygous recombinant individuals were used for the inoculation test. The inoculated plants were divided as follows: 4 recombinants for L406-041; 3 for L404-002; 3 for L402-083; 3 for L406-137, 3 for L407-021; 3 for L403-007; 6 for L402-079, 3 for L405-013 and 3 for L402-069. Family L406-034 was discarded due to inconsistent in genotypic data between this round of genotyping and the previous one.

3.4.2 Inoculation tests (2014)

In 2014 the first two inoculation test at adult stage were performed, one for each QTL respectively. In both the experiments the inbred line Bd3-1 showed a higher level of susceptibility than Bd1-1, as indicated by the level of sporulation (**Figure 15**). Reactions were observed in line Bd1-1 with the presence of necrotic flecks/stripes and a little chlorotic haloes, while none of this was observed in line Bd3-1.

Figure 15. Leaf of Bd1-1 (left) showing necrotic stripes and flecks and few mature pustules; leaf of Bd3-1 (right) showing a high number of mature pustules without any other reaction.

For *Rpbq2* inoculation test, the level of infection ranged between the families/lines from resistant (from 10 to 45 pustules and black flecks on the leaf) to fully susceptible (from 90 to 100 pustules without any other symptom) on the first day of observation (tenth day after inoculation); an example of the less and most infected family/lines L301-062 with an average of 8.17 and L305-059 with an average of 100.83, respectively, is shown in **Figure 16**. Reactions related to interaction with the pathogen were found in some homozygous recombinant lines, like L302-025; this phenotype was a chlorotic halo of different size, from small to extensive spots and also the number and shape of the necrotic flecks was different between family/lines. On the second day of observation the number of the pustules was between 36.2 of line L301-062 and 194.2 of line L305-083, the phenotypes observed were in accordance with the previous day.

For *Rpbq3* inoculation test, the level of infection was in accordance with the one saw for the other QTL. Some resistant plants were found with less than 5 pustules/leaf (the minimum score was of family/line L403-007 with 2.33), while some lines were fully susceptible with sixty pustules/leaf observed (the maximum score of 68.12 registered for family/line L404-002). In this experiment the resistant plants showed black flecks of different

size and shape and sometimes the flecks were surrounded by a little chlorotic halo. The first day of counting of the pustules was the tenth after inoculation and in the second day of observation the most resistant family/line was L403-007 with an average of pustules of 3.33; whereas L404-002 was confirmed as the most susceptible with an average score of 106.25.

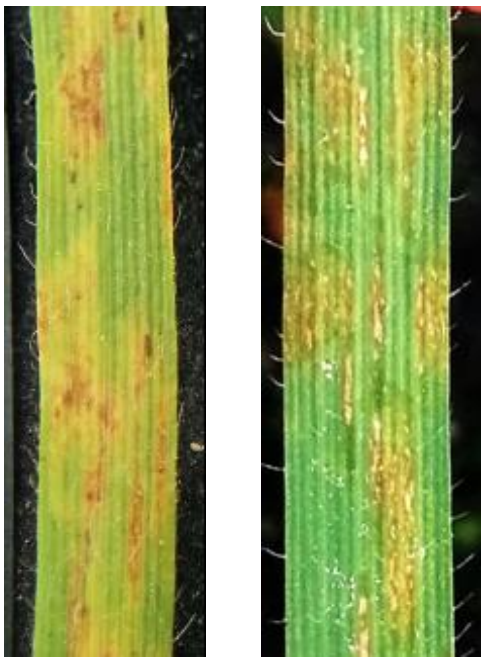


Figure 16. Leaf of L302-025 showing necrotic flecks, chlorotic halos and few mature pustules (left); leaf of L305-059 showing a high number of mature pustules without any other reaction (right).

REML data from disease evaluations confirmed quantitative segregation, suggesting polygenic inheritance of the resistance (Barbieri et al. 2012). Due to inconsistent in phenotypic data box 4 was not further analysed, moreover line L301-001-11 as excluded because it was represented only by one plant. Seeds from L301-001-11 were collected and used for the validation tests. For *Rpbq2* **Table 21** shows the association between the haplotype of every line with the mean of every day of observation and the Fisher's LSD (*P 0.05*), while the distribution of the mean of the number of pustules for every family/line is shown in **Figure 17**.

Table 21. *Rpbq2* **i**noculation experiment. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

Line	bradi3g15660	bradi3g15661	bradi3g15770	bradi3g16010	bradi3g16020	bradi3g16140	bradi3g16141	bradi3g16192	Bradi3g16270	bradi3g16340	bradi3g16341	Mean 1	DMS	Mean 2	DMS
L305-059	b	b	b	a	a	a	a	a	a	a	a	100,83	c	181,2	d
L304-004	b	b	b	a	a	a	a	a	a	a	a	83,17	c	190,8	d
L305-083	b	b	b	b	b	a	a	a	a	a	a	91,25	c	194,2	d
L302-025	b	b	b	b	b	a	a	a	a	a	a	45,83	b	120,3	c
L301-062	b	b	b	b	b	b	b	b	a	a	a	8,19	a	36,2	a
L301-059	a	a	a	b	b	b	b	b	b	b	b	23,38	ab	108	bc
L301-003	a	a	a	b	b	b	b	b	b	b	b	20,87	ab	70,7	bc
L301-016	a	a	a	a	b	b	b	b	b	b	b	19,25	ab	50,4	a

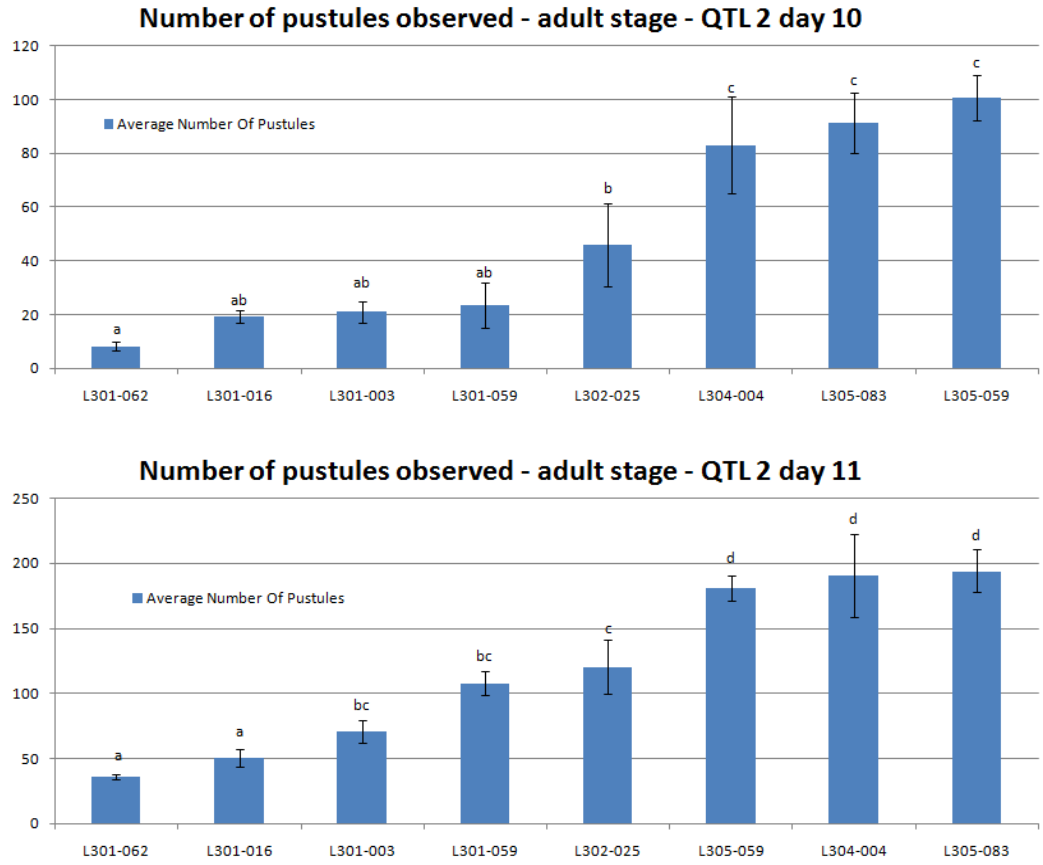
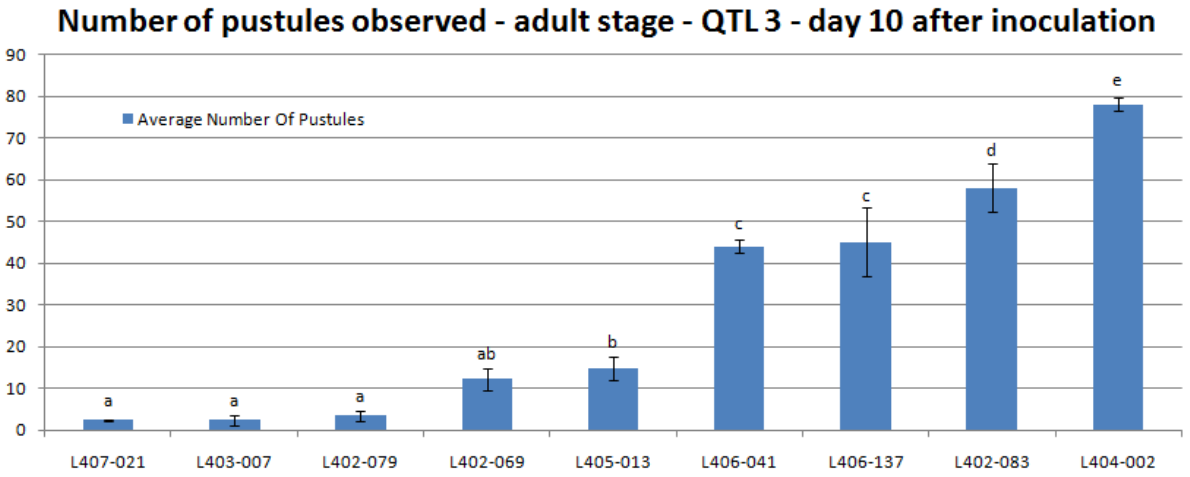


Figure 17. Mean number of pustules for the first day of observation (above) and the second day of observation (below) for every family/line together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported for *Rpbq2*.

As far as *Rpbq3* in regarded, **Table 22** show the association between the haplotype of every line with the mean of every day of observation and the LSD, while the distribution of the mean of the number of pustules for every family/line is shown in **Figure 18**.

Table 22. *Rpbq3* **i**noculation experiment. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

Line	Bradi4g10017	Bradi4g10105	Bradi4g10157	Bradi4g10230	Bradi4g10250	Bradi4g10260	Bradi4g10345	Bradi4g10350	Bradi4g10640	Bradi4g10720	Bradi4g10721	Bradi4g10920	Bradi4g10930	Bradi4g10931	Mean 1	DMS	Mean 2	DMS
L403-007	b	b	b	a	a	a	a	a	a	a	a	a	a	a	2,33	a	3,42	a
L407-021	b	b	b	b	b	b	a	a	a	a	a	a	a	a	2,33	a	3,25	a
L405-013	b	b	b	b	b	b	a	a	a	a	a	a	a	a	14,67	b	22,75	b
L402-079	b	b	b	b	b	b	b	b	a	a	a	a	a	a	3,38	a	5,38	a
L402-069	b	b	b	b	b	b	b	b	a	a	a	a	a	a	12,25	ab	25,75	b
L402-083	a	a	a	a	a	a	b	b	b	b	b	b	b	b	58,08	d	99,17	cd
L406-137	a	a	a	a	a	a	b	b	b	b	b	b	b	b	45,08	c	92,5	c
L404-002	a	a	a	a	a	a	a	a	b	b	b	b	b	b	78,17	d	113,83	d
L406-041	a	a	a	a	a	a	a	a	a	b	b	b	b	b	44,06	c	88,19	c



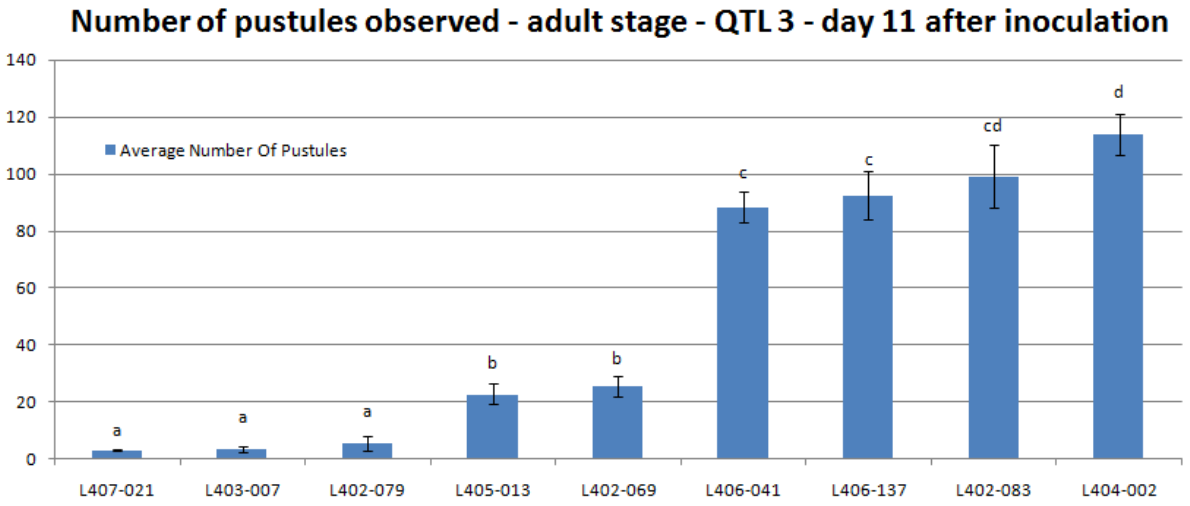


Figure 18. Mean number of pustules for the first day of observation (above) and the second day of observation (below) for every family/line together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported for *Rpbq3*.

For both QTLs a significant difference in number of pustules between the group of resistant plants and the group of the susceptible plants was observed, moreover the mean of the number of pustules of Bd3-1 was 2.13 - 3.71 times bigger than the other parental, the inbred line Bd1-1 (data not shown). As a result it was possible to fine map both the QTLs: *Rpbq2* on a defined interval between bradi3g16010 and bradi3g16140; *Rpbq3* between markers on genes Bradi4g10017 and Bradi4g10345.

3.4.3 Validation of inoculation tests (2015)

The validation trials were conducted at seedling stage, 21 days after sowing, and at adult stage, 35 days after sowing for each QTL. Every validation experiment confirmed the trend of the higher susceptibility of the inbred line Bd3-1, which showed a higher number of mature pustules than Bd1-1 and the lack of reactions in the Bd3-1 lines.

For *Rpbq2* seedling stage validation tests, the level of infection ranged between the mean of 11 pustules observed in line L301-016-03 and 78.75 scored on line L305-059-18 for the first day of observation and the same lines were the less and most infected also at the second day of scoring (35 and 111.75 respectively). The level of infection was in accordance with the first inoculation test and this was confirmed also in the parental lines used as controls. The first day of observation corresponding to the day that the first mature spores showed up was the tenth day after inoculation, as well as in the first trial. Small chlorotic haloes, surrounding the black flecks or the mature pustules were found in some resistant family/line at seedling stage.

Concerning seedling stage validation test of *Rpbq3*, the level of infection at the first day of scoring ranged from the mean of 1.75 of family/line L402-079-09 to 41.75 of L406-041-10 and L404-002-06, while for the second day it ranged from 6.76 of the same resistant line to 82.25 of L404-002-06. As for *Rpbq2* few chlorotic halos were detected only surrounding the black flecks of different size and shape. Parental line Bd3-1 showed a higher level of susceptibility than Bd1-1 and this was in accordance with all the other experimental results described before in **Paragraph 3.2.2**.

The validation test at adult stage for *Rpbq2* showed a higher level of infection among the observed lines. Line L302-025-01 was the less infected with a mean of mature pustules of 33, while line L305-059-18 had a mean of 153 (**Figure 19**). It was not possible to score the

second day after sporulation due to the huge amount of mature spores, a percentage of infected area was scored. The behaviour of the two parental inbred lines was confirmed.

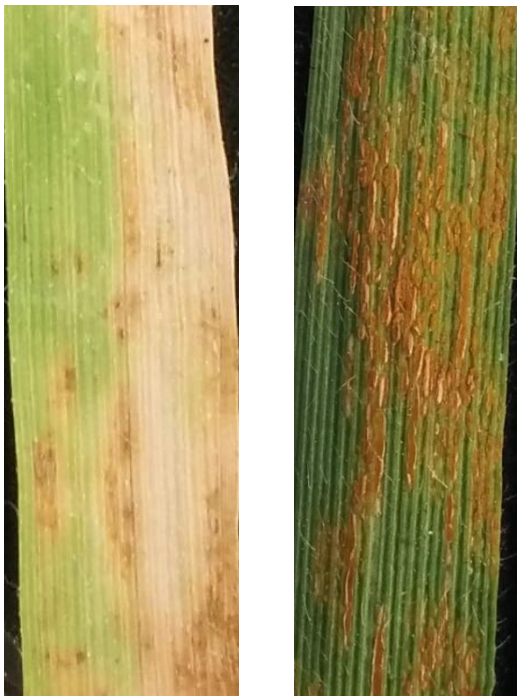


Figure 19. L302-025-01, chlorotic haloes, black flecks and few mature pustules are reported (left). L305-059-18 high number of mature pustules on green background are shown (right).

As far as adult stage validation test of *Rpbq3* is regarded, the level of infection was similar to the first adult stage trial. Line L402-079-09 was the most resistant with the average number of pustules of 1.5, instead line L404-002-06 scored an average of 89.5 during the first day of observation, that was also in this case the tenth day after inoculation. The mature pustules were also counted on the second day and lines L402-079-09 and L404-002-06 confirmed to be the less and most susceptible with an average of 19 and 129.75 mature pustules, respectively. Phenotypes of Bd3-1 and Bd1-1 were confirmed.

The statistical analysis, using the REML, of the data from the disease observations confirmed the results obtained in the first replication. An interesting result is the presence of the resistance given by each QTL in both seedling and adult stage. Is it possible, using the data collected at seedling and adult stage, to discriminate lines that are resistant from lines that are susceptible; moreover lines that are resistant at seedling stage are also resistant at adult stage and *vice versa* with the susceptible lines. The mean of the number of pustules for every family/line is shown from **Figure 20** to **Figure 23**. Genotyping data (haplotypes) and

the phenotyped lines (number of pustules) are shown from **Table 23** to **Table 26**. The results of the validation experiments showed, as in the first year trials a difference in number of pustules between the resistant lines and the susceptible.

Table 23. *Rpbq2* seedling **i**noculation experiment. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

Line	bradi3g15660	bradi3g15661	bradi3g15770	bradi3g16010	bradi3g16020	bradi3g16140	bradi3g16141	bradi3g16192	bradi3g16340	bradi3g16341	Mean 1	DMS	Mean 2	DMS
L305-059-18	b	b	b	a	a	a	a	a	a	a	78,75	c	111,75	d
L302-025-01	b	b	b	b	b	a	a	a	a	a	15,75	a	19,75	a
L301-062-14	b	b	b	b	b	b	b	b	a	a	25,75	ab	38,25	bc
L301-059-07	a	a	a	b	b	b	b	b	b	b	46,5	b	61	c
L301-016-03	a	a	a	a	b	b	b	b	b	b	11	a	35	ab
L301-001-11	a	a	a	a	a	b	b	b	b	b	33	b	47,5	bc
L301-063-03	a	a	a	a	a	a	a	a	b	b	74	c	100,75	d

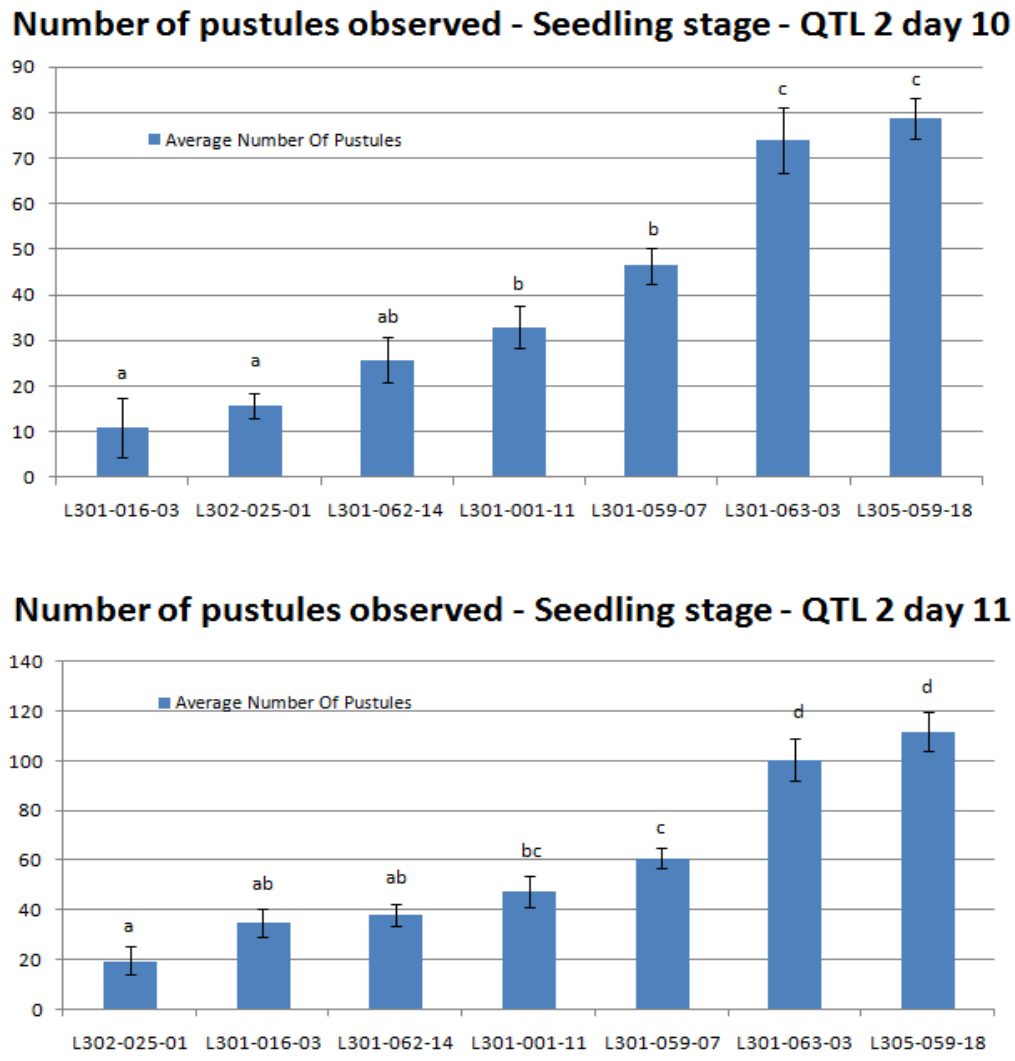


Figure 20. Mean number of pustules for the first day of observation (above) and the second day of observation (below) for every family/line together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported for *Rpbq2*.

Table 24. *Rpbq2* adult **i**noculation experiment. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

Line	bradi3g15660	bradi3g15661	bradi3g15770	bradi3g16010	bradi3g16020	bradi3g16140	bradi3g16141	bradi3g16192	bradi3g16340	bradi3g16341	Mean 1	DMS
L305-059-18	b	b	b	a	a	a	a	a	a	a	153	d
L302-025-01	b	b	b	b	b	a	a	a	a	a	33	a
L301-062-14	b	b	b	b	b	b	b	b	a	a	44	a
L301-059-07	a	a	a	b	b	b	b	b	b	b	69	b
L301-016-03	a	a	a	a	b	b	b	b	b	b	47,5	ab
L301-001-11	a	a	a	a	a	b	b	b	b	b	38	a
L301-063-03	a	a	a	a	a	a	a	a	b	b	104	c

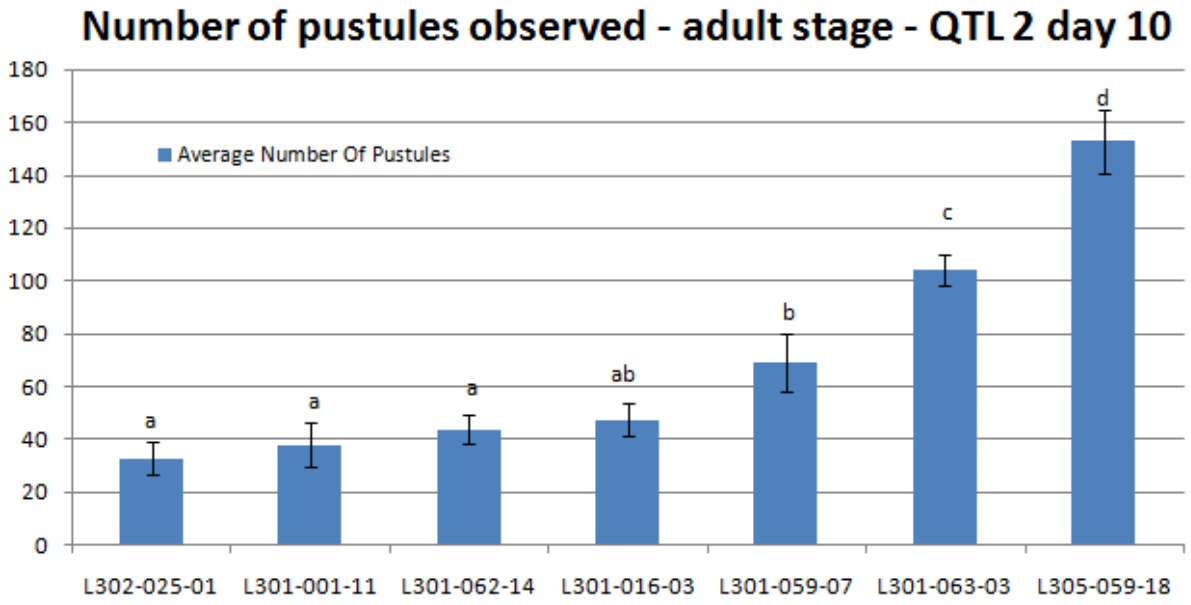


Figure 21 Mean number of pustules for the first day of observation (for every family/line together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported for *Rpbq2*.

Table 25. *Rpbq3* seedling **i**noculation experiment. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

Line	Bradi4g10017	Bradi4g10105	Bradi4g10345	Bradi4g10350	Bradi4g10640	Bradi4g10720	Bradi4g10721	Bradi4g10920	Bradi4g10930	Bradi4g10931	Mean 1	DMS	Mean 2	DMS
L403-007-04	b	b	a	a	a	a	a	a	a	a	2,25	a	14	ab
L403-007-01	b	b	a	a	a	a	a	a	a	a	9,75	ab	26,25	b
L407-021-02	b	b	a	a	a	a	a	a	a	a	13,5	ab	21,25	ab
L407-021-03	b	b	a	a	a	a	a	a	a	a	21,25	b	50	c
L407-021-17	b	b	a	a	a	a	a	a	a	a	18,25	b	45,25	c
L402-079-09	b	b	b	b	a	a	a	a	a	a	1,75	a	6,75	a
L402-069-01	b	b	b	b	a	a	a	a	a	a	13	ab	19,25	ab
L406-137-08	a	a	b	b	b	b	b	b	b	b	34	c	68,5	d
L404-002-06	a	a	a	a	b	b	b	b	b	b	41,75	c	82,25	d
L406-041-10	a	a	a	a	a	b	b	b	b	b	41,75	c	72,5	d

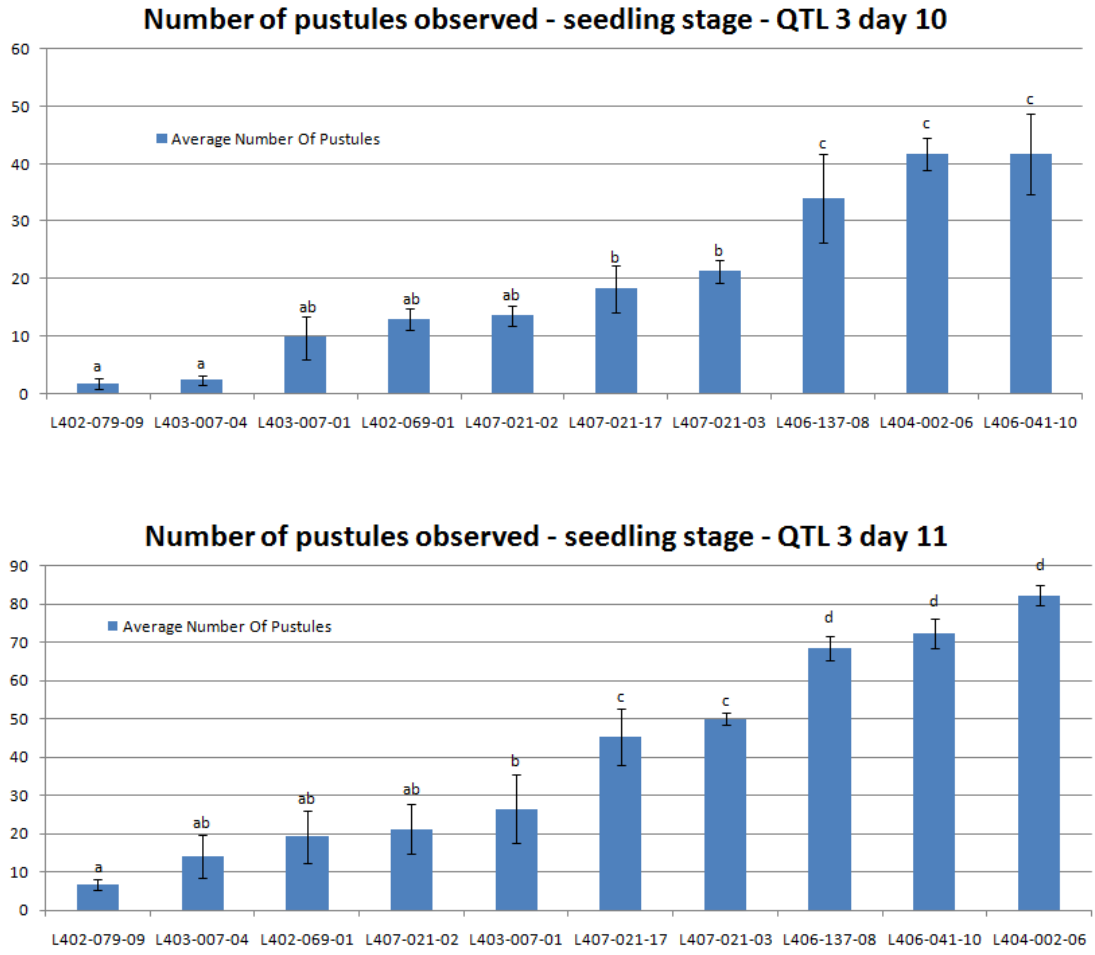
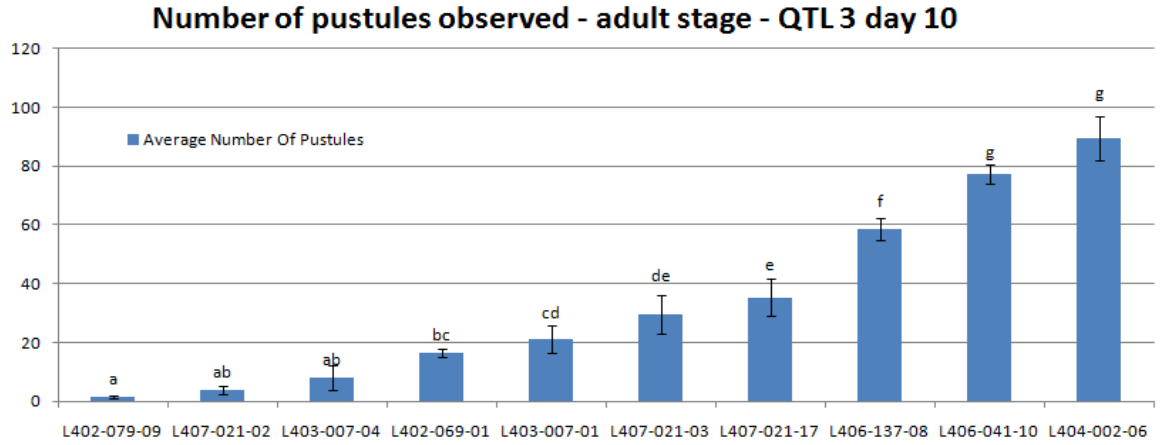


Figure 22. Mean number of pustules for the first day of observation (above) and the second day of observation (below) for every family/line together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported for *Rpbq2*.

Table 26. *Rpbq3* adult **i**noculation experiment. Names of the lines and origin, mean of the mature pustule number scored in the three days of observation and Fisher's Least Significant Differences (LSD, *P 0.05*) used to examine the significance differences and represented with letters are reported.

Line	Bradi4g10017	Bradi4g10105	Bradi4g10345	Bradi4g10350	Bradi4g10640	Bradi4g10720	Bradi4g10721	Bradi4g10920	Bradi4g10930	Bradi4g10931	Mean 1	DMS	Mean 2	DMS
L403-007-04	b	b	a	a	a	a	a	a	a	a	8	ab	32,25	a
L403-007-01	b	b	a	a	a	a	a	a	a	a	21,25	cd	53,5	b
L407-021-02	b	b	a	a	a	a	a	a	a	a	3,75	ab	35,5	a
L407-021-03	b	b	a	a	a	a	a	a	a	a	29,5	de	63,25	bc
L407-021-17	b	b	a	a	a	a	a	a	a	a	35,25	e	76	c
L402-079-09	b	b	b	b	a	a	a	a	a	a	1,5	a	19	a
L402-069-01	b	b	b	b	a	a	a	a	a	a	16,5	bc	54	b
L406-137-08	a	a	b	b	b	b	b	b	b	b	58,5	f	95,25	d
L404-002-06	a	a	a	a	b	b	b	b	b	b	89,5	g	129,75	e
L406-041-10	a	a	a	a	a	b	b	b	b	b	77,25	g	104,75	d



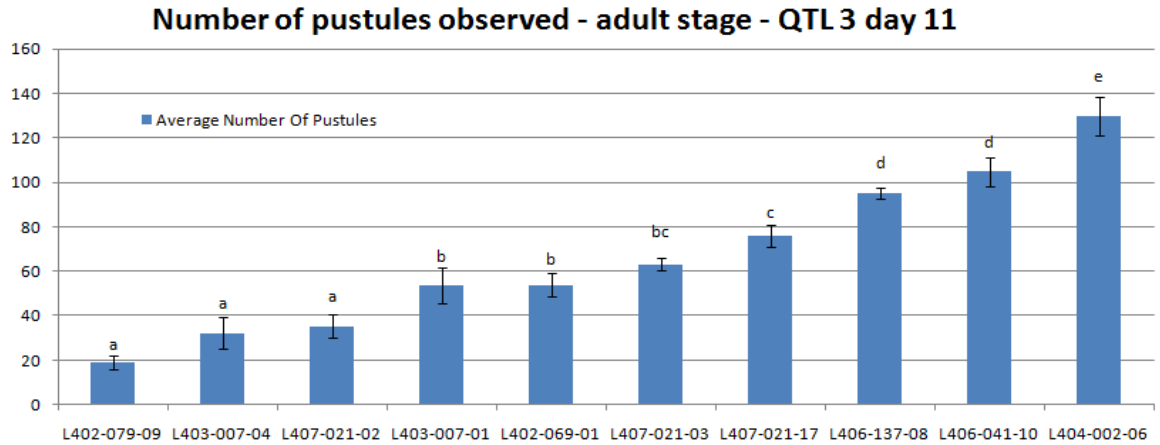


Figure 23. Mean number of pustules for the first day of observation (above) and the second day of observation (below) for every family/line together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported for *Rpbq3*.

As far as parental inbred lines Bd3-1 and Bd1-1 are regarded, the validation data confirmed the trend of the first experiment, done in adult stage. Bd3-1 is the susceptible line not only at adult stage but also at seedling stage. The data of the parental are not shown because a specific experiment on the behaviour of the parental it is described in **Paragraph 3.2.2** of this chapter and the result is the same obtained here with the parental used as controls.

All the validation data confirmed the results obtained in first instance; moreover for *Rpbq2* it was possible, due to the use of line L301-001-11, to reduce the segment of interest. The new QTL *Rpbq2* is now between markers Bd3g16020 and Bd3g16140 and is one gene smaller than the previous interval. *Rpbq3* data are confirmed and the interval of interest is the same as before.

Correlations between first experiments and validation experiments were performed using GenStat 12.1. The data obtained between and within the *Rpbq2* and *Rpbq3* experiments were analysed showing high correlation for all the trials performed (**Table 27**, **Table 28**).

Table 27: Correlation calculated for data obtained in *Rpbq2* experiments. P1-2 indicates the day of observation; A-S indicates adult or seedling stage; 2014-2015 indicates the experiment (P1A2014 stands for pustules scored in the first day of observation at adult stage of the experiment done in 2014)

Experiments	P1A2015	P1S2015	P1A2014	P2A2014	P2S2015
P1A2015	-				
P1S2015	0,90	-			
P1A2014	0,89	0,87	-		
P2A2014	0,87	0,92	0,94	-	
P2S2015	0,94	0,95	0,89	0,90	-

Table 28: Correlation calculated for data obtained in *Rpbq3*experiments. P1-2 indicates the day of observation; A-S indicates adult or seedling stage; 2014-2015 indicates the experiment (P1A2014 stands for pustules scored in the first day of observation at adult stage of the experiment done in 2014)

Experiments	P1A2014	P1A2015	P1S2015	P2A2014	P2A2015	P2S2015
P1A2014	-					
P1A2015	0,90	-				
P1S2015	0,86	0,91	-			
P2A2014	0,97	0,91	0,88	-		
P2A2015	0,84	0,95	0,89	0,89	-	
P2S2015	0,82	0,92	0,92	0,87	0,93	-

3.4.4 Genotyping in the fine mapped regions

Once the new fine mapped regions for both the QTLs of resistance to the leaf rust pathogen *Puccinia brachypodii* had been obtained, the next step was to reduce this interval, exploiting molecular marker. The aim was to cut one of the two far ends of the fine mapped segment to reduce the number of the genes inside the QTL.

Genotyping of Rpbq2

To reduce *Rpbq2* interval DNA from resistant lines L301-001-11, L302-025-01 and from susceptible lines L305-083-17, L305-083-20 and L305-083-22 was extracted. As shown in **Table29** in which the results of the genotyping are highlighted, the five CAPs molecular marker were all polymorphic between Bd3-1 and Bd1-1 and it was possible to reduce the interval of this QTL due to the difference in the position of the recombination points between the resistant and susceptible lines. The region of the QTL obtained was composed of only three genes.

Table 29. Annotation of genes within QTL interval. The marker names, the gene names, the protein families and haplotypes of the lines genotyped for *Rpbq2*are reported. Bd3-1 susceptible and Bd1-1 resistant alleles are reported with letter "a" and letter "b", respectively. Lines L302-025-01 and L301-001-11 are resistant, while lines L305-083-17, L305-083-20 and L305-083-22 are susceptible.

Line	Bradi3g16020	Bradi3g16040	Bradi3g16060	Bradi3g16091	Bradi3g16110	Bradi3g16130	Bradi3g16140
L302-025-01	b	b	b	b	b	b	a
L301-001-11	a	b	b	b	b	b	b
L305-083-17	b	b	b	b	a	a	a
L305-083-20	b	b	b	b	a	a	a
L305-083-22	b	b	b	b	a	a	a

Genotyping of Rpbq3

DNAs from seven homozygous recombinant families: L403-007-01; L403-007-04; L403-007-05; L407-021-02; L407-021-03; L407-021-17 and L406-137-08 were extracted and genotyped in order to define *Rpbq3* interval. A set of 5 SSR gene-based markers was used, four out of five markers were placed above the flanking marker due to the fact that the fine mapped segment was on the top of the QTL and there was the need to find the recombination point also above the gene Bradi4g10017. Only two of markers were polymorphic between the parental lines Bd3-1 and Bd1-1. These two SSR were placed above the flanking marker Bd4g10017, on genes Bradi4g09970 and Bradi4g09997 respectively. L403-007-01; L403-007-04; L403-007-05;L407-021-02; L407-021-03; L407-021-17 are fixed with Bd1-1 allele at the Bradi4g09997 locus, while with the exception of L403-007-05 that keep the same trend, all the other lines such as L403-007-01; L403-007-04;L407-021-02; L407-021-03; L407-021-Seven markers; four STS and three CAP markers were added between genes Bradid4g10017 and Bradi4g10345 with the aim of reducing the Bradi4g10345 far end. Five out of seven markers showed to be polymorphic between the parental lines and were used to genotype the resistant and susceptible lines listed before.

Sibling lines L403-007-01; L403-007-04; L403-007-05 showed the same recombination point and with the alleles of Bradi4g10230, Bradi4g10250 and Bradi4g10260 coming from the susceptible parental inbred line Bd3-1. Using this information the QTL was positioned to a new interval between Bradi4g09970 and Bradi4g10230 (**Table 30**).

Table 30. Haplotypes within QTL interval. The line names, the marker names and haplotypes of the lines genotyped for *Rpbq3* are reported. Bd3-1 susceptible and Bd1-1 resistant alleles are reported with letter "a" and letter "b", respectively.

Line	Bradi4g09970	Bradi4g09997	Bradi4g10017	Bradi4g10105	Bradi4g10158	Bradi4g10230	Bradi4g10250	Bradi4g10260	Bradi4g10345
L403-007-01	h	b	b	b	b	a	a	a	a
L403-007-04	h	b	b	b	b	a	a	a	a
L403-007-05	b	b	b	b	b	a	a	a	a
L407-021-02	h	b	b	b	b	b	b	b	a
L407-021-03	h	b	b	b	b	b	b	b	a
L407-021-17	h	b	b	b	b	b	b	b	a
L406-137-08	a	a	a	a	a	a	a	a	b

3.5 Conclusions

Two novel segregating populations of 462 and 420 heterozygous recombinant plants for *Rpbq2* and *Rpbq3* were obtained respectively; 34 and 31 of homozygous recombinant individuals were obtained from these plants for the two QTLs respectively and inoculated using *P. brachypodii* isolate *Kistápé*, the same pathogen used in the first mapping experiments (Barbieri et al. 2012). Seeds harvested from these genotyped populations were used in the validation experiment.

As far as the quality of the genetic map and of the molecular markers are regarded, the result obtained are supported by the accordance between the gene model order, available on Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Bdistachyon), and the order of the markers obtained in the designing of the map.

A significant difference in the number of pustules scored on the leaves together with phenotypic macroscopical differences between the resistant plants and susceptible plant were observed. A lower number of pustules together with the presence of necrotic flecks, spots, patched, stripes and sometimes with sometimes chlorotic haloes were presented in resistant plants, whereas a higher number of mature pustules and lack of response to the infections were shown by susceptible plants.

These results were confirmed in a validation experiment, performed one year later in the same facilities, moreover the second year experiments showed the resistance both at seedling and at adult stage.

A consistent reduction of the interval of the two QTLs arose from the combination of the genotypic and the phenotypic data obtained from the infection experiments. The major reduction was obtained for *Rpbq2*, the region of interest was restricted to only three genes. As far as *Rpbq3* is regarded, the initial interval covering 78 genes was reduced to 26. However, on the other hand, QTL position moved slightly above the previous flanking marker Bd4g10017 and furthermore in the present study it was not possible to exclude that the QTL could further extend in this direction.

A short list of candidate genes for *Rpbq2* and *Rpbq3* was drafted and genes with difference in the protein domain between Bd1-1 (resistant line) and Bd3-1 and Bd21 (susceptible lines) were identified.

As far as the parental lines Bd3-1 and Bd1-1 are regarded, a lower level of infection together with a reaction induced by the plant-pathogen interaction was observed on leaves of

Bd1-1 line, whereas a higher number of pustules on a green background was shown by leaves of Bd3-1. The same results were obtained consistently not only in the experiments involving directly the two parental lines, but also in all the other experiments in which lines Bd1-1 and Bd3-1 were used as controls. Brachypodium-*Puccinia brachypodii* pathosystem may be considered to be a useful model plant to investigate the interaction between *Puccinia* and its host, as a starting point for translational genomics to more economically relevant temperate cereals.

Chapter 4

Comprehensive evaluation of the Brachypodium-*P. brachypodii* pathosystem

4.1 Evaluation of the isolate-specificity

4.1.1 Materials and methods

4.1.1.1 Plant material

In order to investigate the isolate-specificity of the fine mapped QTLs, 6 families were chosen to be inoculated with four different isolates of the leaf rust pathogen *Puccinia brachypodii*. Four lines were chosen for the QTL *Rpbq2*, L305-059-1 and L302-025-01 a susceptible and resistant homozygous recombinant lines, respectively, and the homozygous lines L305-083-07 and L302-025-23, susceptible and resistant, respectively, as controls. *Rpbq3* was represented by the homozygous recombinant line L406-137-08 susceptible to the leaf rust pathogen and the homozygous line L406-137-02 that showed previously to be resistant (**Chapter 3**). Different reactions between inbred lines Bd3-1 and Bd1-1 in response to the pathogen *Puccinia brachypodii* were already reported (Barbieri 2011). For this reason Bd1-1 and Bd3-1 were transplanted in boxes for the inoculation as controls as well.

For each of the line used for *Rpbq3,* 4 plants were transplanted; the same number of individuals was transplanted for homozygous recombinant family chosen for *Rpbq2*; while 3 plants were used for the controls homozygous plants of *Rpbq2* and 2 individuals for the parental line controls Bd3-1 and Bd1-1.

4.1.1.2 Pathogen multiplication

Five different isolates of *Puccinia brachypodii*: *Kistápé* (collected in Kistápé, Tolna, Hungary; July 2006 - abbreviation *Ki*), *Delden* (collected in Delden, Overijssel, The Netherlands; May 2007 - abbreviation *De*), *Fleurines* (collected in Fleurines, Oise, France; August 2008 - abbreviation *Fl*), *Vijlen* (collected in Vijlen, Limburg, The Netherlands; October 2008 - abbreviation *Vij*) and *La Colle-Sur-Loup* (collected in La Colle-Sur-Loup, Alpes Maritimes, France, June 2011 - abbreviation *Co*) were used for this experiment.

Fresh spores were available only for isolate *Kistápé*, while isolates *Fleurines*, *Delden*, *Vijlen* and *La Colle-Sur-Loup* were used directly from the liquid nitrogen. A defrosting procedure that consist in submerging the ampoule in luke warm water (around 35 °C) for

about 3 minutes is required to perform an inoculation of *Puccinia brachypodii* spores from liquid nitrogen; after this procedure the spores were ready to be used for the inoculation and the inoculation was performed as described in **Chapter 3**. Due to bad germination of the spores from isolates *Delden, Vijlen* and *La Colle-Sur-Loup,* the first experiment was repeated. Before repeating the experiment, isolates *De, Vij* and *Co* were multiplied on Bd3-1 inbred line in order to obtain fresh spores ready to be inoculated. Isolate *Fl* had a good germination from spores stored in liquid nitrogen and for both the two experiments this kind of spores was used.

4.1.1.3 Inoculation tests

All the plant materials were transplanted in a total of 5 inoculation trays (33.5 x 42 cm), one for isolate; each tray contained 26 seedlings divided in 3 columns: two columns of 10 plants and one of 6 individuals.

For the inoculation of isolate *Kistápé,* 1.3 mg of fresh spores were used with the methodology described in the previous chapter (**Chapter 3).** As far as, *Delden, Vijlen* and *La Colle-Sur-Loup* are regarded 3 mg of fresh spores, were used for the inoculation in both experiments, while the quantity of inoculum for *Fleurines* isolate 3.5 mg of -80°C stored spores.

The inoculations were carried out in two different days in order to avoid contaminations between the isolates. The incubation time and infection procedure were the same as described in **Chapter 3** with the difference that these experiments were performed only at seedling stage, 21 days after sowing.

4.1.1.4 Statistical analysis

All the data coming from these experiments were analysed using Linear Mixed Model REML with GenStat 12.1 (VSN International Ltd. 2009, Hemel Hempstead, UK), using the same methodology described in **Chapter 3**. The number of pustules was the Y-variate, the family/line were the Fixed Models, with the difference that for these experiments was used one inoculation tray so there was no Random Model. This method allowed to estimate missing values, make predicted means and calculate the Fisher Least Significant Difference at 5% (LSD). Following the fine mapping experiments the correlations were performed using GenStat 12.1.

4.1.2 Results

This experiment was conceived with the purpose to understand if the resistances conferred by *Rpbq2* and *Rpbq3* are isolate-specific or not. In addition, a box inoculated with the isolate *Kistápé* was added in the first replication to validate the single experiment of fine mapping conducted at seedling stage (**Chapter 3).** The result of the box inoculated with the isolate *Ki* is in accordance with the fine mapping trials. Lines L302-025-01 and L406-137-02 might be classified as resistant in comparison with lines L305-059-18 and L406-137-08 because of a statistical significant difference between the number of spores that they developed. Line L302-025-01 showed the lowest average number of mature pustules on the observed leaves for all the days of scoring starting from the 10th day after inoculation, while L305-059-18 developed the highest average number of mature pustules for the first and third day. During the second day of observations the greatest sporulation on line L406-137-08 was observed (**Figure 1**).

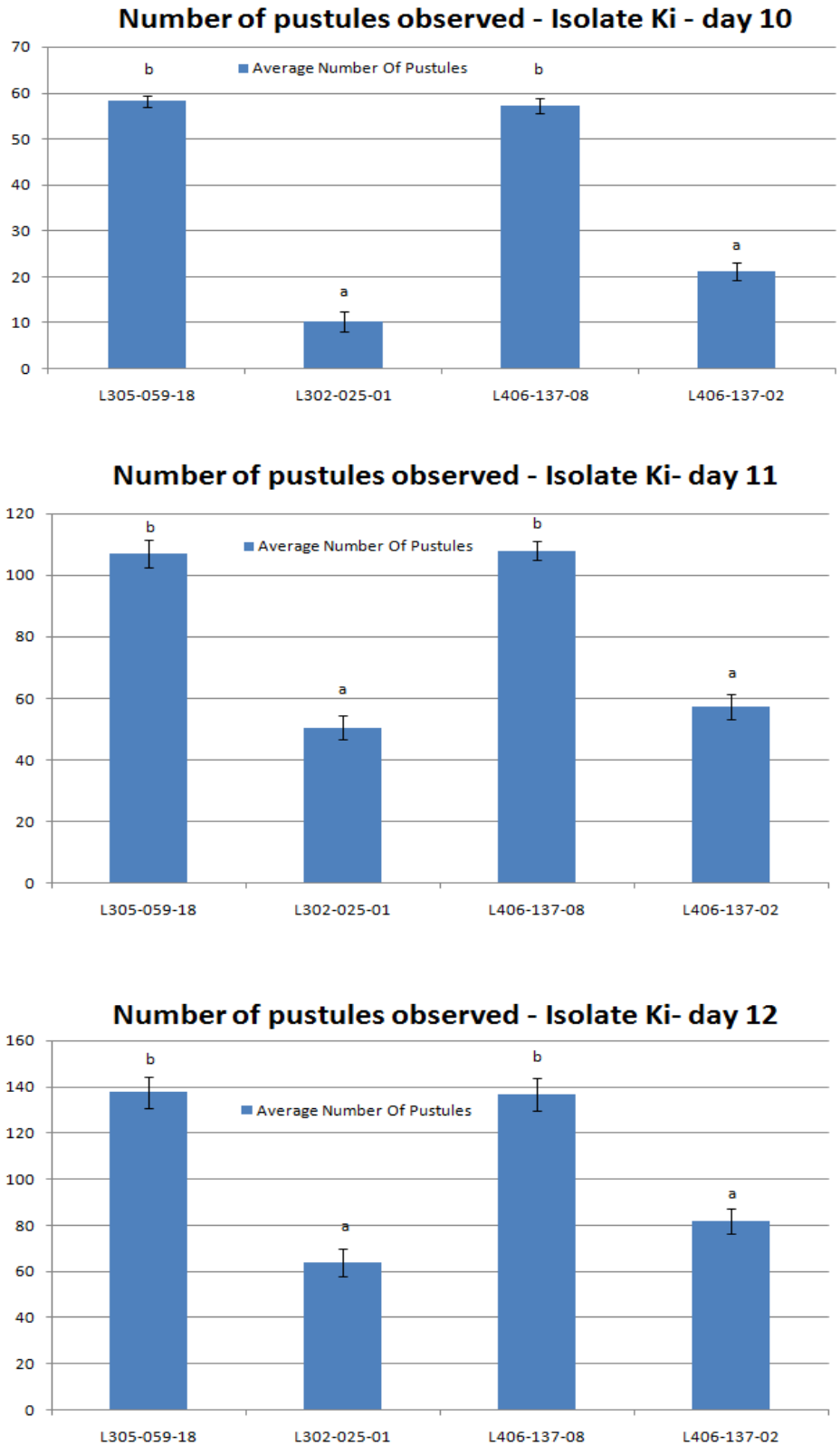


Figure 1: Mean number of pustules of isolate *Ki* for the first, second and third day of observations (above, middle, below, respectively) for *Rpbq2* lines (first two from left) and *Rpbq3* lines (last two) together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported.

As a general rule, the lines object of this study, inoculated with isolates *Fleurines*, *Delden* and *Vijlen* presented the same result as inoculated with isolate *Kistápé*. A statistical significant difference was observed between lines L302-025-01, L406-137-02 and lines L305-059-18, L406-137-08 resistant (allele b) and susceptible (allele a) to all these isolates, respectively, however with a different levels of infection was observed.

Only the isolate *Fl* had a similar number of pustules as isolate *Ki* (**Figure 2**).

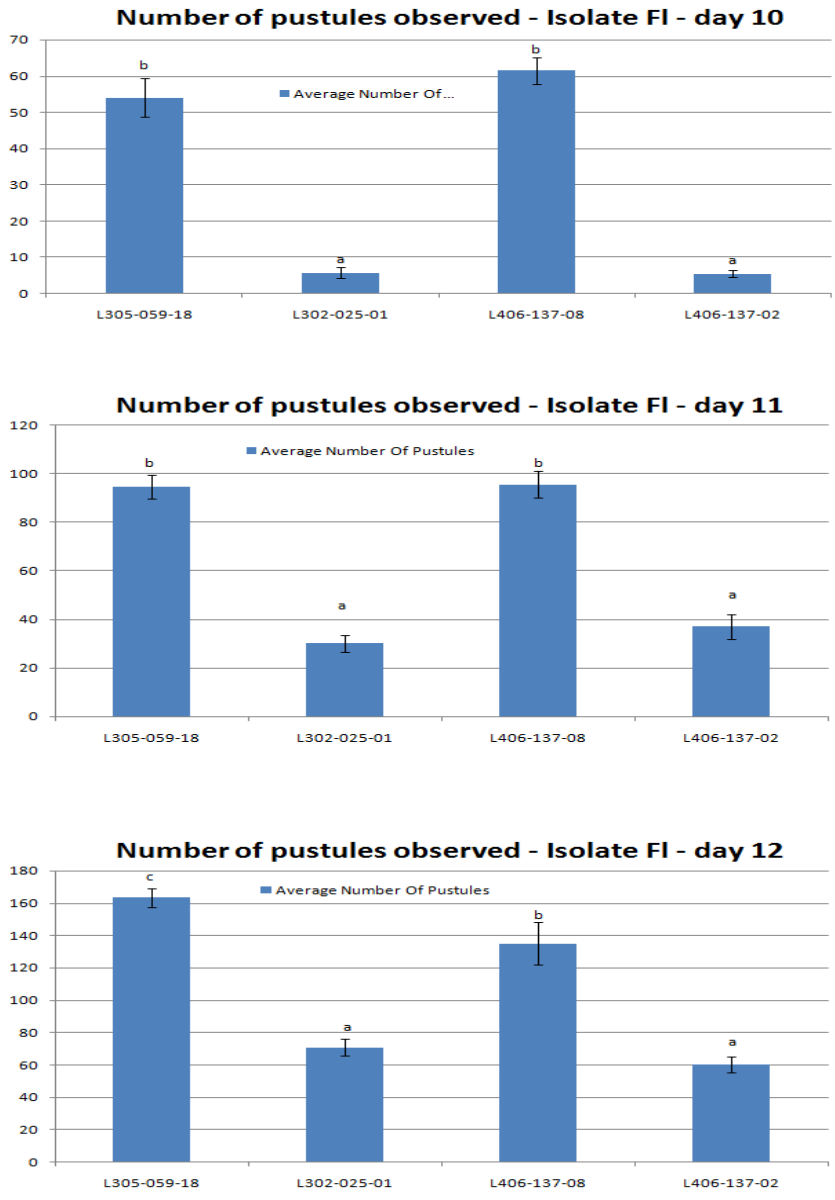


Figure 2: Mean number of pustules of isolate *Fl* for the first, second and third day of observations (above, middle, below, respectively) for *Rpbq2* lines (first two from left) and *Rpbq3* lines (last two) together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported.

Lower number of spores in both susceptible and resistant lines were scored in the experiments in which isolate *De* was used (**Figure 3**).

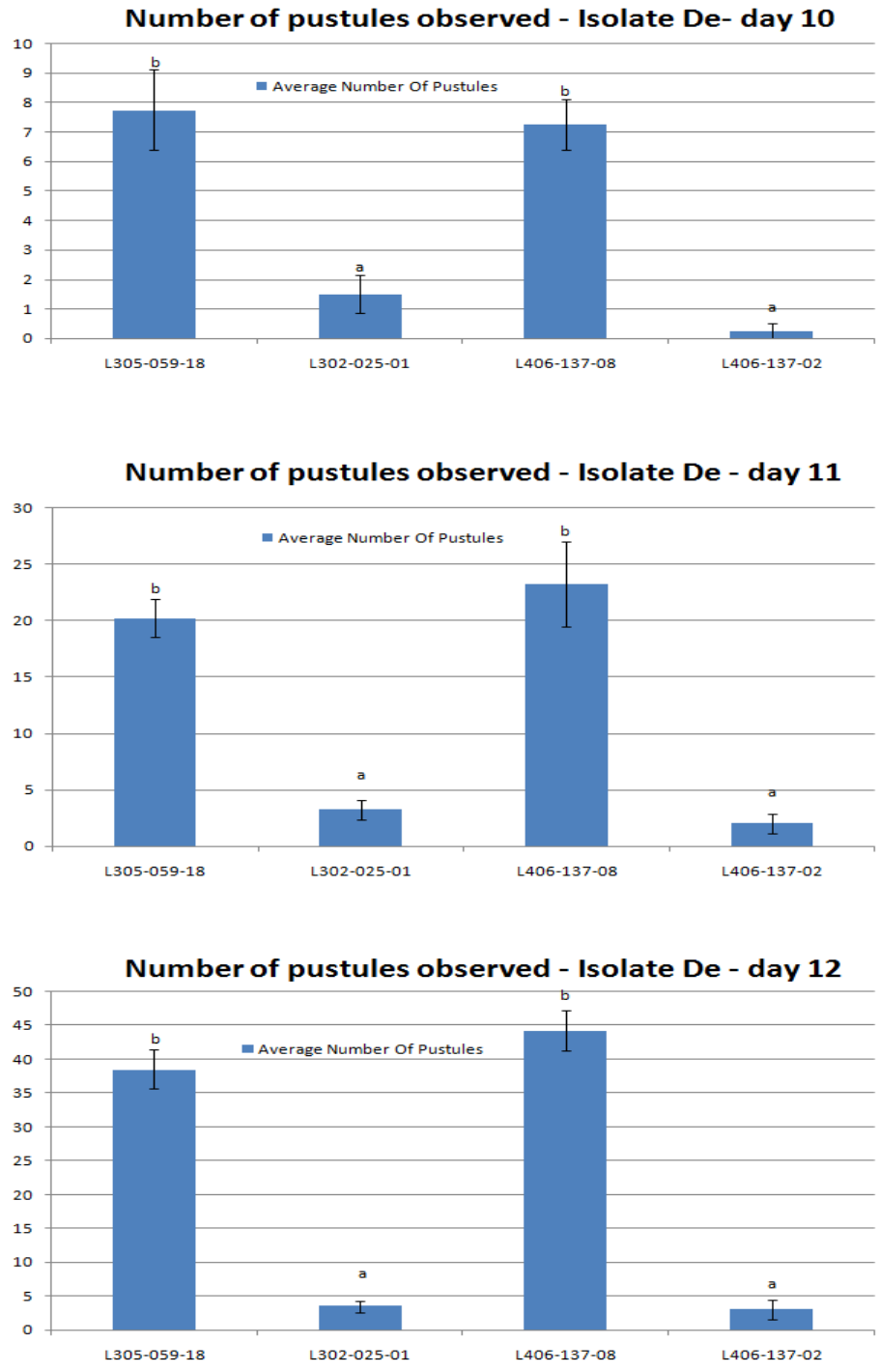


Figure 3: Mean number of pustules of isolate *De* for the first, second and third day of observations (above, middle, below, respectively) for *Rpbq2* lines (first two from left) and *Rpbq3* lines (last two) together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported.

Line L406-137-02 and the inbred line Bd1-1 showed an immune phenotype for the first two days of observations (with only a very low number of pustules developed on the third day), when inoculated with *Vij* isolate (**Figure 4**).

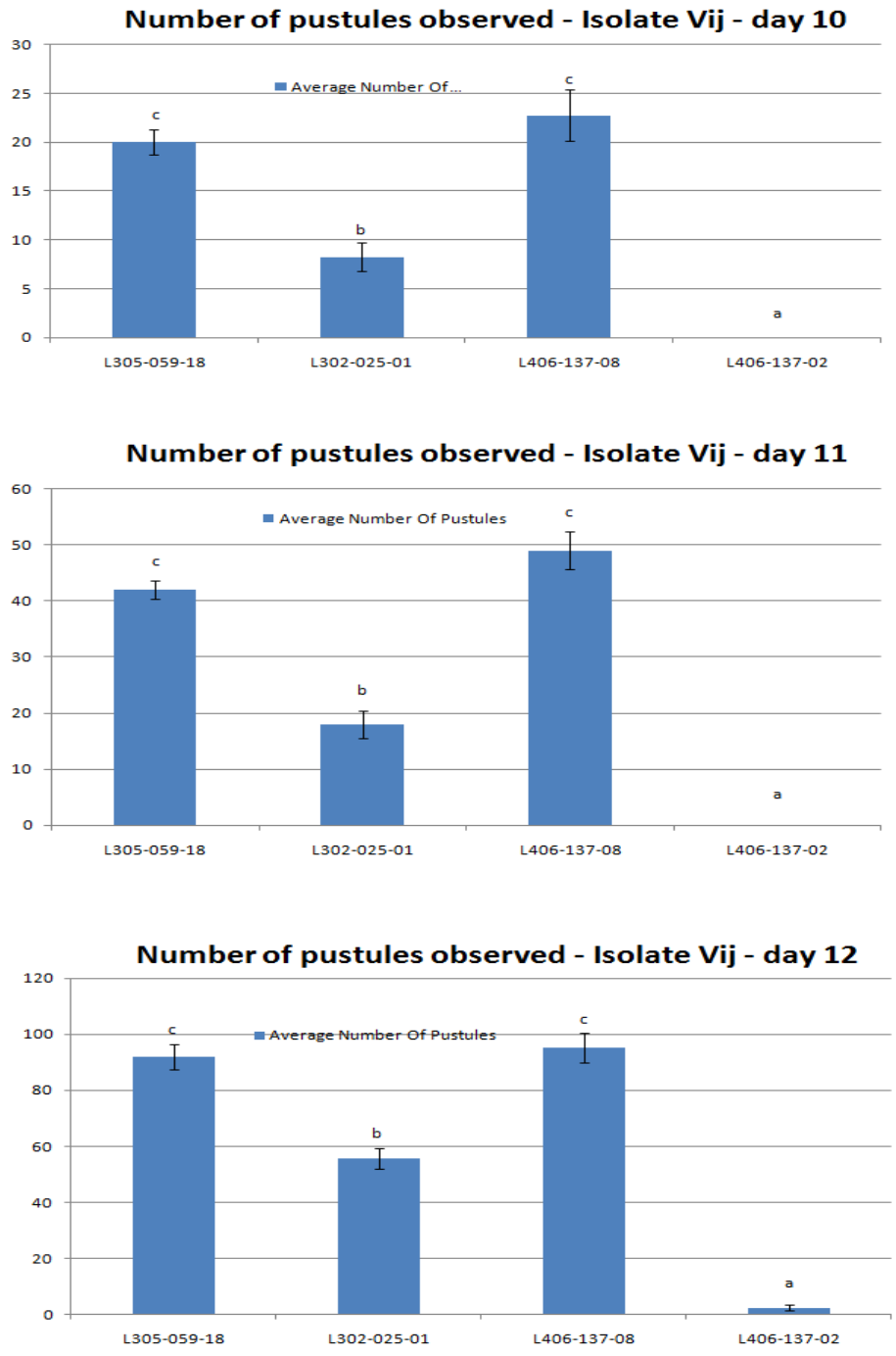


Figure 4: Mean number of pustules of isolate *Vij* for the first, second and third day of observations (above, middle, below, respectively) for *Rpbq2* lines (first two from left) and *Rpbq3* lines (last two) together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported.

No significant difference was found between the resistant and susceptible lines in the experiment in which isolate *Co* was used (**Figure 5**).

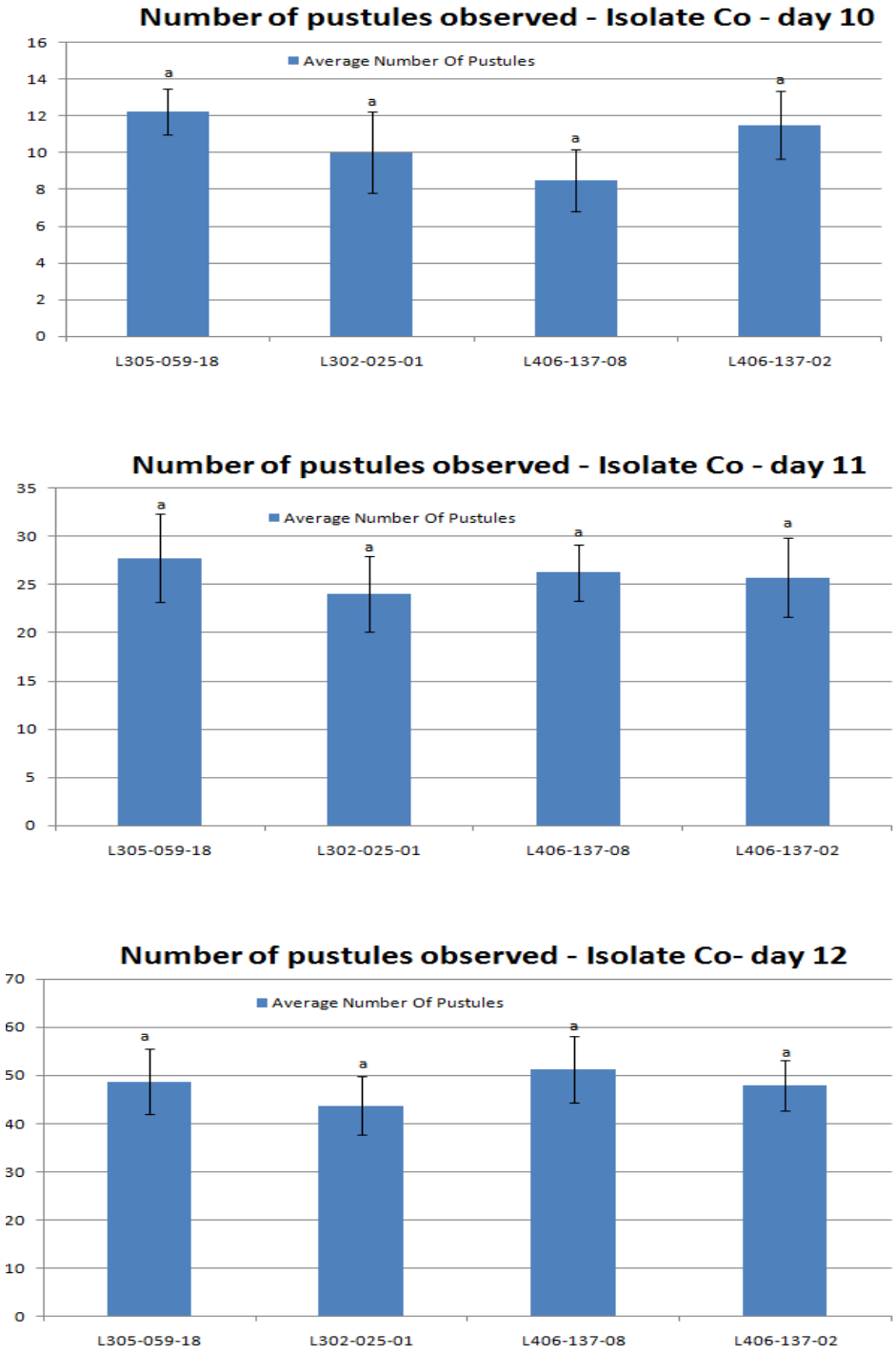


Figure 5: Mean number of pustules of isolate *Ki* for the first, second and third day of observations (above, middle, below, respectively) for *Rpbq2* lines (first two from left) and *Rpbq3* lines (last two) together with the Fisher's Least Significant Differences (LSD, *P 0.05*) are reported.

For each isolate, high correlation between the two experiments was found, exception for the experiments with isolate *Co* in which the correlation was medium-low (**Table 1, Table 2, Table 3, Table 4**).

Table 1. Correlation calculated for data obtained in isolate *Fl* experiments. P1-2-3 indicates the day of observation; A-S indicates adult or seedling stage; A-B indicates the experiment.

Experiments	P1SA	P1SB	P2SA	P2SB	P3SA	P3SB
P1SA	-					
P1SB	0,90	-				
P2SA	0,97	0,92	-			
P2SB	0,90	0,95	0,91	-		
P3SA	0,92	0,89	0,94	0,88	-	
P3SB	0,88	0,93	0,88	0,98	0,85	-

Table 2. Correlation calculated for data obtained in isolate *De* experiments. P1-2-3 indicates the day of observation; A-S indicates adult or seedling stage; A-B indicates the experiment.

Experiments	P1SA	P1SB	P2SA	P2SB	P3SA	P3SB
P1SA	-					
P1SB	0,76	-				
P2SA	0,93	0,78	-			
P2SB	0,83	0,95	0,83	-		
P3SA	0,89	0,89	0,94	0,91	-	
P3SB	0,85	0,94	0,87	0,99	0,92	-

Table 3. Correlation calculated for data obtained in isolate *Vij* experiments. P1-2-3 indicates the day of observation; A-S indicates adult or seedling stage; A-B indicates the experiment.

Experiments	P1SA	P1SB	P2SA	P2SB	P3SA	P3SB
P1SA	-					
P1SB	0,89	-				
P2SA	0,98	0,93	-			
P2SB	0,89	0,97	0,93	-		
P3SA	0,93	0,86	0,96	0,89	-	
P3SB	0,92	0,95	0,95	0,90	0,91	-

Table 4. Correlation calculated for data obtained in isolate *Vij* experiments. P1-2-3 indicates the day of observation; A-S indicates adult or seedling stage; A-B indicates the experiment.

Experiments	P1SA	P1SB	P2SA	P2SB	P3SA	P3SB
P1SA	-					
P1SB	0,50	-				
P2SA	0,74	0,29	-			
P2SB	0,36	0,82	0,22	-		
P3SA	0,72	0,37	0,74	0,27	-	
P3SB	0,26	0,79	0,14	0,93	0,23	-

Moreover, there was a remarkable variation in phenotypes. When inoculated with isolate *Fl*, resistant lines and Bd1-1 showed necrotic stripes, whereas susceptible lines and Bd1-1 had no response or in some cases a collapsed leaf (**Figure 2**)

Figure 2. Leaf of resistant line (left) showing necrotic stripes and flecks and few mature pustules; leaf of susceptible line (right) showing a high number of mature pustules without any other reaction.

Resistant lines and Bd1-1 presented necrotic patches and/or necrotic stripes when inoculated with isolate *De*, while Bd3-1 had necrotic patches and the susceptible lines had no response (**Figure 3**).

Figure 3. Leaf of resistant line (left) showing necrotic patches and stripes and few mature pustules; leaf of susceptible line (right) showing a high number of mature pustules without any other reaction.

Isolate *Vij*, Bd1-1 line and the resistant lines had necrotic spots as a response to the inoculation; on the other hand Bd3-1 and the susceptible lines had no symptoms showing mature pustules on green background on the leaves (**Figure 4**).

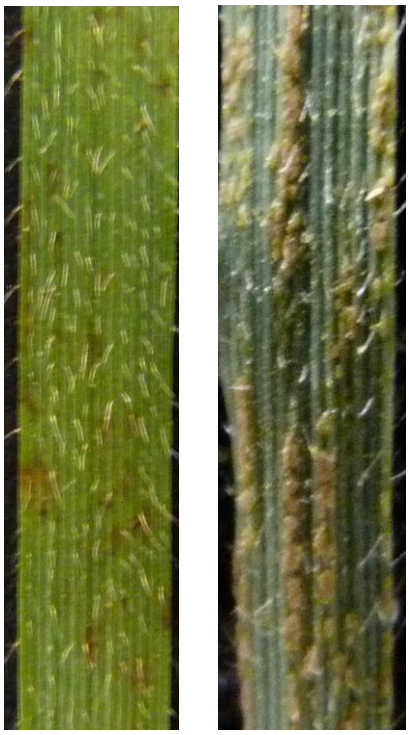


Figure 4. . Leaf of resistant line (left) showing immune phenotype with few necrotic flecks; leaf of susceptible line (right) showing a high number of mature pustules without any other reaction.

Finally all the plants inoculated with isolate *Co* had as a response to the pathogen a sporulation on the leaf without any other symptom (**Figure 5**).

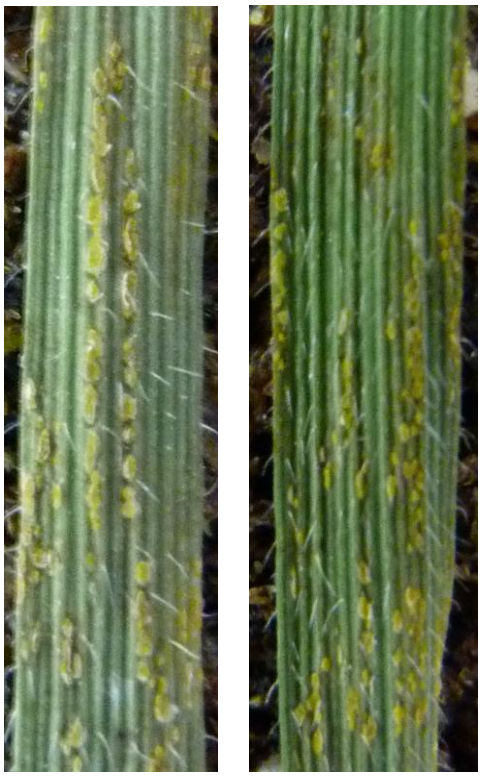


Figure 5. Leaf of putative resistant line (left) and susceptible line (right). Level of infection and symptoms are the same: a high number of mature pustules without any other reaction.

4.2 Host response to *Puccinia brachypodii* isolate *Kistápé* in 50 representative *Brachypodium distachyon* accessions

4.2.1 Materials and methods

4.2.1.1 Plant material

As a complementary approach to the fine mapping, a diversity panel of 50 different accessions of *Brachypodium distachyon* collected around the world (**Table 5)**, and objects of a re-sequencing project lead by John Vogel of the United States Department Of Energy Joint Genome Institute, was screened for response to *Puccinia brachypodii* isolate *Ki*.

Seeds were requested from John Vogel and Sean P. Gordon, United States Department Of Energy Joint Genome Institute collection and Margaret A. Gollnick, USDA-ARS Western Regional Plant Introduction Station & Seed Testing (WRPIS).

Table 5. List of the 50 *Brachypodium distachyon* accession used for this study with their place of collection, latitude and longitude.

Accession	Collection Location	Latitude	Longitude
ABR2	Octon, Herault, France	43° 36' 15.33"N	3° 15' 46.58"E
ABR3	Huesca, Aisa , Spain	42° 41' 22.20"N	0° 36' 44.95"E
ABR4	Huesca, Arens, Spain	40° 59' 33.81"N	0° 16' 17.64"W
ABR5	Huesca, Jaca, Spain	42° 34' 12.99"N	0° 32' 52.59"W
ABR6	Navarra, Los Arcos, Spain	42° 34' 32.10"N	2° 11' 39.03"W
ABR7	Valladolid, Otero, Spain	42° 0' 59.94"N	6° 8' 28.88"W
ABR9	Ljubjana, Croatia	-	-
Adi-2	Adiyaman	37° 46' 14.5"N	38° 21' 8.2"E
Adi-10	Adiyaman	37° 46' 14.5"N	38° 21' 8.2"E
Arn1	Arens, Huesca, Spain	42° 0' 59.94"N	6° 8' 28.88"W
Bd1-1	Turkey	39° 11' 27.44"N	27° 36' 28.59"E
Bd18-1	Turkey	39° 22' 4.25"N	33° 43' 48.91"E
Bd2-3	Iraq	33° 45' 39.18"N	44° 24' 11.07"E
Bd3-1	Iraq	33° 45' 39.18"N	44° 24' 11.07"E
Bd30-1	Spain	36° 59' 25.76"N	3° 33' 31.44"W
BdTR10C	Turkey	37° 46' 41.64"N	31° 53' 5.68"E
BdTR11A	Turkey	38° 25' 0.42"N	28° 1' 52.75"E
BdTR11G	Turkey	41° 25' 17.86"N	27° 28' 36.81"E
BdTR11I	Turkey	39° 44' 17.39"N	28° 2' 24.71"E
BdTR12C	Turkey	39° 44' 53.45"N	34° 39' 1.15"E
BdTR13a	Turkey	39° 45' 23.35"N	32° 25' 56.46"E
BdTR13C	Turkey	39° 24' 46.28"N	32° 59' 17.24"E
BdTR1i	Turkey	38° 5' 35.03"N	28° 34' 59.02"E
BdTR2B	Turkey	40° 4' 55.55"N	31° 19' 52.01"E
BdTR2G	Turkey	40° 23' 37.13"N	32° 59' 7.32"E
BdTR3C	Turkey	36° 46' 58.92"N	32° 57' 46.71"E
BdTR5i	Turkey	40° 23' 37.13"N	32° 59' 7.32"E
BdTR7A	Turkey	39° 44' 53.45"N	34° 39' 1.15"E
BdTR8i	Turkey	37° 6' 31.87"N	34° 4' 17.06"E
BdTR9K	Turkey	39° 45' 10.62"N	30° 47' 19.07"E
Bis-1	Bismil	37° 52' 35.6"N	41° 0' 54.3"E
Foz-1	Foz de Lumbier, Navarra, Spain	42° 38' 11.43"N	1° 18´ 17.42"W
Gaz-8	Gaziantep	37° 7' 39.8"N	37° 23' 26.9"E
Jer1	Ermita de San Jeromimo, Huesca, Spain	42° 3´ 16.56"N	0° 0´ 44.56"W
Kah-1	Kahta	37° 44' 2.3"N	38° 32' 0.2"E
Kah-5	Kahta	37° 44' 2.3"N	38° 32' 0.2"E
Koz-1	Kozluk	38° 9' 8.2.6"N	41° 36' 34.8"E
Koz-3	Kozluk	38° 9' 8.2.6"N	41° 36' 34.8"E
Luc1	Ermita de Santa Lucia, Berdun, Huesca, Spain	42° 36' 36.18"N	0° 53' 35.48"W
Mig3	San Miguel de Foces, Huesca, Spain	42° 8' 52.76"N	0° 11' 41.89"W
Mon3	Puetro de Pallaruelo, Castejon de Monegros. Zaragoza, Spain	41° 39' 4.75"N	0° 12' 37.51"W
Mur1	Castillo de Mur, Lerida, Cataluna, Spain	-	-
Per-1	Puerto de Perdom, Navarra, Spain	42° 44´ 13.34"N	1° 44´ 58.59"W
S8iiC	Spain	-	-
Sig2	Sigues, Zaragoza, Spain	42° 36' 46.54"N	1° 0' 52.38"W
Tek-2	Tekirdag	41° 0' 40.1"N	27° 31' 8.8"E
Tek-4	Tekirdag	41° 0' 40.1"N	27° 31' 8.8"E
Uni2	University of Zaragoza, Huesca	42° 7' 3.97"N	0° 26' 42.81"W
Bd29-1	Krym, Ukraine	44° 30' 55"N	33° 33' 23"E
Bd21 (control)	Salakudin, Iraq	33° 45' 39.18"N	44° 24' 11.07"E

4.2.1.2 Pathogen multiplication

The multiplication of isolate *Ki* was carried out with the same method described in **Chapter 3** order to obtain fresh spores for the inoculation tests.

4.2.1.3 Inoculation test

Three individual for each accession for a total of 150 seedlings were transplanted in 5 boxes for the inoculation (33.5 x 42 cm). Every tray contained 30 individuals divided in 3 columns of 10 seedlings each. The inoculation procedure was the same described in **Chapter 3** and the experiment was repeated for validation.

4.2.1.4 Statistical analysis

Phenotypical data were analysed using Linear Mixed Model REML with GenStat 12.1 (VSN International Ltd. 2009, Hemel Hempstead, UK), using the same methodology described in the previous chapter. The number of pustules was used as the Y-variate, the accessions were Fixed Models, and boxes were treated as Random Model. This method allowed to estimate missing values, make predicted means and calculate the Fisher Least Significant Difference at 5% (LSD). Correlations were calculated using GenStat 12.1.

4.2.2 Results

Wide screening infection using 50 accessions of Brachypodium distachyon

The inoculations were conducted only at seedling stage, 21 days after sowing, due to the fact that the effect of the two fine mapped QTLs was significant in both seedling and adult

stage. A great variability between the 50 accessions infected was recorded in the period between 10 and 13 days after inoculation in each trial, as reported in the illustrative **Figure 6**.

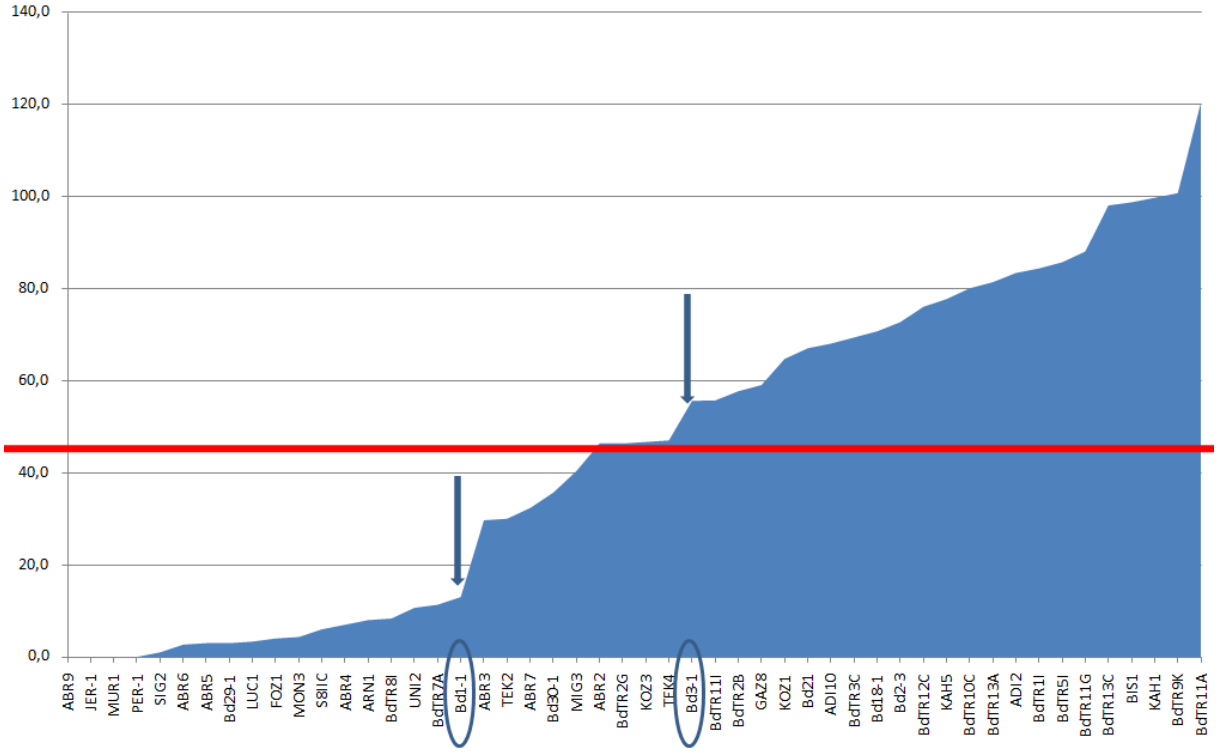


Figure 6. Average number of spores scored in the third day of observation, 13 days after inoculation (24 days after sowing), on the 50 representative *B. distachyon* accessions. Red line is the average number of pustules scored. Parental lines Bd3-1 and Bd1-1 are highlighted by blue arrows.

The first day of observation, the level of infection in the first replication ranged between the average number of 0 pustules observed on accessions ABR3, ABR4, ABR5, ABR6, Bd29-1, FOZ1, JER-1, LUC1, MON3, MUR1, PER-1, S8IIC, SIG2, UNI2 and 39,66 pustules scored on accession BdTR11A. On the second day the means ranged between 0 pustules of accessions ABR4, ABR5, ABR6, Bd29-1, JER-1, S8IIC and SIG2, while the maximum score was annotated again on line BdTR11A with 108. On the third and last day of observation, accessions ABR5,ABR6, JER-1 and SIG2 had still an immune response with 0 pustules, whereas BdTR11A was still the most susceptible. The mean number of pustules recorded on the first, second and third day of observations are reported in **Table 6.**

Table 6 Fifty Accessions used for the isolate *Ki* inoculation. Mean of the mature pustule number is reported for each of the three days of observation.

Accession	Mean 1	Mean 2	Mean 3
ABR5	0,00	0,00	0,00
ABR6	0,00	0,00	0,00
JER-1	0,00	0,00	0,00
SIG2	0,00	0,00	0,00
S8IIC	0,00	0,00	3,67
ABR4	0,00	0,00	5,00
LUC1	0,00	2,00	5,33
Bd29-1	0,00	0,00	7,67
ABR3	0,00	1,33	8,00
UNI2	0,00	1,67	8,67
MUR1	0,00	0,67	10,00
PER-1	0,33	1,00	4,33
Bd1-1	0,33	7,00	13,00
FOZ1	1,00	1,67	2,67
ARN1	1,00	5,67	13,00
BdTR7A	2,67	7,00	12,00
MON3	3,00	5,33	9,33
BdTR8I	4,33	7,67	11,67
ABR9	6,67	12,00	16,33
Bd30-1	7,67	21,00	55,33
ABR2	8,33	16,67	40,33
Bd3-1	8,67	29,67	55,67

MIG3	9,33	28,67	58,67
BIS1	9,67	35,00	72,00
TEK4	10,33	31,67	45,33
BdTR5I	11,67	42,00	67,00
GAZ8	12,00	29,00	58,33
ABR7	12,33	23,00	38,00
BdTR3C	13,00	35,33	54,00
KOZ1	13,33	38,33	61,33
TEK2	14,00	22,67	36,67
KOZ3	15,67	44,00	82,00
BdTR10C	16,00	37,67	70,33
ADI10	16,67	40,67	66,67
BdTR13A	18,33	47,33	67,67
BdTR2B	18,33	39,33	73,33
KAH5	18,33	44,67	79,67
BdTR2G	19,33	42,33	62,67
Bd18-1	19,33	49,00	77,33
BdTR12C	20,67	56,33	76,33
Bd21	22,33	45,33	67,00
Bd2-3	22,67	43,67	68,33
BdTR9K	26,67	51,33	73,33
ADI2	27,33	60,33	79,33
BdTR13C	27,33	53,67	81,67
BdTR1I	27,33	58,33	83,33
BdTR11I	28,67	55,33	91,67

BdTR11G	29,67	60,67	88,67
KAH1	32,67	67,33	88,00
BdTR11A	39,67	77,33	108,00

As far as the second experiment is regarded, the level of infection for the first day of observations (10 dpi) ranged between the average of 0 mature pustules on plants ABR3, ABR4, ABR9, JER-1, LUC1, MUR1, PER-1, S8IIC and 49 of KAH1. The second day of observation ABR4, ABR9, JER-1 and LUC1 presented no symptoms, on the other hand KAH1 scored an average number of pustules of 71,66. On the third day every accession developed spores on the leaves, the lowest amount of spores was registered on ABR4 with a mean of 0,33 pustules, while KAH1 had the maximum amount with 108 The mean number of mature pustules for each day of scoring for all the 50 accessions inoculated Are reported in **Table 7.**

Table 7. Fifty Accessions used for the isolate *Ki* inoculation. Mean of the mature pustule number is reported for each of the three days of observation.

Accession	Mean 1	Mean 2	Mean 3
ABR4	0,00	0,00	0,33
ABR9	0,00	0,00	1,67
JER-1	0,00	0,00	1,67
LUC1	0,00	0,00	1,67
MUR1	0,00	0,33	2,67
PER-1	0,00	0,33	2,67
S8IIC	0,00	1,67	7,00
ABR3	0,00	1,67	10,33
SIG2	0,67	2,00	6,00
UNI2	1,00	1,67	6,00
FOZ1	1,33	2,33	5,00

ABR6	1,33	2,33	6,33
ARN1	1,33	3,67	8,00
MON3	1,67	3,33	7,67
BdTR7A	2,00	7,67	14,33
Bd1-1	2,00	12,67	31,33
ABR5	2,33	3,00	4,67
Bd29-1	2,33	4,33	8,00
BdTR8I	2,33	8,33	21,67
ABR7	3,67	14,67	40,67
Bd30-1	5,00	21,33	41,33
GAZ8	5,33	32,67	61,00
MIG3	6,00	17,00	42,33
ABR2	6,67	22,67	50,00
TEK2	10,33	32,33	56,33
BdTR5I	12,00	33,67	61,33
KOZ3	15,33	35,00	53,67
BdTR13A	15,67	47,33	71,00
BdTR2G	18,33	30,67	42,67
BdTR11A	18,67	27,67	45,33
TEK4	19,00	43,33	61,00
BdTR3C	19,67	43,67	64,67
Bd18-1	20,00	44,67	69,33
KAH5	20,33	46,33	64,00
BdTR2B	21,00	45,00	67,33
Bd3-1	22,00	45,67	82,33

ADI10	22,33	45,67	81,33
BdTR12C	22,67	46,67	83,00
KOZ1	23,67	47,00	65,33
BIS1	23,67	50,33	81,33
BdTR13C	24,00	51,33	92,67
Bd2-3	25,33	49,00	69,67
ADI2	25,67	53,00	79,67
BdTR11I	26,33	51,67	74,33
Bd21	29,00	56,33	83,00
BdTR10C	30,33	54,67	86,67
BdTR1I	34,00	47,67	76,00
BdTR11G	38,00	63,00	95,33
BdTR9K	40,67	63,67	95,00
KAH1	49,00	71,67	108,00

High correlation between the two experiments was observed as shown in **Table 8**.

Table 8 Correlation calculated for data obtained in the 50 accessions panel experiments. P1-2-3 indicates the day of observation; A-S indicates adult or seedling stage; A-B indicates the experiment.

Experiments	P1SA	P1SB	P2SA	P2SB	P3SA	P3SB
P1SA	-					
P1SB	0,76	-				
P2SA	0,93	0,78	-			
P2SB	0,83	0,95	0,83	-		
P3SA	0,89	0,89	0,94	0,91	-	
P3SB	0,85	0,94	0,87	0,99	0,92	-

4.3 Conclusions

As a comprehensive evaluation of the Brachypodium-*P. brachypodii* pathosystem, an investigation of the isolate-specificity of the fine mapped QTLs and the host response to isolate *Kistápé* in fifty representative *B. distachyon* accessions was performed.

Inoculations with four different isolates of *Puccinia brachypodii*, *De*, *Vij*, *Co* and *Fl* of resistant and susceptible lines coming from the fine mapping populations were done. *Rpbq2* and *Rpbq3* were demonstrated to have a putative role in the partial resistance to all but one isolates, the exception was the isolate *Co* in which no statistical difference between the mean number of pustules scored was found. On the contrary, the inoculation of the parental inbred lines Bd1-1 and Bd3-1 using isolate *Co* caused a statistical difference in sporulation where Bd1-1 presented a lower number of mature pustules.

A panel of fifty accessions representative of the genetic variability of *Brachypodium distachyon* lines available, was inoculated with the isolate *Kistápé*, used for the fine mapping experiments. A high variation in phenotypes was observed, with some lines like ABR4 and JER-1 extremely susceptible to *Puccinia brachypodii* isolate *Kistápé*, and other, namely BdTR11A and KAH1, highly susceptible.

Bd3-1 and Bd1-1, parentals of the two fine mapping populations were included in the panel and showed a moderately susceptible and resistant phenotypes, respectively. This final result is in accordance with all the previous experiment described in the present study.

Chapter 5

General discussion and future perspective

5.1 General discussion

Leaf, stripe and stem rust are caused by biotrophich obligate fungal agents gathered in the phylum of Basidiomycota, order *Pucciniales* (former known as Uredinales). Rust pathogens are one of the most dangerous threats to grain cereal production.

Nowadays there is the need to find new sources of resistance to these pathogens. In the last years, especially in Africa, rust pathogens infecting wheat, such as *P. striiformis* f. sp. *tritici*, *P. graminis* f. sp. *tritici* and *P. triticina*, agents of the wheat stripe rust, stem rust and leaf rust, respectively, have overcome genetic resistances in the common cultivated variety of wheat due to the development of new races (Leonard and Szabo 2005; Bolton et al. 2008). Moreover, these new races of stripe rust (Milus et al. 2009), stem rust (Stokstad 2007) and leaf rust (Singh et al. 2004) propagate worldwide in a shorter time than the discovery and delivery of new genetic resistance in elite cultivars. Wheat is one of the most precious resources for human nutrition; in fact wheat itself is known to provide more of the 20 % of the calorific intake worldwide. In eastern African countries as well as South Africa, Zimbabwe, Iran, Sudan and Yemen, the *Ug99* stem rust race of *P. graminis* f. sp. *tritici* is destroying most of the wheat fields due to the susceptibility of the 90% of the wheat cultivated varieties developed and cultivated across the world (Singh et al. 2011).

The increasing of intensity and frequency of pathogen incidence arise in many ways: one of them can be a spontaneous mutation of the pathogen that allows it to overcome the plant resistance and this phenomenon was helped in the past by the thoughtless use of pesticides.

The genetic strategies used to identify disease resistances and exploited them for breeding are focused on the discovery of both resistance genes (R genes) and partial resistance genes that are detected by quantitative genetic approaches.

Although big steps forward were done in finding R genes in barley and wheat with more than 20 genes referred to *P. striiformis*, 40 R genes referred to *P. graminis* f. sp. *triticii* and 40 genes involved in resistance to *P. triticina* mapped in barley or wheat (McIntosh et al. 1995; Ayliffe et al. 2008), only a small number of genes were functionally characterized and cloned. Only four R genes were cloned in wheat: *Lr10* (Feuillet et al. 2003), *Lr21* (Huang et al. 2003), *Sr35* (Saintenac et al. 2013) and *Sr33* (Periyannan et al. 2013); while three R genes conferring rust resistance were cloned in barley: *Rpg1* (Brueggeman et al. 2002) and the complex

Rpg4/*Rpg5* (Brueggeman et al. 2008, 2009; Wang et al. 2013). On the other hand quantitative resistance arises as a the result of additive effect of many loci with a small effect (Young 1996). However, usually, in a quantitative resistance, two to six loci acts as major genes and gives the disease resistance, as reported for example in barley for some leaf rust resistant loci (Qi et al. 1998b; Marcel et al. 2007b). Partial resistance genes are laborious to be identified due to the quantitative genetic approaches that are requested, in fact only two loci conferring partial resistance have been cloned till now: *Lr34* (Krattinger et al. 2009) and *Yr36* (Fu et al. 2009).

As already known, R genes can be easily overcome by evolving pathogens; for this reason one of the most adopted strategies to counter the adaptability of rust fungi is to combine qualitative resistance given by R genes with quantitative resistance genes. Plants harbouring this two kind of resistances, have the advantage to carry a more durable resistance and thus, are believed to have both a broader spectrum of resistance, and to apply a lower pressure of selection on pathogen (Singh et al. 2004). In the complex genome of temperate cereals, the genetic dissection of genes giving quantitative resistance is barely impossible to comply; moreover, the number of resistance genes cloned is very small and the lack of knowledge of the mechanisms of interaction between plant host and pathogen make the rust a difficult opponent to challenge.

The *Puccinia* genus have about 3000 species (van der Merwe et al. 2007) and due to a high diversity inside the genus, rust pathogens have been proven to have most of the cereals as host (e.g. maize, wheat, oat, triticale, millet and barley), however, no rust pathogen affecting rice, former model plant for cereals, has been identified yet (Ayliffe et al. 2011).

In contrast to rice, the purple false brome grass has been proven to be a host to rusts, in particular to *P. brachypodii* which infects majority of *Brachypodium spp* (Barbieri et al. 2011b).

In this scenario the purple false brome grass *B. distachyon* arose as an excellent model to evaluate the quantitative genetic basis of plant-pathogen interaction due to being host of a broad range of pathogens infecting economically relevant temperate cereals (Draper et al. 2001; Routledge et al. 2004; Garvin et al. 2008; Barbieri et al. 2011a; Peraldi et al. 2011; Cui et al. 2012; Figueroa et al. 2013; Peraldi et al. 2014).

The main aim of the present study was the fine mappings of two genomic regions associated to the leaf rust resistance in order to obtain a precise targeting of candidate genes and reach the cloning of the *Rpbq2* and *Rpbq3* QTLs. The second aim of this work was to

unravel and validate the Brachypodium-*P. brachypodii* pathosystem. The results obtained using the purple false brome grass model plant and presented in this work are important for the improvement of the knowledge on the genomic bases of rust resistance and for its exploitation in relevant crops such as wheat and barley by means of translational genomic.

In order to fine map *Rpbq2* and *Rpbq3* QTLs a thorough phenotyping was performed. As far as the inoculation method is regarded, many approaches have been described in literature. To score quantitative resistance, especially in the polycyclic field experiments, the Area Under Disease Progress Curve (AUDPC) is used, while the evaluation of the Latency Period (LP), is mostly used in greenhouses trials with monocyclic experiments (Qi et al. 1998a, 1999; Kicherer et al. 2000; Xu et al. 2005; Jafary et al. 2006; Marcel et al. 2007b, 2008; Barbieri et al. 2011b, 2012). However the visual scoring (Bade and Carmona 2011) remains the most widely used method of assessment and it has been used recently in *B. distachyon* to score the severity of infection caused by the cereal pathogens *Oculimacula spp*. agent of the eyespot and the *Ramulariacollo-cygni*, agent of the ramularia leaf spot (Peraldi et al. 2014). The choice of the most suitable evaluating system of infection was the priority in the present study in order to perform efficient and repeatable experiments. The visual assessment was chosen due to the macroscopical difference in level of infection observed between the Bd1-1 and Bd3-1 and their derived fine mapping population. This methodology, in association with the REML statistical analysis was able to distinguish a resistant line from a susceptible. The high and the continuously increasing number of pustules observed, especially on line Bd3-1, made AUDPC and the LP assessment difficult and not appropriate to use.

An interesting result was obtained by phenotyping a different level of infection was observed for the parental lines Bd3-1 and Bd1-1, with Bd3-1 line more susceptible than Bd1-1, both at seedling and in adult stage. This data is in agreement with previous studies (Barbieri et al 2011, 2012) as far as adult stage is regarded. However different phenotypic response had been obtained by Barbieri et al (2012) at seedling stage in which Bd1-1 was considered as susceptible due to AUDPC values. The results obtained for *Rpbq2* in the present work were thus in partial discordance with Barbieri and colleagues (et al 2012), to our knowledge this QTL had an effect both at seedling and adults stage with the resistant alleles harboured by Bd1-1. Similar results to the present study for leaf rust resistance in barley and wheat were reported (Leonova et al. 2006; Naz et al. 2008), as well as divergent results in barley and wheat in which quantitative resistance with an effect at seedling stage was losing effect at

adult stage and *vice versa* were observed (Qi et al. 1998a; Marcel et al. 2007a; Shankar et al. 2008; Herrera-Foessel et al. 2011; Azzimonti et al. 2014).

The main result of the present work is the fine mapping of two QTLs (Barbieri et al., 2012) and the identification of candidate genes for quantitative resistance to *P. brachypodii*. A consistent reduction of the interval of the QTL *Rpbq2* and the reduction and redefinition of the interval of *Rpbq3* were obtained.

As far as *Rpbq2* is regarded, the region of interest was restricted to only three genes: The first codifies for a protein containing 2-oxoglutarate(2OG)-Fe(II)oxygenase, the second encodes a protein that has a SWIM zinc finger, a FAR1 binding and a MULE transposase domains and the third codifies for a protein having an ATP- binding cassette (ABC) transporter domain.

Transposase-derived transcriptors are reported to have a role in light response in the model plant Arabidopsis (Lin et al. 2007), whereas is more interesting that both 2-oxoglutarate(2OG)-Fe(II)oxygenase and ABC-Transporter domain have a reported role in plant-pathogen interaction. Recently, the Arabidopsis *DMR6* defence-associated gene was found to encode for a putative 2OG-Fe(II). *DMR6* was reported to be required for susceptibility to downy mildew and a non sense mutation caused the resistance of a T-DNA line compared to wild type (Van Damme et al. 2008). *Lr34* have been cloned recently (Krattinger et al. 2009) and it is supposed to code for a protein with an ATP- binding cassette (ABC) transporter domain. It is extremely interesting that one of the few locus cloned in wheat for resistance to leaf rust encodes a protein with the same domain as one of the three genes in restricted interval of *Rpbq2* QTL, even if the orthologous *Lr34* gene was not found in Brachypodium, maize and barley due to a possible independent deletion of these ABC transporter genes (Krattinger et al. 2011).

There are the two strong candidate genes for *Rpbq2* QTL being both associated with resistance to pathogens, and the search for candidate genes described in **Chapter 3** highlighted sequence difference in functional domains of one of them between lines Bd3-1 and Bd1-1. However this should not exclude the other one from the final candidate gene list as a further investigation is required to understand the *Rpbq2* resistance.

As far as *Rpbq3* is regarded, QTL interval moved slightly above the previous flanking marker Bd4g10017 and furthermore in the present study it was not possible to exclude that the QTL could even further extend in this direction. What is interesting, a presence of a deletion of about 131000 bp was found in the susceptible line Bd3-1 was identified through

the analysis of the Bd3-1 against Bd1-1 sequences. This region harboured eight genes: 6 codify for proteins with CNL domain, one corresponds to a reverse transcriptase and one with no functional annotation.

In last years, rising attention has been given by researchers to Nucleotide-binding site leucine-rich repeat gene family (NBS-LRR) due to its association to resistance in plant pathogen interaction. Several works were focused on identification, localization, characterization and genome wide analysis of NBS-LRR in different model plant and other plant species such as Arabidopsis, Brachypodium, apple tree, barrel clover, finger millet, kiwifruit, *Lotus japonicus* L., maize, orange tree, popolar, potato, rice, sorghum and tomato (Meyers et al. 2003; Li et al. 2010; Tamura and Tachida 2011; Luo et al. 2012; Tan and Wu 2012; Jupe et al. 2013; Andolfo et al. 2014; Arya et al. 2014; Fraser et al. 2015; Wang et al. 2015; Saha and Rana 2016).

What is interesting is that one out of three barley stem rust resistance genes cloned: *Rpg5* (Brueggeman et al. 2008) and two out of three wheat leaf rust resistance genes cloned: *Lr10* (Feuillet et al. 2003) and *Lr21* (Huang et al. 2003) encode NBS-LRR genes. In Brachypodium 126 regular NBS-encoding genes were identified and it was observed that they tend to clusterize together. One positional supercluster with 11 NBS genes, located on the terminal of chromosome 4 was identified, and the majority of these NBS-encoding genes clusterized as well in a phylogenetic tree (Tan and Wu 2012); all those eleven genes are located in the *Rpbq3* QTL interval, moreover, six of them are inside the deleted region in line Bd3-1.

These information might point out a smaller region of interest inside the QTL, giving as candidate genes to prioritize for further investigation the 6 NBS-encoding genes inside this interval.

The second aim of the present study was the comprehensive evaluation of the Brachypodium-*P. brachypodii* pathosystem. To reach this goal, isolate-specificity experiments of the fine mapped QTLs and inoculation experiments on 50 different accessions of *Brachypodium distachyon* collected around the world were performed.

In order to investigate the isolate-specificity, four different isolates of the leaf rust pathogen *Puccinia brachypodii* were used: *Delden*, *Fleurines*, *Vijlen* and *La Colle-Sur-Loup*.

A similar study, performed only in adult stage (35 days after sowing), was reported for these three isolates by Barbieri and colleagues (et al. 2011). The novelty of the present work compared to a previous work by Barbieri et al (2011) are: different plant material inoculated

(the population developed for the fine mapping) and time point (21 days after sowing) of the inoculation and the addition of the *P. brachypodii* isolate *Co*.

Plants inoculated with *De*, *Fl*, and *Vij* showed the same significant difference in response to pathogen (resistant vs. susceptible) after inoculation with isolate *Ki* with, in detail, a different mean number of pustules observed for each isolate. Bd3-1 and isolate *Ki* susceptible lines were moderately susceptible to the *De*, *Fl* and *Vij* isolates, while after *Fl* inoculation Bd1-1 and isolate *Ki* resistant lines were moderately resistant. After the inoculation with *De* all the plants used in the experiments showed some mature pustules, in contrast with the immune phenotype reported by Barbieri et al (2011), however in both studies *De* inoculation caused less symptoms than all other isolates. As far as *Co* isolate is regarded, no statistic significance was observed among all the lines developed for fine mapping, however, a significant difference was observed between Bd3-1 (susceptible) and Bd1-1 (resistant). This difference in phenotype between the two inbred lines could be caused by the presence of other QTL not object of the present study.

In conclusion, the results of the isolate-specificity trials highlighted effects of *Rpbq2* and *Rpbq3* QTLs affecting resistance to four out of five isolates of *P. brachypodii* used and confirming the importance of using together quantitative resistance with major resistance genes that are easily overcome by rust pathogens (Leonard and Szabo 2005; Singh et al. 2011).

As a complementary approach to the fine mapping, a panel of 50 accessions of *B. distachyon*, collected around the world and representative of its natural variation was inoculated with isolate *Ki*. High variation in phenotypes was observed: immune, resistant, susceptible and highly susceptible accessions were found. Bd3-1 and Bd1-1 were included in the panel in order to evaluate their level of infection in contrast with the other 48 accessions. As a result Bd1-1 was confirmed in the resistant plant group, while Bd3-1 was placed in the susceptible group.

On the basis of the fine mapping results that included a reduction of *Rpbq2* interval to three genes and the discovery of the 6 NBS-LRR deletion in Bd3-1 in the interval of *Rpbq3*, the potential of *B. distachyon* as a model grass and Brachypodium-*P.brachypodii* as a suitable pathosystem was confirmed.

5.2 Future perspective

From the fine mapping results, many approaches can be adopted in order to functionally validate the candidate genes and reach the final aim of cloning *Rpbq2* and *Rpbq3*.

As described in **Chapter 1**, the efficiency achieved in *Agrobacterium tumefaciens*-mediated transformation of Brachypodium is 45% with the amount of 100 transgenic lines ready every week (Bragg et al. 2012) and a project founded by the DOE has created till now more than 23,000 T-DNA Brachypodium lines and is still ongoing. The T-DNA lines obtained might be useful resources to search for a mutant harboring a mutation in a candidate gene. However, nowadays no T-DNA mutants are available for the candidate genes identified in the present study. Moreover, Virus Induced Gene Silencing (VIGS) is already available in the model plant *B. distachyon* (Demircan and Akkaya 2010).

Small interfering RNA (siRNA) was one of the biggest discoveries of the late nineties. Nowadays, the RNA interference (RNAi) is exploited in research due to the ability of the double strand RNA to interfere with the expression of specific genes causing sequence-specific gene knockdown (Whitehead et al. 2009). In Brachypodium this technique has already been used and is ready to help researchers (Wei et al. 2009; Zhang et al. 2009; Lucas et al. 2014). The genome editing is seen as the next generation system for modification of target genes (Mikami 2014). Both TALENs (Shan et al. 2013) and CRISPR/Cas9 (Brutnell et al. 2015) systems are successful in Brachypodium, and the two techniques can be exploited due to the high potential in high-troughtput application to study and modify the functionality of the candidate genes found in the present study.

All those techniques could be applied for further studies of the candidate genes identified in the two QTLS. Moreover, expression profiling studies are widely used to investigate the molecular mechanisms that underlie the plant-pathogen interactions and could be performed in order to observe Brachypodium gene modulation in response to rust, at the two time point identified in the present study (21 and 35 days after sowing) using real time PCR and RNAseq.

R gene enrichment and sequencing (RenSeq) is a NBS-LRR gene-targeted technique that allows discovery and annotation of this gene family known to be related to resistance to pathogens. To our knowledge this method was never used for the model plant B. distachyon, however recently has been used in important crops such as wheat, tomato and potato (Jupe et al. 2013; Andolfo et al. 2014; Wulff and Moscou 2014). This innovative technique might be

applied for the future investigation of *Rpbq3* QTL in order to unravel the role of the CNL presents in the QTL interval.

For *Rpbq3* a new large population could be developed following the methods used in the present study, in order to obtain another high resolution map and reduce the interval of the QTL.

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