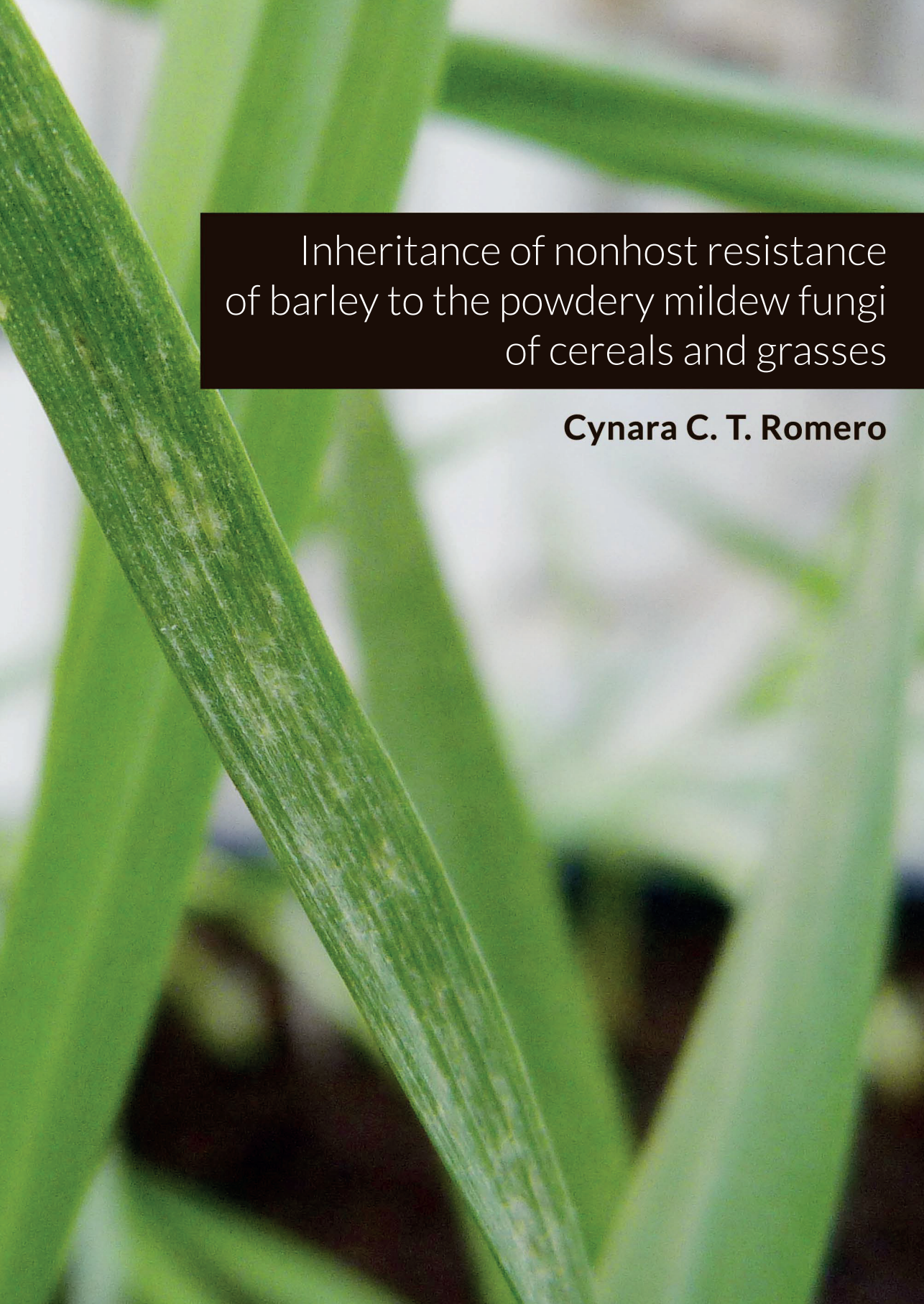

Inheritance of nonhost resistance of barley to the powdery mildew fungi of cereals and grasses

Cynara C. T. Romero

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Cynara C.T. Romero

Thesis

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Contents

Chapter 1

General introduction

The vast majority of microorganisms in the environment do not cause plant diseases. Nevertheless, pathogenic classes of viruses, bacteria, fungi and oomycetes have evolved to infect particular plant species, acquiring nutrients to grow and reproduce. Plant pathogens have the potential to cause epidemics that devastate crop production and result in substantial economic losses. Up to 20% of the world's harvest yield is lost to pathogens, with fungi and oomycetes being the most threatening to global food security (Bebber and Gurr 2015; Oerke 2005). In this context, breeding for resistant crop varieties has become increasingly important, since this is acknowledged to be an effective and environmentally sustainable approach (Wenzel 1985).

In the last century, breeding efforts resulted in the discovery and deployment of disease resistance (*R*) genes for most crop species, often originating from sexually compatible wild relatives (Dangl et al. 2013). *R*-gene-based resistance (also referred to as "vertical" or "race-specific") confers complete resistance to specific pathogen genotypes. This qualitative resistance is typically governed by single, major resistance genes (monogenic) and is relatively easy to handle in breeding programmes (Poland et al. 2009). However, this type of resistance is often not durable, since it favours the selection in the pathogen for mutations impairing recognition of effectors by the cognate R-protein (the *R*-gene-encoded protein in charge of specific pathogen effector recognition) (Poland et al. 2009; Zhang et al. 2013). Rapidly evolving pathogens have the potential to quickly break down the *R*-gene effectiveness.

In contrast to qualitative disease resistance, there is quantitative disease resistance. The term "quantitative" may refer to the *phenotypic* aspect, since this type of resistance is only partial and not complete, or to the *genetic* aspect – since quantitative resistance is conditioned by multiple genes, each contributing for a small amount to the total effect (Niks et al. 2015). Quantitative resistance is also called "horizontal", "partial", "non-specific" or simply "basal resistance", among others. It is the general experience that quantitative resistance is more durable than *R*-gene mediated resistance. As pointed out by Niks et al. (2015), an aspect that should be considered as an explanation for the durability of quantitative resistance is the possibility that adaptation by the pathogen requires a rather particular gain-of-function mutation to overcome a resistance gene. A gain-of-function mutation is far rarer than a loss-of-function mutation necessary to breakdown an *R*-gene resistance. Lasting quantitative resistance is the result of the combined effect of a number of minor-effect genes. A certain pathogen variant overcoming a single quantitative gene will only have a minor advantage, leading to a small selective advantage (Niks et al. 2015; Poland et al. 2009). Besides, there is a great diversity of such minor genes among commercially grown cultivars. Overcoming a minor gene in one cultivar confers a quite small advantage for the pathogen variant in that cultivar, but no advantage in other cultivars in which that minor gene does not occur (Niks et al. 2015).

It is possible to increase the level of partial resistance in crops by recurrent selection against susceptibility. There is an abundance of minor genes for quantitative resistance, coming from different genotypes. By crossing genotypes with moderate level of partial resistance, transgressive segregation will often be observed; accumulation of resistance genes by recurrent selection leads to higher levels of resistance in the progeny than any of the donor parents. This was successfully achieved in barley for resistance to barley leaf rust and powdery mildew (Aghnoum and Niks 2011; Parlevliet and van Ommeren 1988). Although the improvement of crop resistance on the basis of phenotypic selection is feasible, knowledge about loci and underlying genes is important and may enable more efficient ways of exploiting quantitative resistance in breeding programs. Quantitative trait loci (QTLs) for disease resistance have been mapped in numerous studied pathosystems. Marker-assisted selection (MAS) has been applied in breeding programs for transferring and pyramiding resistance QTLs (Pilet-Nayel et al. 2017; St Clair 2010).

Quantitative (basal) resistance to adapted pathogens is argued to reside on similar principles as nonhost resistance – the most durable and effective form of resistance in nature (Niks and Marcel 2009). Nonhost resistance is suggested as a potential novel source of disease resistance (Fan and Doerner 2012; Lee et al. 2016; Thordal-Christensen 2003). However, despite the importance of nonhost resistance and its potential for application in agriculture, the mechanisms and molecular framework underlying this type of resistance are still poorly understood (Delventhal et al. 2017; Lee et al. 2016). Barley is a very useful model crop for studying both quantitative host (e.g. Parlevliet 1979) and nonhost resistance (Aghnoum et al. 2010; Atienza et al. 2004; Jafary et al. 2008). Nonhost resistance in barley to non-adapted rusts (*Puccinia* ff.spp.) and powdery mildews (*Blumeria graminis* ff.spp.) was shown to be based on multiple genes (Dracatos et al. 2016; Jafary et al. 2008; Romero et al. 2018). Nevertheless, not much is known about the function of these genes determining the host or nonhost status of a plant species to a would-be pathogen (Niks 2014). A thorough understanding of plant innate immunity and of genes and mechanisms underlying nonhost resistance will support crop improvement.

The plant immune system

Plants, unlike animals, do not have a mobile immune system to defend themselves from potentially harmful microbes. Instead, they depend on the ability of each cell to recognize infection attempts and trigger defense responses that will prevent pathogen proliferation. Plants have evolved two strategies to detect invasion, governed by interconnected layers of receptors located outside and inside the plant cell (Dangl et al. 2013; Dodds and Rathjen 2010). The first strategy, that constitutes the first layer of defense, is based on the action of cell

receptor proteins called pattern recognition receptors (PRRs) located on the external surface of the plant cell. PRRs recognize molecules known as pathogen-associated molecular patterns (PAMPs, or the equivalent "microbe-associated molecular patterns", MAMPs), which are conserved structural components of whole pathogen taxa. Typical examples of PAMPs are flagellin and peptidoglycans from bacteria, and chitin from fungi (Boller and Felix 2009). PRRs may also recognize damage-associated molecular patterns (DAMPs), endogenous molecules that might be released during pathogen invasion, like oligogaracturonides released from plant cell walls upon fungal infection, for example (Macho and Zipfel 2014). Activation of PRRs leads to downstream signalling events that mobilize a complex array of defense responses, inducing a basal resistance response known as PAMP-triggered immunity (PTI) (Couto and Zipfel 2016; Macho and Zipfel 2014). PTI activation confers broad-spectrum disease resistance and represents the first phase of the widely accepted 'zig-zag' model of plant immunity (Jones and Dangl 2006).

Pathogens have adapted to particular host genotypes by evolving virulence factors called effectors, many of which are delivered into the host cell to interfere with PTI (Bent and Mackey 2007; Cui et al. 2015). Bacterial effectors are released into the host cell through a specialized structure known as type-III secretion system (TTSS) (Cunnac et al. 2009), while oomycetes and many pathogenic fungi deliver effectors through the haustoria, which are specialized feeding organs (Koeck et al. 2011). To counter the action of effectors, plants evolved a second strategy of defense, particularly against biotrophic pathogens, which involves intracellular receptors that recognize and intercept particular effectors. Plant intracellular receptors typically contain a nucleotide-binding (NB) and a leucine-rich repeat (LRR) domain. The activation of NB-LRR proteins induces effector-triggered immunity (ETI), often associated with hypersensitive cell death response (HR), which stops infection (Cui et al. 2015). The zig-zag model postulates continued co-evolution between the pathogen, evolving novel effectors in order to suppress ETI (or losing effectors in order to evade ETI), and the host, evolving new *R* gene alleles in order to re-establish ETI (Jones and Dangl 2006). Another option, not represented in the zig-zag model, is that plants slightly change the interaction site of molecules to which the effectors bind. Slight changes in that site may prevent the effector to increase the pathogenicity and would require a gain-of-function mutation by the pathogen (Niks et al. 2015).

PAMPs are classically seen as widely conserved structural molecules, essential for pathogen fitness, while effectors are specific to a single or few related species, highly diversified, rapidly-evolving and dispensable (Dangl et al. 2013). Although these definitions might hold true in many cases, the strict separation between PAMPs and effectors, as well as between PRRs and R proteins do not serve as a general rule. There are examples of effectors that have a wide distribution and PAMPs that are only narrowly conserved (Thomma et al. 2011).

Plant immune receptors and downstream responses

There are two known types of plant PRRs: receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Macho and Zipfel 2014). RLKs have an extracellular domain responsible for ligand-binding, a transmembrane domain and the intracellular kinase domain. The structure of RLPs is similar, although the intracellular kinase domain is lacking. The extracellular domains (or "ectodomains") will determine the ligand-binding nature of the PRR (Couto and Zipfel 2016). In order to activate immune signalling, both RLKs and RLPs form complexes with regulatory receptor kinases at the plasma membrane. The type of regulatory receptor kinase recruited seems to depend on the type of PRR ectodomain. As soon as the PRR complex is activated in response to pathogen perception, it initiates an active defense response called basal immunity (or PTI). A signalling cascade is triggered to confer local and systemic defense (Boller and Felix 2009). The early events include rapid ion-flux (H^+, K^+, Cl^- and Ca^{2+}) at the plasma membrane – which mediates the elevation of Ca^{2+} levels in the cytoplasm – and generation of reactive oxygen species (ROS) in an oxidative burst. Other key events in PTI response are the activation of calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) cascades, which transfer the signal to the nucleus triggering changes in gene expression. The high cytosolic concentration of Ca^{2+} activates CDPKs, which, in turn, phosphorylates a group of WRKY transcription factors. Several transcription factors downstream of CDPKs and MAPKs coordinate the transcriptional changes that results in the synthesis of enzymes, compounds or hormones playing a role in plant defense (Couto and Zipfel 2016). Upon fungal attack, a typical marker of PTI is the deposition of callose between the plasma membrane and the cell wall to form cell wall reinforcements called *papillae* at sites of attempted penetration (Zhang et al. 2013).

Acting on the second line of defense, intracellular NB-LRR (or NLR) proteins may contain an N-terminal TIR (Toll, interleukin-1 receptor, resistance protein) or a coiled-coil (CC) domain. TIR-NB-LRR receptors are found only in dicot plants (Cui et al. 2015; Dodds and Rathjen 2010). The specific recognition of effectors may occur via direct interaction between NB-LRR and effector, or indirectly, via an accessory protein that is recognized by the receptor after being modified by effector activity (Dodds and Rathjen 2010; Zhang et al. 2013). Intramolecular interactions keep the NB-LRRs in a restrained conformation ("off" state) before effector activation ("on" state). Upon direct or indirect effector activation, a series of conformational changes leads the protein to the "on" state by opening the molecule and exposing the N-terminal domain for interaction with signalling components (Cui et al. 2015). Activated NB-LRRs induce an array of immune responses, many of which are in common with PTI. Both layers of defense share the downstream signalling machinery to a great extent. Nevertheless, immune responses triggered during ETI are more prolonged in comparison to PTI

response (Tsuda and Katagiri 2010). The genetic overlap existing between basal (PTI) and specific (ETI) resistance responses implies that the purpose of signalling mediated by NB-LRRs is to activate defense mechanisms shared by both layers in a more efficient and rapid way (Dangl and Jones 2001). The defense responses triggered during ETI include ROS bursts, Ca^{2+} influx, MAPK cascades, transcriptional reprograming, hormonal changes and hypersensitive response (HR) (Cui et al. 2015; Zhang et al. 2013).

Nonhost resistance

One of the most popular definitions of nonhost resistance states that it is "the resistance shown by an entire plant species to a specific parasite or pathogen" (Heath 2000). Considering the big picture of all possible interactions between plant and microorganisms, disease is certainly the exception. Nonhost resistance confers robust and durable protection against pathogenic microorganisms, and is therefore proposed as a novel source of resistance mechanism for crop improvement (Fan and Doerner 2012; Lipka et al. 2005; Thordal-Christensen 2003).

To cope with the diversity of microorganisms, nonhost resistance relies on a multitude of defense components, either pre-formed or induced (Fan and Doerner 2012). The majority of nonadapted pathogens fail to overcome pre-formed physical or chemical barriers such as cuticle and the plant cell wall. The chemical composition of leaf waxy cuticle is an important determinant of whether the microorganism will develop infection structures and hence these are potentially relevant factors in nonhost resistance (Lee et al. 2017; Thordal-Christensen 2003; Tsuba et al. 2002). Induced pre-invasive defenses such as cytoskeleton rearrangements occur in response to penetration attempts, leading to cytoplasmic aggregation and cell-wall apposition at the penetration attempt sites (Lee et al. 2017). Secondary metabolite synthesis can also mediate nonhost resistance: while phytoanticipins are constitutively produced, phytoalexin production is stimulated through pathogen perception, often mediated by the MAPK cascade.

Nonhost resistance appears to be governed by several genes. Transcriptomic and metabolomic studies provided evidence that nonhost resistance shares signalling components with basal host resistance (Lee et al. 2017). A topic of debate is whether both PTI and ETI contribute to nonhost resistance. One possibility is that, in nonhost pathosystems, PTI is sufficient to confer durable resistance because non-adapted pathogens lack appropriate (adapted) effectors to subvert this first line of defense, therefore immunity will be the ultimate outcome. A second model for nonhost resistance predicts that immunity is actually determined by multiple *R*-genes encoding NB-LRRs responsible for perceiving a number of effector proteins. In the latter case, functional redundancy would be responsible for the durability of nonhost resistance (Lipka et al. 2010; Schweizer 2007). According to the evolutionary model for nonhost

resistance (Schulze-Lefert and Panstruga 2011), the relative contribution of ETI or PTI varies according to the phylogenetic distance between the host and nonhost plants. In cases where the nonhost is relatively closely related to the host, ETI would have a higher contribution than PTI. If, however, the host and nonhost plant diverged a longer time ago, nonhost resistance would predominantly be due to the pathogen not able to suppress PTI (due to the lack of appropriate effector targets).

Early investigations on the genetics of nonhost resistance utilized the interaction between Arabidopsis and non-adapted powdery mildews as a model system. Two independent and complementary layers of defense were identified, at pre-haustorial (pre-invasion) and post-haustorial (post-invasion) levels (Lipka et al. 2010). Arabidopsis mutants for the genes *PENETRATION1* (*PEN1*), *PEN2* and *PEN3* show impaired pre-invasive defenses, allowing enhanced haustorium formation by the non-adapted *B. graminis* f.sp. *hordei* (*Bgh*) and *Erysiphe pisi* (*Ep*), the barley and pea powdery mildew, respectively (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). Pre-penetration defense responses are associated with cytoskeletal rearrangements, organelle transport and papilla formation. The few successful penetration attempts are stopped by post-invasion resistance, when attacked cells would often undergo HR-like cell death (Lipka et al. 2010). While the *PEN* genes play a role in pre-invasion resistance, EDS1, PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101) act on the post-penetration level of resistance, inhibiting hyphal growth (Lipka et al. 2010; Lipka et al. 2005; Thordal-Christensen 2003).

Identification of genes underlying the specificity of (non)host status

Although several genes associated with nonhost resistance have been identified, a question that remains to be elucidated is: which are the genes that actually determine the specificity of host and nonhost interactions? Mutagenesis and gene expression studies are useful in identifying the genes that are involved in defense response against non-adapted pathogens. Nevertheless, such genes are normally involved in general plant defense mechanisms and act downstream of the actual gene(s) that determine whether or not the plant will be infected by the non-adapted pathogen (Niks 2014; Niks and Marcel 2009). It is proposed that inheritance and mapping studies and, ultimately, cloning are necessary to reveal the genes responsible for the specificity of the nonhost status (Niks and Marcel 2009). According to this hypothesis, QTLs represent defense network genes that are 'operative targets' of effectors, i.e. plant targets that, once manipulated by the effector, will confer enhanced pathogen fitness. Even subtle motif variations in the underlying genes or their regulatory regions can interfere with the effector affinity, influencing the specificity of the interaction (Niks et al. 2015).

The obvious difficulty in performing inheritance studies for nonhost resistance is that it assumes interspecific crosses between a host and a nonhost plant to generate a segregating population, which is rarely feasible. To overcome this problem, plants showing natural variation on the level of resistance/susceptibility against a particular non-adapted pathogen can be used (Atienza et al. 2004). Barley is considered a near-nonhost to several non-adapted rusts and powdery mildew species, because a few accessions showing moderate or rudimentary susceptibility can be found (Atienza et al. 2004; Niks 2014).

Barley-powdery mildew as a model system

The study of barley-powdery mildew interaction serves as an experimental model system for other plant-pathogen interactions for biological and practical reasons (Collinge et al. 2008). Traditionally, studies of powdery mildew interaction with the respective host species wheat and barley allowed genetic analyses that revealed major *R* genes (Jørgensen and Wolfe 1994), many of which have been introgressed into near isogenic lines (NILs). This fungal pathogen colonizes the epidermal cell layer of the plant, thus allowing easy visualization by light microscopy of cellular events taking place in the host during the interaction, as well as the successive developmental stages of the pathogen. Besides, this interaction is also well suited for single-cell analysis, due to the cell-autonomous nature of the defense response against invasion (Huckelhoven and Panstruga 2011).

Studies of nonhost resistance were also performed using the barley-powdery mildew pathosystem (Aghnoum and Niks 2010; Delventhal et al. 2017; Douchkov et al. 2014; Romero et al. 2018; Zellerhoff et al. 2010). Although almost all barley genotypes are immune to the non-adapted wheat powdery mildew, *Blumeria graminis* f. sp. *tritici* (*Bgt*), a few exotic accessions show a rudimentary susceptibility to this *f.sp*. This natural variation was used to accumulate susceptibility factors to *Bgt* and develop two barley lines with increased susceptibility, $SusBgt_{SC}$ and $SusBgt_{DC}$ (Aghnoum and Niks 2010). These two experimental lines enabled studies that provided valuable insights on the specificity of the nonhost status in barley (this thesis, chapters 2 and 3). Inoculation of SusBgt lines, as well as SusPtrit, with a high inoculum density of *Bgt* results in the formation of microcolonies, which appear as small white dots over the leaf surface. Microcolonies formed by this non-adapted pathogen contrast with colonies formed by the adapted pathogen, *Bgh*, which are much larger and produce a great number of conidia (Fig. 1).

Transient gene expression assays based on ballistic transformation are common for studying cereal-powdery mildew interaction. Because powdery mildew infection is restricted to the epidermal cell layer of the host, transient overexpression (Tox) and transient-induced gene silencing (TIGS) can be successfully employed to test whether candidate genes influence the

susceptibility or resistance of barley to powdery mildew (Douchkov et al. 2005; Ihlow et al. 2008; Panstruga 2004).

Fig. 1 Barley line SusPtrit, 10 d after inoculation with the wheat powdery mildew *B. graminis* f.sp *tritici* (*Bgt*, ~50 conidia/mm^2), showing the formation of microcolonies (small white dots over the surface of the leaves). The arrows indicate colonies formed by the adapted barley powdery mildew *B. graminis* f.sp. *hordei* (*Bgh*).

Barley

Cultivated barley (*Hordeum vulgare* ssp. *vulgare* L.) belongs to the Triticeae tribe of the grass family, Poaceae. Other economically important cereals are also part of the Triticeae tribe: wheat (*Triticum*), rye (*Secale cereale*) and triticale (*Triticosecale*). The genus *Hordeum* comprises 32 species and 45 taxa and occurs in most temperate areas of the world (Pourkheirandish and Komatsuda 2007; von Bothmer and Komatsuda 2010). Cultivated barley and its wild progenitor *H. vulgare* ssp. *spontaneum* belong to the same biological species. Because there are no crossing barriers between ssp. *vulgare* and ssp. *spontaneum*, a high frequency of introgression occurs, particularly in areas where wild and cultivated barley are in close contact (Pourkheirandish and Komatsuda 2007). For this reason, the wild ancestor has potential to be a good source of exotic germplasm in breeding programs (von Bothmer and Komatsuda 2010).

Barley domestication is estimated to have occurred about 10.000 years ago in the Fertile Crescent (Zohary et al. 2012). Among the key traits that were selected by ancient farmers to domesticate barley as a crop for agricultural production were: non-brittle rachis, six-rowed spikes and naked caryopsis (Pourkheirandish and Komatsuda 2007; Salamini et al. 2002). The non-brittle rachis mutant was essential because the spikes remained attached to the plant after maturation, enabling efficient harvest. In ancient times barley was supposedly used as human food, but nowadays it is mostly employed as animal feed (50-60% of its crop production) and substrate for malting (30-40% of crop production). The use of barley in human diet remains relevant in some cultures, particularly in Asia and north Africa, and several health benefits are acknowledged due to the nutritional composition of the grains (Baik and Ullrich 2008). In 2014, barley was the fourth most produced cereal crop worldwide, with 144.5 million tonnes, after maize, rice and wheat (http://www.fao.org/faostat/) (Fig. 2).

Barley is a diploid autogamous crop traditionally used as a model for genetic studies in cereals, and especially a good genomic model for hexaploid bread wheat (Schulte et al. 2009). The barley genome is large and abundant in repetitive DNA: of its 5.1 Gb (haploid size), it is estimated that 84% is composed of mobile elements or other repeat structures (IBSC 2012). Only recently a high-quality reference barley genome assembly sequence has been released, with data from the spring barley cultivar Morex (Mascher et al. 2017). The current version of the barley reference genome represents 4.79 Gb (~ 95%) of the genome, and contains 6.347 "super-scaffolds" composed of merged assemblies of individual Morex BACs, ordered and assigned to chromosomes based on genetic map information (Mascher et al. 2017; Mascher et al. 2013). Based on transcriptome data and protein sequences from other plant species, 83.105 putative gene loci were identified.

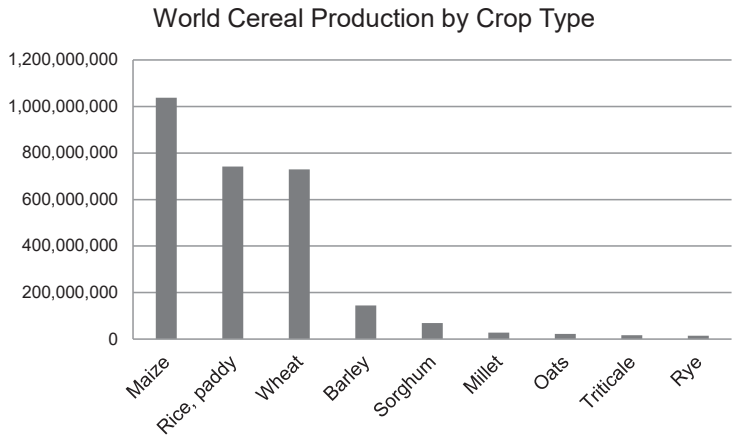

Fig. 2 World cereal production by crop type (in tonnes). Data from http://www.fao.org/faostat/ accessed on October 2017.

Powdery mildew

Powdery mildew is a widespread disease caused by obligate biotrophic Ascomycetes from the Erysiphales order. A number close to 10,000 species of angiosperms can be infected by mildews, including several economically important crops (Glawe 2008). Powdery mildews of cereals and grasses (subfamily Pooideae) are caused by *Blumeria graminis* (DC.) Speer (formerly *Erysiphe graminis*). Due to the impact on wheat and barley production worldwide, *B. graminis* was ranked among the top 10 most harmful fungal plant diseases (Dean et al. 2012; Spanu 2014). In addition to the significant economic impact on crop production, the importance of *B. graminis* relies on the fact that they serve as experimental models in plant-pathogen interaction studies, as well as developmental morphology, cytology, and molecular biology (Glawe 2008). The

pathogen colonizes the epidermal cell layer, enabling easy inspection of cellular interactions by light microscopy observations (Huckelhoven and Panstruga 2011).

Life cycle

B. graminis can reproduce sexually and asexually. Sexual recombination occurs between two individuals of opposite mating types: their hyphae fuse and form a chasmothecium. The chasmotecia are structures capable of surviving for long time under adverse conditions. When conditions are favourable, the chasmothecia releases the ascospores (Spanu 2014). Most commonly observed for this pathogen is asexual reproduction, which consists of short cycles that can repeat several times in the course of a crop growing season. Asexual cycles are, therefore, the most important for the spread of the disease (Spanu 2014) and starts when a wind-dispersed conidium or an ascospore lands on a susceptible plant and germinates, forming a primary germ tube (PGT) approximately 1-2 h after inoculation (hai). The formation of the PGT is unique to *B. graminis*, and has been implicated in the recognition of the host surface (Yamaoka et al. 2007). A small structure called cuticular peg is produced by the PGT that penetrates the cuticular layer of the plant and is believed to be involved in the attachment of the conidium to the host (Glawe 2008). After sensing the host surface, the conidium develops an appressorial germ tube (AGT), which differentiates into an appressorium (APP), from which a protrusion emerges about 12 hai: the penetration peg (PP). As the name suggests, the penetration peg is in charge of penetrating the host cell. It was demonstrated that the fungus makes use of cell-wall degrading enzymes, as well as mechanical forces to breach the cell wall (Glawe 2008; Spanu 2014). The plant will generally respond by producing papilla, cell wall appositions that prevent invasion in most of penetration attempts. Papilla-based resistance occurs in nonhost barley-*Bgt* interaction as well as in quantitative (race non-specific) and *mlo*-mediated host resistance against *Bgh* (Trujillo et al. 2004).

In case of successful cell wall penetration, the penetration peg grows and differentiates to form the haustorium, a multi-digitate structure responsible for nutrient uptake. The extrahaustorial membrane, derived from the host plasma membrane, surrounds the haustorium and separates it from the cytoplasm (Huckelhoven and Panstruga 2011). Following haustorial establishment, fungal growth continues on the leaf surface, forming secondary hyphae to further attack the neighbouring cells forming additional haustoria. Three to five days after inoculation (dai), conidia begin to be produced on conidiophores, structures that grow perpendicularly to the leaf surface from secondary hyphae (Glawe 2008; Spanu 2014). At this point, colonies are macroscopically visible. It is estimated that a single colony is able to produce up to 200.000 conidia (Zhang et al. 2005).

Taxonomy and host range

Fungi from the *B. graminis* species are classified into *formae speciales* (*ff.spp.)* based on host specialization (Wyand and Brown 2003). Eight *ff.spp.* were officially described for *B. graminis*: for the cultivated cereals barley (f.sp. *hordei*), wheat (f.sp. *tritici*), rye (f.sp. *secali*), oat (f.sp. *avenae*); and for wild grasses from the genera *Poa* (f.sp. *poae*), *Elymus* (f.sp. *agropyri*), *Dactylis* (f.sp. *dactylidis*) and *Bromus* (f.sp. *bromi*) (Oku et al. 1985; Troch et al. 2014). It is known that *B. graminis* infect some other grass species not officially described as hosts to named *ff.ssp.* (Troch et al. 2014), e.g. *Lolium perenne* (common name: ryegrass), *Hordeum murinum* (wall barley) and *H. secalinum* (meadow barley). The taxonomic classification into *ff.spp.* has raised some discussion due to evidence that the host range extends to more than one genus and may vary depending on the geographical origin of the isolate (Eshed and Wahl 1970; Wyand and Brown 2003). Troch et al. (2014) proposed that the *f.sp.* classification should no longer be applied for the isolates of most wild grasses, because many are polyphyletic and not highly specialized to their host species. For *B. graminis* infecting cultivated crops, however, the *f.sp*. classification should be kept.

Genome

The genomes of *B. graminis* f.sp *hordei* and f.sp *tritici* have been sequenced, and to a large extent assembled and annotated (Spanu et al. 2010; Wicker et al. 2013). The *B. graminis* genomes contain a high amount of repetitive DNA, attributed to retrotransposon activity, making the powdery mildews the most repetitive fungal genomes sequenced to date (Spanu 2014). Transposable element sequences account for more than 90% of the *Bgt* genome (Wicker et al. 2013). Comparative analysis between a few *Bgt* and *Bgh* loci showed a high degree of conservation and synteny (Oberhaensli et al. 2011), and over 90% of predicted genes in *Bgt* have homologs in the barley powdery mildew (Wicker et al. 2013). Genes encoding candidate effectors are abundant and diverse: in the *Bgh* genome 7% of the protein coding genes are candidate secreted effector proteins (CSEPs) and in *Bgt* 9.2% of genes are putative effector genes (including CSEPs and CEPs – candidate effector proteins). CSEP-encoding genes are closely associated with retro-transposon DNA, and it was suggested that transposable elements have promoted the multiplication and proliferation of CSEPs, therefore facilitating powdery mildew evolution (Pedersen et al. 2012). Such a role of transposons in effector diversification was first discovered in *Phytophtora* species, where transposon activity is associated with expansion and diversification of the effector repertoire (Haas et al. 2009; Stassen and Van den Ackerveken 2011).

Scope and outline of the thesis

In the present thesis, the main goal was to investigate the genetic factors responsible for the specificity of the (non)host status in barley to powdery mildews of cereals and grasses.

In chapter 2, two recombinant inbred lines (RIL) mapping populations were developed from the crossing of each of the SusBgt lines with barley cultivar Vada. The genotyping-by-sequencing technology was used to create a high-density genetic map for each of the populations. QTL mapping analyses allowed, for the first time, the identification of resistance factors against non-adapted powdery mildew in barley. QTL mapping for resistance to the adapted *Bgh* allowed us to verify whether the same genetic factors are implicated in nonhost resistanceas in basal resistance to the adapted powdery mildew fungus.

In chapter 3 we describe the fine-mapping of a major effect QTL for nonhost resistance to powdery mildew. This QTL, *Rbgnq1*, was mapped in both of the SusBgt populations in chapter 2, and again in this chapter, in the Vada x SusPtrit barley mapping population. We established the physical map for the QTL locus of cultivar Vada, the resistance allele. Candidate genes in the interval were tested by transient transformation, using the sequences of whole BAC clones.

Chapter 4 reports on a seed quality condition severely affecting lines of three barley mapping populations that have the experimental barley line SusPtrit in their parentage. The SusPtrit mapping populations, used in chapter 3, represent a valuable resource for the study of basal and nonhost resistance to rust and powdery mildew. Affected grains showed a dark discolored and shrivelled appearance, and germination was impaired – therefore compromising the multiplication and maintenance of the populations. No biotic agent was recognized as the fundamental cause of the problem. Based on seed quality scores QTLs were mapped in the three mapping populations. We compared the symptoms of our seeds with symptoms described in the relevant literature for cereal grain discoloration.

Finally, in chapter 5 the results obtained in previous chapters are discussed and contextualized.

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Chapter 2

Mapping resistance to powdery mildew in barley reveals a large-effect nonhost resistance QTL

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Abstract

The durability and effectiveness of nonhost resistance suggests promising practical applications for crop breeding, relying upon elucidation of key aspects of this type of resistance. We investigated which genetic factors determine the nonhost status of barley (*Hordeum vulgare* L.) to powdery mildews (*Blumeria graminis*). We set out to verify whether genes involved in nonhost resistance have a wide effectiveness spectrum, and whether nonhost resistance genes confer resistance to the barley-adapted powdery mildew. Two barley lines, $SusBgt_{SC}$ and $SusBgt_{DC}$, with some susceptibility to the wheat powdery mildew *B. graminis* f.sp. *tritici* (*Bgt*) were crossed with cv Vada to generate two mapping populations. Each population was assessed for level of infection against four *B. graminis* ff.spp, and QTL mapping analyses were performed. Our results demonstrate polygenic inheritance for nonhost resistance, with some QTLs effective only to non-adapted mildews, while others play a role against adapted and non-adapted forms. Histology analyses of nonhost interaction show that most penetration attempts are stopped in association with papillae, and also suggest independent layers of defense at haustorium establishment and conidiophore formation. Nonhost resistance of barley to powdery mildew relies mostly on non-hypersensitive mechanisms. A large-effect nonhost resistance QTL mapped to a 1.4 cM interval is suitable for map-based cloning.

Introduction

Plants are exposed to an infinity of microorganisms during their lifespan, many of which are potentially harmful. The evolution of a sophisticated and dynamic immune system has enabled plants to protect themselves against most infectious microorganisms. For a pathogen to be successful in infecting a host plant, it must be adapted to overcome several layers of defense (Jones and Dangl 2006; Nurnberger and Lipka 2005; Thordal-Christensen 2003). The most common outcome of infection attempts by potential pathogens on plants is failure, making by far most plant species nonhosts. Nonhost resistance has been defined as immunity of an entire plant species against all races of a particular non-adapted pathogen (Heath 2000; Lipka et al. 2010; Mysore and Ryu 2004). The typical durability and effectiveness of nonhost resistance suggests promising practical applications in breeding programs (Heath 2000; Lee et al. 2016; Niks 1987; Nurnberger and Lipka 2005). Two models were proposed in the last decade to explain nonhost resistance (Jones and Dangl 2006; Schweizer 2007). According to the first model, plant cell surface receptors (known as pattern-recognition receptors, PRRs) perceive pathogen-associated molecular patterns (PAMPs, also referred to as microbial-associated molecular patterns, MAMPs) or endogenous damage-associated molecular patterns (DAMPs) and trigger the first layer of defense response known as PAMP-triggered immunity (PTI). Non-adapted pathogens fail to suppress PTI because their effector repertoire is not adapted to nonhost plant targets to undermine defense. The second model presupposes that nonhost resistance is the result of active participation of intracellular receptors, mainly nucleotide-binding – leucine-rich repeat (NB-LRR) proteins encoded by resistance (*R*) genes. Effector molecules released by the pathogen to undermine PTI would be perceived by NB-LRRs, triggering a second layer of defense known as effector-triggered immunity (ETI) (Stam et al. 2014; Zhang et al. 2013). There are several examples of the participation of PTI and ETI on nonhost resistance (reviewed in Lee et al. 2017), and, although distinction between PAMPs and effectors may not be strict (Thomma et al. 2011), it is still an issue whether nonhost resistance relies mainly on PTI or on ETI. Schulze-Lefert and Panstruga (2011) hypothesized that, for cases where host and nonhost plant species are phylogenetically closely related, the contribution of ETI to nonhost resistance would be relatively higher than that of PTI.

Despite the undeniable practical relevance of nonhost resistance, the genetic mechanisms governing the (non)host status of a plant to a potential pathogen species are yet to be elucidated. It is known that basal resistance, defined as the "resistance activated by virulent pathogens on susceptible hosts" (Jones and Dangl 2006), and nonhost resistance share several aspects (Gill et al. 2015; Niks and Marcel 2009). Many studies using reverse genetics approaches have identified genes involved in basic plant metabolism as contributing to nonhost resistance

(Lee et al. 2016). Such genes, mostly components of general plant defense mechanisms, are widely conserved among plant species, and therefore their identification by mutagenesis or transcriptomics is not sufficient to explain the nonhost status of a plant (Niks 2014; Niks and Marcel 2009). Inheritance and mapping studies are, for this reason, necessary to reveal which genes determine host-nonhost status to a potential pathogen. The fundamental problem in studying the inheritance of nonhost resistance is its dependence on host x nonhost interspecific crosses, which are usually not interfertile (Niks and Marcel 2009). It was proposed that studying the genetics of the resistance in plants that have an intermediate status between host and nonhost could provide useful insights (Atienza et al. 2004; Zhang et al. 1994). Some plant species may be regarded as 'near-nonhosts' or 'marginal-hosts' with a few accessions being somewhat susceptible to a normally non-adapted pathogen (Niks 1987). This susceptibility may be true solely during seedling stage and/or when under high inoculum density.

Barley (*Hordeum vulgare* L.) is a near-nonhost to many non-adapted pathogens of cereals and grasses, including rust and powdery mildew fungi. Aghnoum and Niks (2010) tested 439 barley accessions for resistance to the non-adapted *Blumeria graminis* f.sp. *tritici* (*Bgt*), the wheat powdery mildew fungus. The great majority of the accessions were immune, but at least six showed a low degree of susceptibility. Four of those were selected to be inter-crossed and to develop two lines with increased susceptibility to *Bgt* at seedling stage. These lines, named $SusBgt_{SC}$ and $SusBgt_{DC}$, allowed a relatively high rate of haustorium formation by *Bgt* and three other non-adapted *B. graminis* forms. In barley, nonhost resistance to powdery mildews is typically due to formation of localized cell wall reinforcements, called papillae, preventing haustorium formation (Trujillo et al. 2004). Papilla formation is also a main feature of basal host resistance, as in barley with *mlo* resistance or with high gene dose of quantitative resistance to *B. graminis* f.sp. *hordei* (*Bgh*) (Aghnoum et al. 2010; Niks and Rubiales 2002). Although *Bgt* is not able to form as large colonies on barley leaves as it would on its host, the germlings that are able to penetrate the cell and establish an haustorium can grow enough mycelium to form microcolonies: tiny white dots on the epidermal layer of young leaves. Microcolonies depend mostly on one successful haustorium, or on several haustoria in one successfully colonized plant cell. Further attempts to penetrate additional plant cells were generally not successful (Aghnoum and Niks 2010).

The main goal of our research was to map the gene(s) underlying nonhost and basal host resistance in barley against four *ff.spp*. of *B. graminis* (three non-adapted and the adapted form). We determined whether genes involved in nonhost resistance may have a wide spectrum of effectiveness, with the same gene(s) having effect to multiple powdery mildew forms, and whether nonhost resistance genes may also confer resistance to the barley-adapted *Bgh*. Two mapping populations were developed by crossing the SusBgt lines with the barley cv Vada: Vada

x $SusBgt_{SC}$ and Vada x $SusBgt_{DC}$. We developed a high-density genetic map for each SusBgt population, using the genotyping-by-sequencing technology (Elshire et al. 2011; Poland et al. 2012). The QTL mapping results bring us one step further in the identification of genes responsible for the specificity of (non)host status.

Materials and methods

Plant material & DNA extraction

Two barley lines selected for relatively high susceptibility to the non-adapted mildew *Bgt* ($SusBgt_{SC}$ and $SusBgt_{DC}$) were crossed with cv Vada to develop two Recombinant Inbred Line (RIL) mapping populations. The Vada x $SusBgt_{SC}$ (VxS_{SC}) population consists of 110 RILs (104 RILs in F_7 generation and 6 in F_8) and the Vada X $SusBgt_{DC}$ (VxS_{DC}) population consists of 115 RILs (14 RILs in F_6 generation, 8 in F_7, 86 in F_8, and 7 in F_9). Genomic DNA of the RILs from both populations was extracted from leaf tissue of 16 days-old seedlings (one seedling per RIL), using a modified version of the CTAB method (Fulton et al. 1995). DNA samples were RNase-treated and column-cleaned using the Quiagen DNeasy Plant Midi kit. DNA concentrations were quantified using the QubitBR kit (Thermofisher Scientific) and diluted to a final concentration of 20-25 ng/µL. The integrity of DNA samples was confirmed on a 0.8% agarose gel with 1% Ethidium Bromide.

Genotyping and genetic map construction

Both mapping populations were genotyped using the Genotyping-by-sequencing (GBS) approach (Elshire et al. 2011) following a two-enzyme protocol (Poland et al. 2012) essentially as described previously (Wendler et al. 2014). For sequencing-by-synthesis (single read, 1x100 cycles), the Illumina HiSeq2500 device (IPK Gatersleben, Germany) was employed (Wendler et al. 2014). Illumina adapters were trimmed from the raw reads using Cutadapt version 1.8.1 (Martin 2011). Trimmed reads were aligned to the whole-genome shotgun assembly of barley cv Morex (International Barley Genome Sequencing Consortium 2012) using BWA-MEM version 0.7.12 (Li 2013). After conversion to BAM format with SAMtools (Li et al. 2009), the resulting alignments were sorted and indexed with Novosort (http://www.novocraft.com/products/novosort/). SNP calling was performed with SAMtools version 1.3 (Li 2011) using the commands 'samtools mpileup –DV' and 'bcftools call –c –f GQ'. The resulting VCF file was filtered with the AWK script gen_call.awk provided by Mascher et al. (2013b). Only SNPs with a minimum quality (QUAL) of 40 were considered. Genotype calls were set to missing if their coverage was below 2 (4) and their quality score (GQ) below 5 (10) for homozygous (heterozygous) calls. Genetic maps were calculated separately for both populations. Only SNPs with a minor allele frequency of at least 30 % and missing rate below 10

% were considered for map construction. Linkage maps were built with MSTMAP (Wu et al. 2008) using the population type 'RIL8' and a cut-off p-value of 10^{-12}. Correctness of the maps was assessed by comparison to the POPSEQ reference map (Mascher et al. 2013a) using R scripts (R Core Team 2016).

A set of markers homogeneously distributed along the chromosomes at distances of ~ 3 cM was extracted from the SNP matrices, with the condition that they were polymorphic for both populations – that would facilitate later comparison of QTL positions among populations. Selected markers were used to build a skeletal map for each mapping population for QTL mapping analysis.

Inoculum material and inoculation trials

Four isolates, each belonging to a different *forma specialis* (*f.sp.*) of *Blumeria graminis* were tested: the powdery mildew fungus of wheat, *B. graminis* f.sp. *tritici* (*Bgt*, Swiss field isolate FAL92315), two isolates collected from wild grasses (*Hordeum murinum* and *H. secalinum*) near Wageningen-NL, and referred to in this paper as: *B. graminis* f.sp. *hordei-murini* (*Bghm*), and *B. graminis* f.sp. *hordei-secalini* (*Bghs*), respectively; the fourth *f.sp*. was the adapted powdery mildew of barley, *B. graminis* f.sp. *hordei* (*Bgh*, collected in Wageningen). The mildew isolates were continuously propagated on their respective host plants (for wheat: cv Vivant; for barley: cv Manchuria).

Each population was phenotyped for level of infection in two consecutive experiments, with two seedlings/RIL per experiment. The whole set of RILs was grown in boxes (40 x 60 cm), together with the parent Vada and both $SusBgt_{SC}$ and $SusBgt_{DC}$ lines as references. Also the host plants (either wheat cv Vivant, *H. murinum* or *H. secalinum*, depending on the inoculation experiment) were included in the trays to verify the viability of the inoculum. Compost soil was used as a substrate. The seedlings grew up in a controlled growth chamber (18-20°C day time, 15°C night time, 40-60% relative humidity, 16h photoperiod) until they were *c*. 13 days-old. The first leaf of each seedling was pinned horizontally to the substrate with the adaxial side up, using metal pins; remaining emerging leaves were removed. Inoculations were performed in a settling box (100 cm x 120 cm x 87 cm height), where the entire population was placed to be inoculated at once. Fresh conidia from heavily sporulating host leaf segments were blown into the settling box using compressed air, until the aimed density was reached. For the non-adapted forms (*Bgt*, *Bghm* and *Bghs*), the density was 20-30 conidia/mm^2; for the adapted pathogen (*Bgh*), around 5 conidia/mm^2. Metal pins were kept on the leaves until the next day. Inoculated seedlings were transferred to a second compartment (same conditions as previous one) where they were kept until the moment of evaluation. Macroscopic evaluation occurred 7 d after inoculation (dai) with the non-adapted mildew, when seedlings were assessed for level of

infection. Non-adapted fungi can only grow enough to form microcolonies, visible as small white spots over the surface of the leaf. A relative scale was set, in which the score of each RIL was always given in comparison to the references Vada (resistant, no microcolony formation; score 1) and SusBgt lines (susceptible, high degree of microcolony formation; score 5; Fig. 1a). Some RILs showed more fungal growth than the respective SusBgt parent, and therefore were given a score higher than 5. Phenotyping of the *Bgh*-inoculated plants occurred 4 dai, by assessing infection frequency (number of colonies formed in a 2 x 1 cm^2 area) using a metal frame with a rectangular opening of 1 cm^2 and a magnifying glass. Seven days after inoculation with *Bgh*, the presence of necrotic reaction was also assessed, on a scale of 1 to 4 (1: no necrotic reaction observed; 4: highest necrosis reaction observed in the population).

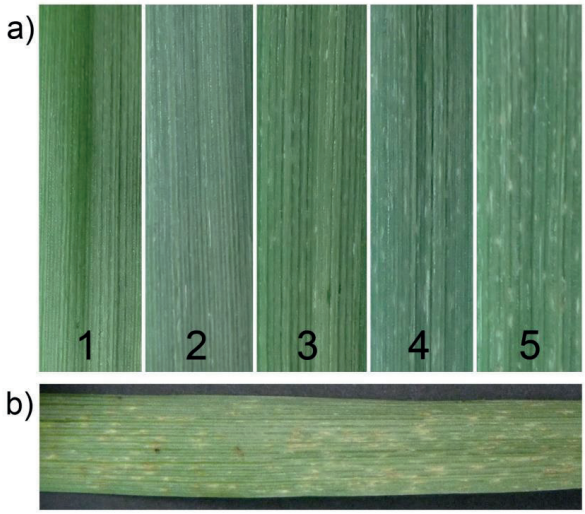

Fig. 1 (a) Illustration of the relative scale values used to assess the degree of microcolony development on the surface of barley (*Hordeum vulgare*) leaves 7 d after inoculation with *Blumeria graminis* f.sp. *tritici* (*Bgt*) or f.sp. *hordei-murini* (*Bghm*); **(b)** Barley line SC-28 8 d after inoculation with *Blumeria graminis* f.sp. *tritici* (*Bgt*), showing mild necrotic reaction phenotype

Microscopic evaluation of barley-Bgt interaction

Seven RILs from the VxS_{SC} population and 13 RILs from the VxS_{DC} population scoring higher than 3 during the macroscopic phenotyping with *Bgt* were sampled to assess microscopically the number of established microcolonies/cm^2 and the conidiation rate (percentage of microcolonies that produced at least one conidiophore). The parental lines were included in the microscopic analysis to serve as references.

Leaf segments of *c*. 4 cm were transferred to tubes containing a solution of acetic acid-ethanol (1:3 v v^{-1}) to be cleared for at least 1 day. For staining, segments of *c*. 1 cm were cut and immersed for 25 min in a solution of 15% trichloroacetic acid and 0.6% Coomassie Brilliant

Blue in 99% methanol (1:1 v v^{-1}) (Wolf and Fric 1981). Leaf segments were then transferred to a solution of acetic acid : glycerol : Milli-Q water (5:20:75) for 5-10 min to remove the excess of dye. Microscope slides were prepared by embedding the stained leaf segments in 100% glycerol, with the adaxial side up. Slides were screened using bright field microscopy with a total 100x magnification under a white light microscope. Germlings showing secondary elongating hyphae were considered as established (here called microcolonies). For each sample, the number of microcolonies was counted and expressed in microcolonies/cm^2. For each barley line, segments of 2 leaves were assessed per inoculation experiment. Statistical analyses were performed using Genstat (VSN International 2015). An ANOVA followed by a Fisher's unprotected LSD ($P < 0.05$) was performed to test for significant differences in the rates of establishment and conidiation.

QTL mapping

QTL analyses for both mapping populations were performed using MapQTL 6 software (Van Ooijen 2009). The skeletal maps of VxS_{SC} and VxS_{DC} contained 354 and 372 markers, respectively. QTL mapping was performed independently for the two replicate experiments and for the average of both. The mapping analysis was done in three steps. First, an interval mapping (IM) was performed using a mapping step size of 5 cM. A LOD threshold of 2.9 was set (estimated with a permutation test at 1000 permutations, using a significance level of $P < 0.05$) to declare QTLs. The identified QTL peak markers were chosen as cofactors for the subsequent mapping steps, multiple-QTL mapping (MQM) and restricted multiple-QTL mapping (rMQM) (Jansen 1993; Jansen and Stam 1994).

Graphical maps of both populations were constructed using MapChart v2.3 (Voorrips 2002), to indicate the regions where QTLs were found. The averages of macroscopic disease scores for QTL allele combinations were compared and tested for significant differences with an ANOVA following a Fishers' unprotected LSD ($P < 0.05$) using Genstat 18th edition (VSN International 2015).

Conidia viability test

The viability of *Bgt* conidia produced on barley plants was tested for the ability to re-infect its natural host, wheat. For this trial, $SusBgt_{SC}$, $SusBgt_{DC}$, Vada and one RIL of each population were selected: SC-45 and DC-02. Both RILs had an average macroscopic score slightly higher than their susceptible parent. Three seeds per genotype were sown, and 12 days later seedling leaves were inoculated with *Bgt* to a density of 22 conidia/mm^2, following the same inoculation procedure previously described. Ten days after inoculation, the infected barley leaves were detached and rubbed against the leaves of 11 days-old wheat cv. Vivant plants. Each of the three barley seedlings per genotype was gently rubbed against the first and second leaves of an

individual wheat plant, only in areas delimited by a marker pen. Wheat seedlings not treated with any barley leaf were also added to the experiment as negative controls. The growth of *Bgt* colonies on wheat seedlings was assessed 5 days later.

Results

Phenotyping of the resistance to non-adapted Blumeria forms

The two mapping populations were evaluated macroscopically for degree of microcolony formation by the non-adapted forms *Bgt*, *Bghm* and *Bghs*. For *Bghs* it was not possible to observe any microcolony on the parents nor on a subset of 50 random RILs, even 14 dai (Fig. S1a). Samples of leaves from the parental lines inoculated with *Bghs* were examined under the microscope. We observed 2.2 and 4.5 microcolonies/cm^2 for $SusBgt_{SC}$ and $SusBgt_{DC}$, respectively, which was apparently too low to result in macroscopically visible infection.

For *Bgt*, RILs were scored on a scale of 1 to 5, having the phenotypes of the parental lines as references (Fig. 1a, Fig S1b). This phenotyping method proved reliable, as indicated by the high correlation of scores between inoculation experiments (Table S1). The majority of RILs showed no macroscopic symptom to *Bgt* infection, and were given the lowest score '1' (Fig. S2 a-b). The highest scores were assigned to lines with a similar level of fungal growth as on the SusBgt parent. A one-way ANOVA followed by Fisher's unprotected LSD ($P < 0.01$) on the top 50 RILs with highest scores in each population showed that 7 out of 110 (6.36%) in the VxS_{SC}, and 10 out of 115 (8.69%) in the VxS_{DC} population scored not significantly lower than the respective SusBgt parent. Scores for 41 out of 110 RILs for VxS_{SC} and 41 out of 115 RILs for the VxS_{DC} population were continuously distributed between 1.5 and 5.

The distribution of the macroscopic disease scores for *Bghm* was similar to those for *Bgt*: more than 75% of RILs in both populations scored lower than '2', and only a small number of RILs had scores above '4' (Fig. S2 c-d, Table S1). Average infection scores for *Bgt* and *Bghm* were highly correlated ($r > 0.7$ for both populations). The shape of the frequency distributions, with the vast majority of RILs showing a resistant phenotype, suggests that several genes are involved in the nonhost resistance. The very skewed frequency distributions suggests that resistance alleles at one of the loci already results in a substantial level of resistance. A limited hypersensitive reaction (HR) was observed in association with microcolonies development, in some RILs (Fig. 1b).

Genetic map construction and QTL mapping

The SusBgt mapping populations were genotyped using the Genotyping-by-sequencing (GBS) approach (Elshire et al. 2011) following a two-enzyme protocol (Poland et al. 2012; Wendler et

al. 2014). We obtained on average 1.7 million reads per sample (min: 0.5 million; max: 14.8 million). Read mapping against the whole-genome shotgun assembly of barley cv Morex (International Barley Genome Sequencing Consortium 2012), SNP calling and linkage map construction were performed following a previously published pipeline (Mascher et al. 2013b).

The high-density genetic maps contained a total of 6,966 (VxS_{SC}) and 7,422 (VxS_{DC}) SNP markers (available with the online version of this publication, Romero et al. 2018). The largest gap between two adjacent loci was 6.21 cM in the VxS_{SC} population on chromosome 1H (from 100.2 cM to 106.4 cM) and 6.42 cM on chromosome 2H (from 68.8 cM to 75.3 cM) in VxS_{DC}. Total genetic lengths of the linkage maps were 1007 cM for VxS_{SC} and 1023 cM for VxS_{DC}.

Two RILs were excluded from the QTL mapping analysis due to a high percentage of missing genotyping data: DC-26 and DC-101. QTLs mapped in this study for resistance to non-adapted mildews were named '*Rbgnq*' (acronym for Resistance to *Blumeria graminis* nonhost quantitative) and followed by a number, according to the order in which they were mapped. In total, four chromosome regions were associated with nonhost resistance. QTL mapping results based on macroscopic disease scores were similar for *Bgt* and *Bghm* (Fig. 2). Peak markers of QTLs mapped in one population were in general overlapping with the LOD-1 region of a QTL mapped on the other population, and therefore received the same name (Table 1). The two largest effect QTLs (*Rbgnq1* and *Rbgnq2*) are effective to both non-adapted forms. *Rbgnq1*, on linkage group 5H, had the highest LOD scores and estimated additive effects; it appeared consistently over the inoculation experiments in both SusBgt populations and is a major-effect QTL for nonhost resistance to powdery mildew. Another QTL, *Rbgnq3*, has the resistance allele contributed by the susceptible parent. *Rbgnq3* was sometimes mapped with a LOD score slightly below threshold, but the data were still included in Table 1 because the LOD score for resistance to *Bghm* in the VxS_{SC} population was above the threshold. *Rbgnq4*, located near the telomere of the short arm of chromosome 1H was mapped only in the VxS_{DC} population for resistance to *Bghm*.

To look for possible interaction effects of QTLs on the macroscopic infection scores, we grouped the RILs according to the alleles of the nonhost resistance QTLs (Table 2; refer to Table S2 for similar results on the VxS_{DC} population). In general, RILs only show high susceptibility scores when all resistance alleles are absent. Because of the high contribution of *Rbgnq1* to the phenotype, RILs carrying the resistance allele for this locus show the resistant phenotype irrespective of the background QTLs In the absence of *Rbgnq1*, a similar resistant phenotype can be achieved if the resistance allele of the other two QTLs are both present. A couple of VxS_{SC} RILs carrying the Vada allele of the peak marker of *Rbgnq1* showed an unexpected high average score for *Bgt*, so we retrieved from the original high-density genetic map additional markers at this locus.

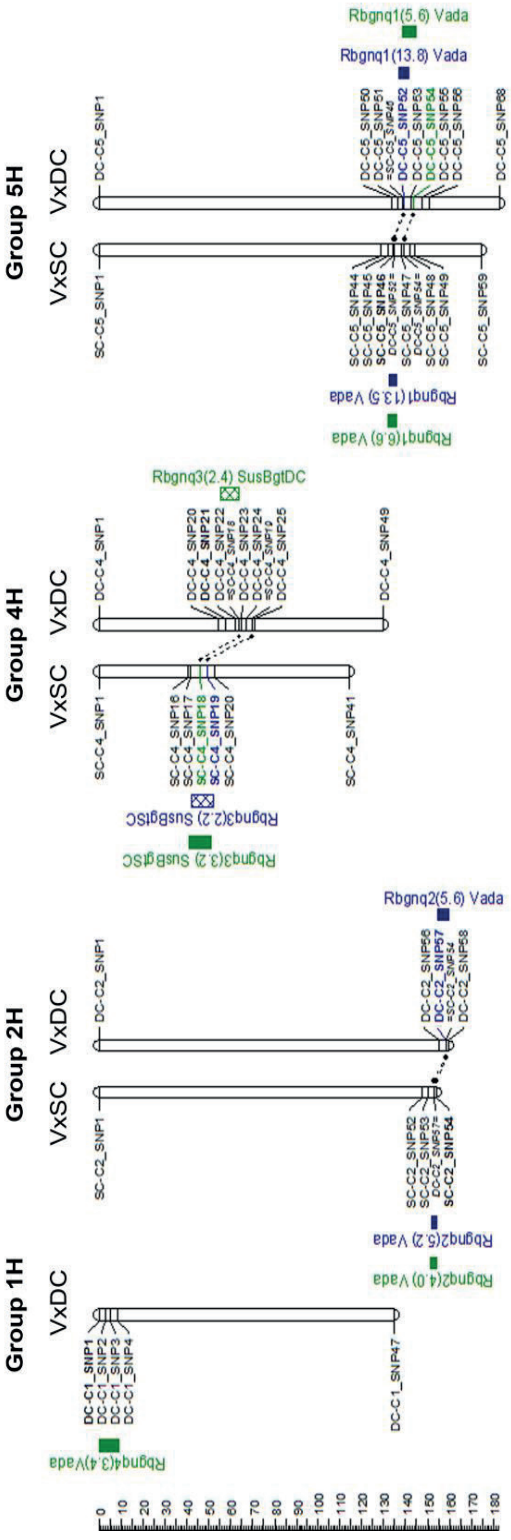

Fig. 2 Localization of QTLs for nonhost resistance to powdery mildew mapped in the Vada x SusBgt$_{SC}$ (VxSC) and Vada x SusBgt$_{DC}$ (VxDC) mapping populations. Bars along each linkage group represent the LOD-1 interval of QTLs mapped for resistance to different *ff. spp.*, indicated in colours: blue = *Blumeria graminis* f.sp. *tritici* (*Bgt*); green = f.sp. *hordei-murini* (*Bghm*). Shaded bars represent QTLs below the LOD threshold. Label to each QTL region mentions name of the QTL, its LOD score and the name of the parent contributing the resistance allele. For each linkage group, only the first and last markers of the skeletal map are represented, plus the markers at the QTL-containing regions. Linkage groups that did not contain significant QTLs were omitted from this figure. The ruler on the left indicates the distance in cM

Based on recombination points located in between the markers used for QTL mapping, it was possible to narrow-down the QTL interval to a window of 1.4 cM (available at Romero et al. 2018). The flanking markers were aligned to the map-based reference genome of cv Morex (Mascher et al. 2017) and delimit an interval containing 188 (VxS_{SC}) and 104 (VxS_{DC}) predicted genes.

Developing near-isogenic lines for a nonhost resistance QTL using RIL DC-04

A considerable difference in phenotypic scores was noticed for RIL DC-04 for the two *Bgt* inoculation experiments. This RIL was in F_8 and hence, harvested from a single F_7 plant. During the first inoculation DC-04 was given the maximum score of 5 (susceptible), while for the second inoculation it was given the minimum score of 1 (resistant). A third inoculation was set up for this line, and among the three DC-04 seedlings, one was susceptible and two were resistant. Probability for a marker or gene in F_8 to be heterozygous is $(0.5)^7$, which is 0.8 %. In a set of 115 RILs it is therefore expected to find about one such a segregating RIL for a particular locus. We found a segment of ~ 8 cM on chromosome 5H segregating for the region of *Rbgnq1* in this particular RIL, explaining the segregation in phenotype. RIL DC-04 therefore is a heterogeneous inbred family (HIF), from which a pair of near-isogenic lines is being developed, as proposed by Tuinstra et al. (1997).

Table 1. Summary of QTL mapping in the SusBgt mapping populations (Vada x $SusBgt_{SC}$, VxS_{SC}; and Vada x $SusBgt_{DC}$, VxS_{DC}) for nonhost resistance to *Blumeria graminis* f.sp. *tritici* (*Bgt*) and f.sp. *hordei-murini* (*Bghm*) and basal resistance to *B. graminis* f.sp. *hordei*. Mapping is based on results from the average of two inoculation experiments

Population	*f.sp.*	Trait[a]	QTL name[b]	Chr[c]	Peak marker	Position (cM)	LOD-1 interval (cM)	LOD[d]	% Expl[e]	Additive[f]	Donor resistance	Mapped for other *f.sp.*[g]
VxS_{SC}	*Bgt*	Microcolonies	*Rbgnq1**	5H	SC_C5-SNP46	134.2	132.9-135.8	**13.5**	41.8	-0.73	Vada	*Bghm*
	Bgt	Microcolonies	*Rbgnq2**	2H	SC_C2-SNP54	154.0	152.1-154.0	**5.2**	13.3	-0.41	Vada	*Bghm, Bgh* (=*Rbghq1*)
	Bgt	Microcolonies	*Rbgnq3*	4H	SC_C4-SNP19	49.5	42.2-52.2	**2.2**	4.9	0.24	$SusBgt_{SC}$	*Bghm*
	Bghm	Microcolonies	*Rbgnq1**	5H	SC_C5-SNP46	134.2	132.3-136.0	**6.6**	20.7	-0.40	Vada	*Bgt*
	Bghm	Microcolonies	*Rbgnq2*	2H	SC_C2-SNP54	154.0	151.6-154.0	**4.0**	11.9	-0.29	Vada	*Bgt*, *Bgh* (=*Rbghq1*)
	Bghm	Microcolonies	*Rbgnq3*	4H	SC_C4-SNP18	46.0	41.2-50.8	**3.2**	9.3	0.25	$SusBgt_{SC}$	*Bgt*
	Bgh	*IF*	*Rbghq1*	2H	SC-C2_SNP54	154.0	153.2-154.0	**11.6**	35.1	-4.794	Vada	*Bgt*, *Bghm* (=*Rbgnq2*)
	Bgh	*IF*	*Rbghq3*	6H	SC-C6_SNP40	116.8	111.5-119.2	**2.9**	7.1	-2.208	Vada	No
	Bgh	*Nec*	*Rbghq1**	2H	SC-C2_SNP54	154.0	153.4-154.0	**26.0**	68	1.084	Vada	*Bgt*, *Bghm* (=*Rbgnq2*)
VxS_{DC}	*Bgt*	Microcolonies	*Rbgnq1**	5H	DC_C5-SNP52	139.2	137.1-141.7	**13.8**	37.7	-0.70	Vada	*Bghm*
	Bgt	Microcolonies	*Rbgnq2**	2H	DC_C2-SNP57	158.3	154.9-159.7	**5.6**	12.9	-0.40	Vada	*Bgh* (=*Rbghq1*)
	Bghm	Microcolonies	*Rbgnq1*	5H	DC_C5-SNP54	143.4	139.0 -144.6	**5.6**	17.0	-0.42	Vada	*Bgt*
	Bghm	Microcolonies	*Rbgnq3**	4H	DC_C4-SNP21	57.4	55.5-63.6	**2.4**	6.3	0.24	$SusBgt_{DC}$	No
	Bghm	Microcolonies	*Rbgnq4*	1H	DC_C1-SNP1	0	0-9.2	**3.4**	9.8	-0.31	Vada	No
	Bgh	*IF*	*Rbghq1**	2H	DC-C2_SNP58	159.7	159-159.7	**8.3**	20.6	-2.738	Vada	*Bgt* (=*Rbgnq2*)
	Bgh	*IF*	*Rbghq2*	7H	DC-C7_SNP2	3.2	0-4.0	**11.8**	31.6	3.406	$SusBgt_{DC}$	No
	Bgh	*Nec*	*Rbghq1**	2H	DC-C2_SNP58	159.7	158.2-159.7	**4.0**	9.8	0.38	Vada	*Bgt* (=*Rbgnq2*)
	Bgh	*Nec*	*Rbghq2*	7H	DC-C7_SNP1	0	0-0.3	**13.2**	39.4	-0.77	$SusBgt_{DC}$	No

[a] For nonhost interactions, the macroscopic infection score ('microcolonies', data correspond to the average of two inoculation experiments) was assessed; for basal resistance, both 'IF' (Infection frequency, data from the average of two inoculation experiments) and 'Nec' (necrosis phenotype, based on the results of the second inoculation experiment) were assessed.

[b] The QTLs mapped for nonhost resistance (Bgt and Bghm) are named '*Rbgnq*' and those mapped for basal resistance (*Bgh*) were named '*Rbghq*,', in both cases followed by a number based on the order and relevance in which they were mapped. The QTL name is followed by an asterisk (*) if its peak marker is located within the LOD-1 region of the QTL that was mapped for the same *f.sp.* on the second population; underlined QTLs were mapped consistently over the two inoculation experiments.

[c] The chromosome (linkage group) in which the QTL was mapped.

[d] The LOD-score of the QTL.

[e] The proportion of phenotypic variance explained by the QTL.

[f] The effect of having one allele from Vada on the macroscopic infection score, infection frequency or necrosis score.

[g] Indicates whether the QTL was also mapped for other *f.sp.*in the same mapping population.

Microscopic evaluation of Bgt-infected lines

Seven RILs from the VxS_{SC} population and 11 RILs from the VxS_{DC} population were selected for microscopic analysis and sampled from the same experiments for which the macroscopic scores were recorded. As expected, most of the infection attempts, in all RILs, were stopped in association with papilla formation and Vada did not show any established microcolonies (Fig. 3c,d; Fig. 4a). For both SusBgt parents, a similar number of around 50-60 established microcolonies/cm^2 was observed (Fig. 3c,d), from the total of *c*. 2550 conidia inoculated per cm^2 area, implying that at most 3% of the applied spores succeeded in establishing haustoria in the barley epidermis. Some variation was observed in the number of established microcolonies/cm^2 between RILs with similar macroscopic infection scores (Fig. 3a-d). This situation is well illustrated by RILs SC-83 and SC-106: their macroscopic scores were 4.3 and 4.7, respectively,

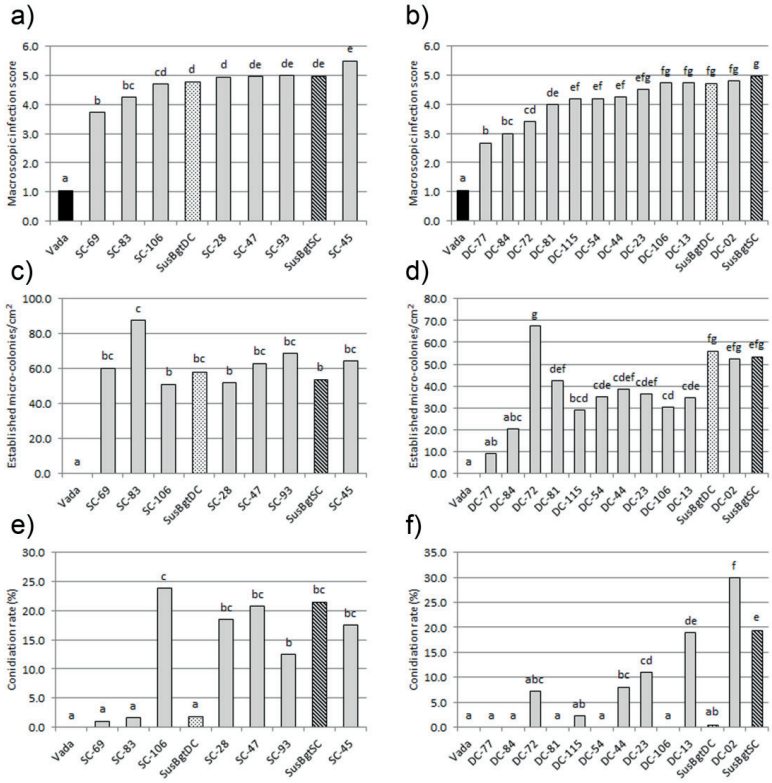

Fig. 3 Microscopic data from the interaction of *Blumeria graminis* f.sp. *tritici* (*Bgt*) with a subset of recombinant inbred lines (RILs) from the VxS_{SC} and VxS_{DC} mapping populations, including the parents. The bars represent average data of two replicate experiments, with 2 leaf segments per experiment. Parental lines are represented by coloured bars: green for Vada, purple for $SusBgt_{DC}$, and red bars for $SusBgt_{SC}$. (a-b) Macroscopic infection scores 7 days after inoculation with *Bgt*. (c-d) Number of established microcolonies/cm^2 counted under the microscope 8 days after inoculation with *Bgt*. (e-f) Conidiation rate: percentage of established microcolonies that formed conidia 8 dai with *Bgt*. Within each chart, bars sharing the same letter are not significantly different ($P < 0.05$)

but the number of established microcolonies/cm^2 differed significantly from 87.5 in the former to 51 in the second (Fig. 3a,c). Also RILs DC-84 and DC-72 (macroscopic scores: 3.0 and 3.4, respectively) differed greatly in the number of germlings that were able to penetrate the cell and form microcolonies: 20.3 microcolonies/cm^2 in DC-84, compared to 67.5 in DC-72 (Fig. 3b,d).

At macroscopic level, the microcolonies evaluated 7 dai differed in appearance: for some RILs, they appeared more floccose than for others with similar score. This is due to different percentage of established microcolonies able to form conidiophores, as seen in SusBgt$_{SC}$ compared to SusBgt$_{DC}$ (conidiation 20% and less than 2%, respectively. Fig. 3c-f; Fig. 4b,c). The results obtained on, for example, SC-83 and SC-106 (Fig. 3c,e) suggest that microcolony formation and conidiation are not correlated. Even though the SusBgt$_{DC}$ parent allowed very low formation of conidiophores, VxS$_{DC}$ RILs segregated for this trait (Fig. 3f). This suggests that Vada may have (a) gene(s) that promote conidium formation.

Table 2. Average macroscopic infection scores for VxS$_{SC}$ recombinant inbred lines (RILs) grouped according to presence (+) or absence (-) of the resistance allele of QTLs mapped for *Blumeria graminis* f.sp. *tritici* (*Bgt*) and f.sp. *hordei-murini* (*Bghm*). Corresponding resistance alleles of each QTL are into brackets (V= Vada; SC= SusBgt$_{SC}$). Values in each column that share the same letter are not significantly different ($P < 0.05$)

Rbgnq1 (V)	*Rbgnq2* (V)	*Rbgnq3* (SC)	Number of RILs*	*Bgt*	*Bghm*
+	+	+	11	1.1 a	1.1 a
+	+	-	10	1.1 a	1.1 a
+	-	+	19	1.1 a	1.2 a
+	-	-	14	1.1 a	1.3 ab
-	+	+	20	1.6 ab	1.1 a
-	+	-	14	2.1 b	2.0 bc
-	-	+	8	3.3 c	2.3 cd
-	-	-	8	4.3 d	3.1 d

*Total number of RILs analysed: 104 for *Bgt* and 105 for *Bghm*. The number of RILs in each group differed slightly (± 2 RILs) between *Bgt* and *Bghm* scores because the peak marker of *Rbgnq3* was different for the two *ff.spp*. or because of missing phenotyping data. Four RILs were excluded from the analysis because there was a recombination point close to the peak marker of *Rbgnq1*.

Viability of Bgt conidia formed on barley leaves

We tested whether the *Bgt* conidia produced on the nonhost plant barley would be viable and therefore able to grow on its natural host, wheat. No fungal growth was observed on the negative controls (not-rubbed with barley leaves) and also not on wheat plants rubbed with Vada-infected leaves (Table S3 a-b). This rules out the possibility that any old spores present in the environment or on the surface of leaves would cause the infection. On wheat seedlings treated with SusBgt$_{DC}$ also no colonies of *Bgt* developed (Table S3 c), which can be explained by

the low conidiation rate observed for this line. Wheat seedlings treated with SusBgt$_{SC}$, SC-45 and DC-02 (Table S3 d-f) all produced mildew colonies, indicating that the *Bgt* conidia formed on the leaves of the nonhost barley plants were viable and fit for infecting their natural host. This confirmed that line DC-02 was able to produce viable conidia, while its parents, Vada and SusBgt$_{DC}$, were not or had a very limited production.

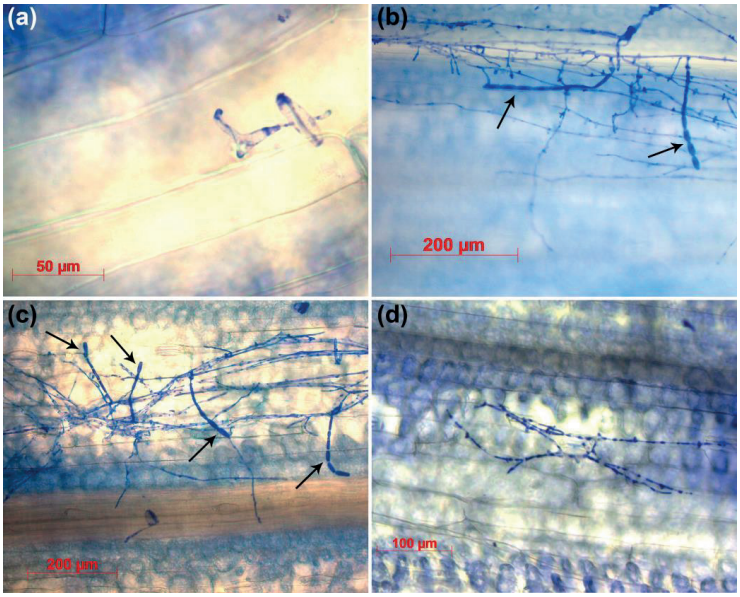

Fig. 4 Infection units of *Blumeria graminis* f.sp. *tritici* (*Bgt*) on barley (*Hordeum vulgare*) plants, 8 d after inoculation. Conidiophores are indicated with an arrow. **(a)** A stopped penetration attempt including papilla formation on Vada **(b)** An established microcolony with conidiophores on the susceptible parent SusBgt$_{SC}$. **(c)** Established microcolony with conidiophores on SC-45 **(d)** Established microcolony without conidia on DC-106.

Phenotyping and mapping QTLs for basal resistance to Bgh

Both mapping populations gave a continuous quantitative distribution for infection frequency (IF), suggesting a polygenic inheritance of the basal resistance to *Bgh*. Transgressive segregation towards resistance and susceptibility was observed, indicating that both parents contributed resistance and susceptibility alleles (Fig S3). As early as 4 dai, when seedlings were phenotyped for IF, it was possible to notice the occurrence of HR in some genotypes. We decided then to evaluate also the necrotic reaction, but only after the first inoculation experiment had already been carried out. For this reason, our results regarding necrotic reaction assessment are based on a single inoculation experiment. SusBgt$_{SC}$ showed high IF without macroscopic necrosis, SusBgt$_{DC}$ and Vada a lower level of IF in association with necrosis, which was more obvious on SusBgt$_{DC}$ than on Vada (Fig. 5).

Three QTLs were detected for IF in the two mapping populations, using the average scores from two inoculation experiments (Table 1). QTLs were named '*Rbghq*', standing for 'Resistance to *Bgh*, quantitative' and the LOD score of 2.9 was set as threshold. *Rbghq1*, at the telomeric region of the long arm of chromosome 2H, seems to play a major role for *Bgh* resistance in both populations. It is associated with the necrotic phenotype at infection sites. *Rbghq1* has the same peak marker as the nonhost QTL *Rbgnq2*. A second QTL, *Rbghq2*, was mapped for IF and necrosis in the VxS_{DC} population. In the VxS_{DC} population, *Rbghq2* is responsible for a higher percentage of explained phenotypic variance for necrotic reaction than *Rbghq1*, and has $SusBgt_{DC}$ as donor of the resistance allele. In VxS_{SC}, HR seems to be mainly governed by *Rbghq1*, with the high LOD score of 26 and accounting for 68% of the explained phenotypic variance. A few additional minor-effect QTLs were detected when the IF data of single experiments for *Bgh* were used (Online Resource 11). One of these minor QTLs, mapped on 4H in the VxS_{SC} population (LOD 3.48), overlaps with the LOD-1 region of the nonhost QTL *Rbgnq3*.

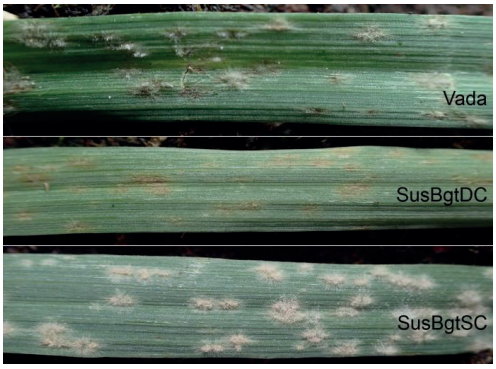

Fig. 5 Phenotype of barley (*Hordeum vulgare*) seedlings 7 d after inoculation with the adapted powdery mildew *Blumeria graminis* f.sp. *hordei* (*Bgh*). $SusBgt_{SC}$ (bottom) shows no necrosis and higher infection frequency in comparison to Vada (top) and $SusBgt_{DC}$ (middle); Vada and $SusBgt_{DC}$ show a conspicuous necrotic phenotype

Discussion

Our study to identify genes that determine nonhost resistance uses natural variation existing among genotypes of a plant species in the level of resistance to a non-adapted pathogen. This is an alternative to the use of interspecific, host x nonhost crosses. Such genetic variation was demonstrated in rice cultivars that, despite being immune to rust fungi, differed in the degree of penetration and haustorium establishment by several cereal rust species (Ayliffe et al. 2011). Other examples include Arabidopsis genotypes for resistance to the bean pathogen *Pseudomonas syringae* pv. *phaseolicola* (Forsyth et al. 2010) and to wheat leaf rust (*P. triticina*)

(Shafiei et al. 2007); wheat for resistance to the barley pathogen *Puccinia striiformis* f. sp. *hordei* (Rodrigues et al. 2004); and barley, considered a near-nonhost to several *Puccinia* spp. (Dracatos et al. 2016; Jafary et al. 2008; Yeo et al. 2014). Studies in the barley-*Puccinia* pathosystem were made possible by SusPtrit, a barley line developed by accumulation of susceptibility genes or effective selection against resistance genes to the wheat leaf rust (Atienza et al. 2004). SusPtrit is at seedling stage as susceptible to *P. triticina* as susceptible wheat accessions. The SusBgt lines were developed following a similar approach, with *Bgt* as target pathogen (Aghnoum and Niks 2010). The resulting SusBgt lines were by far not as susceptible to *Bgt* as wheat. This indicates the existence of some fixed genes for (this type of) resistance in the barley gene pool, at least as far as represented in the germplasm used by Aghnoum and Niks (2010). The uniformity of such shared genetic factors precludes their identification, and therefore a large complement of nonhost resistance in the barley-*Blumeria* pathosystem remains unresolved. The increased susceptibility status of the SusBgt lines was, nevertheless, sufficient to allow genetic analyses to be performed and part of the genetic components of nonhost resistance to be mapped.

We crossed two SusBgt lines with an immune barley cultivar, Vada, to develop plant material segregating for nonhost resistance to non-adapted *B. graminis* forms. The SusBgt mapping populations allowed, for the first time, identification of QTLs associated with resistance of barley against the non-adapted *Bgt* and *Bghm*. We also evaluated the SusBgt mapping populations for resistance to *Bgh*, enabling comparison between sets of QTLs mapped for nonhost and basal host resistance. The six genomic regions mapped in this study fit into three classes: QTLs mapped for nonhost resistance, for basal host resistance and for both nonhost and host resistance. The set of nonhost resistance QTLs detected in the two populations was almost the same, which can be due to the degree of shared ancestry of the SusBgt lines, which have two parental lines in common (Chame 2 and SusPtrit) and also to the fact that both populations share the resistant parent. However, it could also indicate that there is little variation among barley genotypes in genes causing nonhost resistance to powdery mildew. QTLs effective to *Bgt* were typically also effective to *Bghm*, which can be partly attributed to the close relationship between the pathogens, but also points to a wide specificity of genes, simultaneously effective to multiple *ff.spp*.

Nonhost resistance to haustoria-forming biotrophic fungal pathogens is mostly pre-haustorial but the small percentage of germlings able to form haustoria can be prevented from further developing by plant cell death (Lipka et al. 2005). Most RILs in both mapping populations, as well as Vada, do not show any macroscopic symptom upon *Bgt* and *Bghm* inoculation (Fig. S2), and microscopy showed that penetration attempts are stopped in association with papillae (Fig. 4a). This is consistent with earlier reports on the papilla-based

nature of nonhost resistance of barley to *Blumeria* (Aghnoum and Niks 2010; Trujillo et al. 2004). In some of the RILs that were not immune, infection was associated with very mild necrosis (Fig. 1b). Although RILs were not scored for necrotic reaction to *Bgt* and *Bghm*, we speculate here that *Rbgnq2* might be associated with necrotic reaction in RILs where the largest effect nonhost resistance QTL had the susceptibility allele. *Rbgnq2* has the same map position as *Rbghq1* (Table 1) and we presume that both may represent the same gene. Because *Rbghq1* confers some necrotic reaction to *Bgh*, it may also confer necrosis-associated defense against non-adapted *Bgt* and *Bghm*. *Rbgnq2*/*Rbghq1* may actually represent the powdery mildew resistance gene *MlLa*, which was introgressed into barley cultivars from the barley accession '*H. laevigatum*' and confers an intermediate type of reaction associated with HR phenotype to *Bgh* (Giese et al. 1993; Marcel et al. 2007a). Markers (MWG097, MWG2200) that co-segregated with *MlLa* in the study of Marcel et al. (not published) mapped in the LOD-1 interval of *Rbgnq2*/*Rbghq1.* The parent donor of the *Rbgnq2*/*Rbghq1* resistance allele is Vada, known to carry *MlLa* (Marcel et al. 2007a).

Niks and Marcel (2009) proposed that QTLs represent 'operative targets', defined as "host targets that, when manipulated by a pathogen effector, results in enhanced pathogen fitness". Such operative targets are thought to play a role in plant basal defense responses, and interact with effectors in a minor gene-for-minor gene fashion (Gonzalez et al. 2012; Parlevliet and Zadoks 1977). The ability of a potential pathogen to infect a plant species will mostly rely on its array of effectors and whether they fit the target motifs in the plant. Therefore, failure of non-adapted *B. graminis* species to infect barley can be due to the pathogen lacking appropriate effector molecules and/or due to barley lacking matching operative targets (Niks et al. 2015). As proposed by Antonovics et al. (2013), this would be the consequence of pathogen specialization to its 'source host', rather than the result of evolved resistance in the plant. Because barley and wheat evolved from a common ancestor, some barley accessions are expected to still carry variants of operative targets that are compatible with *Bgt* effectors, and such variants may have been accumulated in the SusBgt lines (Aghnoum and Niks 2010). While *Bgt* and *Bghm* manage to partially suppress PTI in the SusBgt lines and establish haustoria, the same is hardly true for *Bghs*, supporting the notion that genes for basal resistance act in a mildew *forma specialis*-specific way (Aghnoum and Niks 2010).

The main determinant of the outcome of barley-*Bgt*/*Bghm* interaction found in this study is *Rbgnq1*. RILs carrying the resistance allele of this QTL are (near) immune to *Bgt* regardless of the background QTLs (Table 2 and Table S2). This is also illustrated by RIL DC-04, segregating for the *Rbgnq1* locus: even though this line carries the susceptibility allele of *Rbgnq2*, *Rbgnq1* seems to be sufficient to confer immunity. The high density of markers available, combined with the large effect of *Rbgnq1* made it possible to delimit the gene to a 1.4

cM interval. The phenotypic effect conferred by this gene should be sufficient to allow map based cloning. The effect size suggests that *Rbgnq1* could rather be called a 'major gene'. A note of caution is due here, since the immunity of Vada (and barley cultivars, in general) to *Bgt* is the result of the action of several genes regulating an infinity of pathways, and *Rbgnq1* only explains a small part of the spectrum from susceptibility to immunity. Barley lines not carrying *Rbgnq1* (i.e. carrying the SusBgt allele) still have a considerable amount of nonhost resistance left, since no colonies as large as those formed on wheat develop. Despite its large effect on establishment of *Bgt* and *Bghm* on barley, *Rbgnq1* did not reduce IF of the adapted *Bgh*.

Whereas *Rbgnq1* seems a good example of a nonhost resistance gene to which *Bgh* has evolutionary adapted, the above mentioned *Rbgnq2* and the minor QTL *Rbgnq3* have larger effectiveness spectra and appear to confer also basal host resistance to *Bgh*. A QTL mapped for resistance to *Bgh* in the VxS_{SC} population (Online Resource 11) overlaps with the LOD-1 region of *Rbgnq3*. Surprisingly, the resistance allele of *Rbgnq3* is contributed by the susceptible parent, suggesting that the SusBgt lines have at least one resistance factor that lacks in Vada. At a similar position on chromosome 4H, Jafary et al. (2008) reported the mapping of a QTL effective to four non-adapted rust species, also with the susceptible parent (SusPtrit) contributing the resistance allele. This chromosomal region is therefore associated with a wide-spectrum resistance against different fungal pathogens. Association of this region with resistance to non-adapted powdery mildews and rusts can be due to the presence of many linked resistance genes or to the same gene. Resistance to several fungal pathogens caused by a single gene has been reported in wheat, for the genes *Lr34* (synonyms: *Yr18*/*Sr57*/*Pm38*), *Lr67* (*Yr46*/*Sr55*/*Pm46*) and *Lr46* (*Yr29*/*Sr58*/*Pm39*). These three broad-spectrum resistance genes are effective against all tested races of the wheat leaf rust, stem rust and stripe rust fungi (*P. triticina*, *P. striiformis* f.sp. *tritici* and *P. graminis* f.sp. *tritici*, respectively) and also the wheat powdery mildew fungus *Bgt* (Ellis et al. 2014; Herrera-Foessel et al. 2014; Kolmer et al. 2008). Two of these genes, *Lr34* and *Lr67*, have been cloned and found to encode for membrane-localized transporter proteins (Krattinger et al. 2009; Moore et al. 2015).

Histological studies are helpful to elucidate certain aspects of the interaction, like the rate of haustorium formation and conidiation. Different numbers of established microcolonies were found for RILs showing similar macroscopic scores. This is probably due to different sizes of microcolonies in different RILs: some RILs may allow more secondary hyphal growth than others or even formation of some conidiophores. This might be caused by variation in gene(s) acting at post-invasion nonhost resistance. We also observed that haustorium establishment is not always associated with successful conidiophore formation (Fig. 3), in agreement with previous reports by Aghnoum and Niks (2010) that barley lines showing similar levels of haustorium establishment by non-adapted *B. graminis* forms differed in conidiation rates. This

indicates that several layers of defense are involved in basal defense, acting at different stages of pathogen development. Conidiation segregated among RILs from VxS_{DC}, even though $SusBgt_{DC}$ had a conidiation rate close to zero (Fig. 3f). This suggests that the immune Vada carries, underneath a very effective pre-haustorial defense, some factors that would allow the pathogen to further develop and complete its life cycle. Due to the limited number of RILs that actually allowed some degree of *Bgt* growth, it was not possible to map the QTL(s) determining conidiation.

Our work is analogous to that of Jafary et al. (2006) and (2008), who mapped QTLs for nonhost resistance to non-adapted rust species in three barley mapping populations. Rusts and powdery mildews are both obligate biotrophs, and nonhost and basal resistance in these two pathosystems are typically pre-haustorial (Niks 1986; Olesen et al. 2003). A high diversity of loci was implicated in resistance to rusts, and immunity in different tested barley accessions was shown to be due to a different combination of genes (Jafary et al. 2008). Some QTLs mapped in the rust study were species-specific, others were effective to more than one rust fungal species. Our results also demonstrate polygenic inheritance for nonhost resistance to *Bgt* and *Bghm*, but because we only used Vada as immune parent, it still remains to be investigated how wide diversity there is to protect barley against non-adapted powdery mildews. Loci mapped for nonhost and basal host resistance to rusts were found to be significantly associated with loci for plant defense gene homologs (Jafary et al. 2008) such as peroxidases (Gonzalez et al. 2010), in agreement with the hypothesis that these two types of resistance rely on similar principles (Aghnoum and Niks 2010; Jafary et al. 2006; Marcel et al. 2007b; Schweizer and Stein 2011). In the present study at least two QTLs are in common for non-adapted and adapted mildew forms (*Rbgnq3* and *Rbgnq2*/*Rbghq1*), also pointing to an overlap on genetic mechanisms mediating nonhost and basal host resistance. The indication that *MlLa* is not only effective to *Bgh* but also against non-adapted mildews is an interesting finding, with no parallel in the barley-rust pathosystem.

This research extends our knowledge on the genetic basis of nonhost resistance. We confirmed the polygenic mode of inheritance in barley to powdery mildew and that plant genetic factors determining establishment by haustorium formation act independently from factors determining level of conidiation. Fine-mapping and complementation studies are necessary to isolate the underlying genes for nonhost resistance to powdery mildew. Types of genes expected to be found may belong to an as diverse array of gene families as found for basal/quantitative host resistance, rather than to one family, as accepted for race specific hypersensitive resistance (Lee and Yeom 2015). In a parallel study, our group is close to cloning the gene responsible for *Rbgnq1* resistance. Fine-mapping resulted in a QTL interval comprising 17 candidate genes (Romero et al. unpublished – Chapter 2). The cloning of nonhost resistance

gene(s) in barley will open up the possibility of transferring this resistance to wheat, where its orthologues are likely to be suppressed by *Bgt* effectors (Douchkov et al. 2014). There are several examples demonstrating successful transfer of nonhost resistance across species (Du et al. 2015; Johnston et al. 2013; Lacombe et al. 2010; Lee et al. 2017; Lee et al. 2016). The QTLs mapped in this study could, in the future, emerge as a valuable resource for Triticeae disease resistance breeding programs.

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Supplementary material

Table S1 Summary of data for the inoculation experiments with *Blumeria graminis* f.sp. *tritici* (*Bgt*) and f.sp. *hordei-murini* (*Bghm*) for the two SusBgt mapping populations.

	Mapping population			
	Vada x $SusBgt_{SC}$		Vada x $SusBgt_{DC}$	
Formae specialis	*Bgt*	*Bghm*	*Bgt*	*Bghm*
Average inoculation density (conidia/mm^2)	28.3	21.1	23.3	23.7
Correlation between reps	0.83	0.69	0.84	0.81
% RILs with score below 2	75.2%	79.1%	76.5%	77.9%
% RILs with score above 4	6.4%	1.8%	8.7%	5.3%

Table S2 Average macroscopic infection scores for VxS_{DC} recombinant inbred lines (RILs) grouped according to presence (+) or absence (-) of the resistance allele of QTLs mapped for *Blumeria graminis* f.sp. *tritici* (*Bgt*) and f.sp *hordei-murini* (*Bghm*). Resistance alleles of all QTLs are from Vada (V). Values in each column that share the same letter are not significantly different ($P < 0.05$).

Rbgnq1 (V)	*Rbgnq2* (V)	*Rbgnq4* (V)	Number of RILs*	*Bgt*	*Bghm*
+	+	+	21	1.1 a	1.1 a
+	+	-	10	1.1 ab	1.5 ab
+	-	+	15	1.2 ab	1.1 a
+	-	-	14	1.1 ab	1.2 ab
-	+	+	7	1.8 ab	1.5 ab
-	+	-	14	1.8 b	2.0 b
-	-	+	8	3.9 c	1.4 ab
-	-	-	12	3.3 c	3.3 c

* Total number of RILs analysed: 101 for *Bgt* and 99 for *Bghm*. The number of RILs in each group differed slightly (±1 RIL) between *Bgt* and *Bghm* scores because of missing phenotyping data. Ten RILs were excluded from the analysis because there was a recombination point close to the peak markers of *Rbgnq1*.

Table S3 Viability test of *Blumeria graminis* f.sp. *tritici* (*Bgt*) conidia produced on barley epidermis: wheat leaves 5 days after being in direct contact with barley leaves of different genotypes inoculated with *Bgt*; **(a)** Negative control; **(b)** Vada; **(c)** $SusBgt_{DC}$; **(d)** $SusBgt_{SC}$; **(e)** SC-45 and **(f)** DC-02.

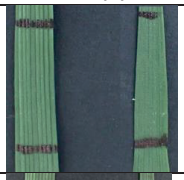
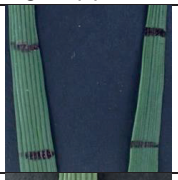
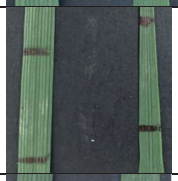
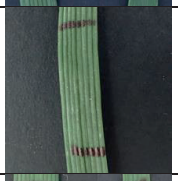
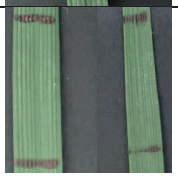
(**a**) Negative control			
(**b**) Vada			
(**c**) $SusBgt_{DC}$			
(**d**) $SusBgt_{SC}$			
(**e**) SC-45			
(**f**) DC-02			

Table S4 QTL mapping results for resistance to *Blumeria graminis* f.sp. *hordei* (*Bgh*) for all inoculation experiments.

Mapping population	Rep[a]	Trait[b]	QTL name[c]	Peak marker	Chr[d]	Position	LOD	% Expl[e]	Additive[f]	Donor[g]	Mapped for NHR?[h]
VxS_{SC}	Rep1	IF	*Rbghq1*	SC-C2_SNP54	2H	153.9	10.97	37.1	-6.069	Vada	*Bgt, Bghm*
	Rep1	Nec	*Rbghq1*	SC-C2_SNP54	2H	153.9	33.02	75.6	1.00	Vada	
	Rep1	Nec		SC-C7-SNP24	7H	64.0	3.12	3.5	0.217	Vada	
	Rep2	IF		SC-C2_SNP9	2H	23.6	3.10	10.2	2.511	SusBgtSC	
	Rep2	IF	*Rbghq1*	SC-C2_SNP54	2H	153.9	8.12	24.1	-3.766	Vada	*Bgt, Bghm*
	Rep2	IF		SC-C4_SNP17	4H	41.9	3.48	9.3	2.346	SusBgtSC	*Bghm*
	Rep2	IF	*Rbghq3*	SC-C6_SNP41	6H	119.2	3.87	10.4	-2.494	Vada	
	Rep2	Nec	*Rbghq1*	SC-C2_SNP54	2H	153.9	26.01	68	1.084	Vada	
	Average	IF	*Rbghq1*	SC-C2_SNP54	2H	153.9	11.62	35.1	-4.795	Vada	*Bgt, Bghm*
	Average	IF	*Rbghq3*	SC-C6_SNP40	6H	116.8	2.87	7.1	-2.208	Vada	
VxS_{DC}	Rep1	IF		DC-C1_SNP39	1H	108.9	3.04	7.8	1.571	SusBgtDC	
	Rep1	IF	*Rbghq1*	DC-C2_SNP58	2H	159.7	4.78	14.5	-2.084	Vada	*Bgt, Bghm*
	Rep1	IF	*Rbghq2*	DC-C7_SNP1	7H	0.0	6.9	21.5	2.572	SusBgtDC	
	Rep2	IF	*Rbghq1*	DC-C2_SNP58	2H	159.7	5.63	15.2	-3.224	Vada	*Bgt, Bghm*
	Rep2	IF	*Rbghq2*	DC-C7_SNP2	7H	3.2	9.88	29.2	4.494	SusBgtDC	
	Rep2	Nec	*Rbghq1*	DC-C2_SNP58	2H	159.7	4.02	9.8	0.380	Vada	*Bgt, Bghm*
	Rep2	Nec	*Rbghq2*	DC-C7_SNP1	7H	0.0	13.24	39.4	-0.770	SusBgtDC	
	Average	IF	*Rbghq1*	DC-C2_SNP58	2H	159.7	8.32	20.6	-2.738	Vada	*Bgt, Bghm*
	Average	IF	*Rbghq2*	DC-C7_SNP2	7H	3.2	11.82	31.6	3.406	SusBgtDC	

[a] The inoculation experiment from which the data was taken for QTL analysis, whether a single experiment from replicate 1, 2, or the average of both inoculation experiments;
[b] Trait analysed, whether infection frequency (IF) or necrosis (Nec);
[c] QTLs were named only when mapped for the average of inoculation experiments;
[d] The chromosome (linkage group) in which the QTL was mapped;
[e] The proportion of phenotypic variance explained by the QTL;
[f] The effect of having an allele from Vada on the macroscopic infection score;
[g] Parent donor of resistance allele
[h] Indicates whether the QTL was mapped for nonhost resistance

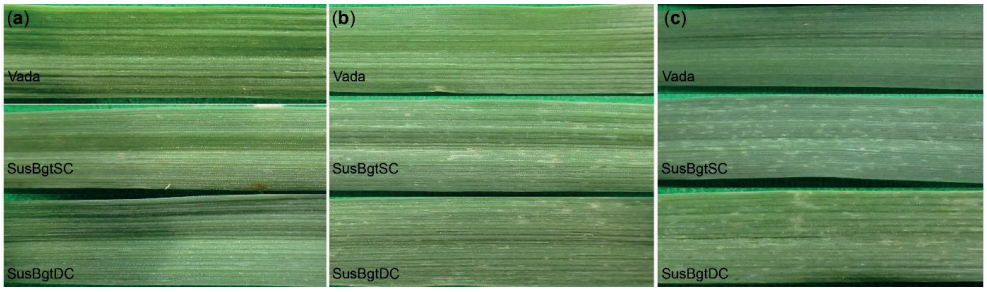


Fig. S1 Macroscopic phenotypes of parental barley (*Hordeum vulgare*) lines Vada, $SusBgt_{SC}$ and $SusBgt_{DC}$ upon inoculation with different *ff.spp.* of *Blumeria graminis*. **(a)** f.sp. *hordei-secalini* (*Bghs*), the pathogen of *Hordeum secalinum*, 14 days after inoculation (dai): no macroscopically visible symptoms on the surface of the leaves. Development of microcolonies is observed on the SusBgt lines 7 dai with **(b)** f.sp. *tritici* (*Bgt*), the pathogen of wheat and **(c)** f.sp. *hordei-murini* (*Bghm*), the pathogen of *H. murinum*

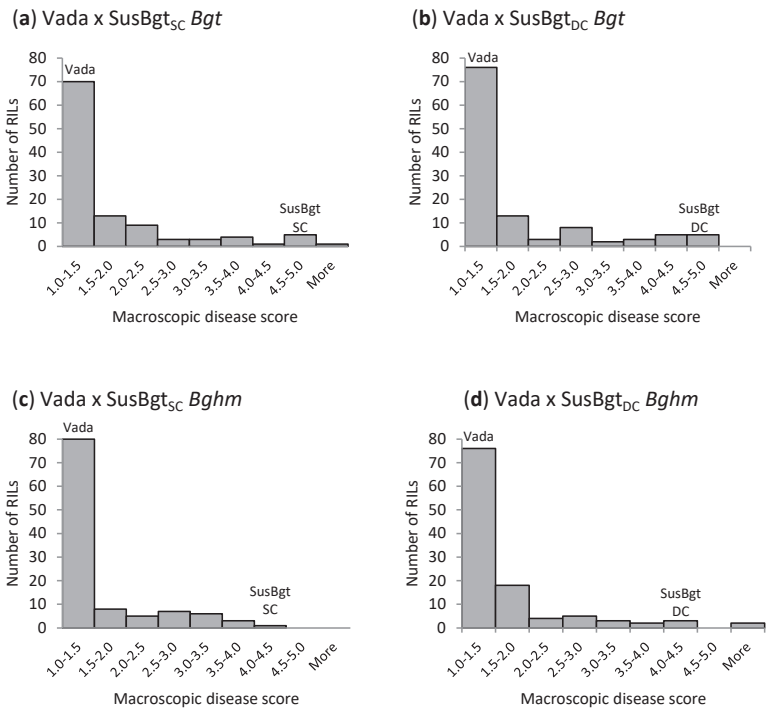


Fig. S2 Frequency distributions of the macroscopic disease scores in two barley mapping populations inoculated with *Blumeria graminis* f.sp. *tritici* (*Bgt*) and f.sp *hordei-murini* (*Bghm*). Values on the y-axis show the number of RILs, and the x-axis represent the classes of disease scores from 1 to 5, or larger than 5. (a) VxS$_{SC}$ population inoculated wth *Bgt*, (b) the VxS$_{DC}$ population inoculated with *Bgt*. (c) VxS$_{SC}$ population inoculated wth *Bghm* and (d) VxS$_{DC}$ population inoculated with *Bghm*.

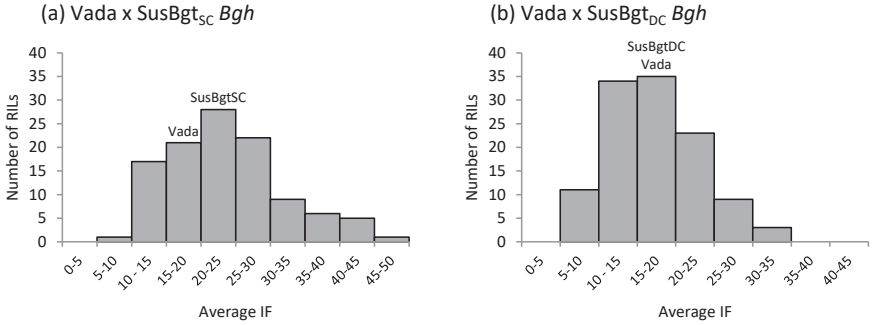


Fig. S3 Frequency distributions of the macroscopic disease scores in the SusBgt populations inoculated with *Blumeria graminis* f.sp. *hordei* (*Bgh*). Values on the y-axis show the number of Recombinant Inbred Lines (RILs), and the x-axis represent average infection frequency (IF) classes (a) Vada x SusBgt$_{SC}$ (b) Vada x SusBgt$_{DC}$.

Chapter 3

Towards map-based cloning of a QTL for nonhost resistance to powdery mildew in barley

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Abstract

Nonhost resistance has been proposed as a promising alternative for durable resistance in plants. The inheritance of nonhost resistance, however, is particularly difficult to study, since it implies interspecific hybridization. The barley line SusPtrit shows rudimentary susceptibility to *Blumeria graminis* f.sp. *tritici* (*Bgt*), the powdery mildew of wheat, enabling studies of near-nonhost resistance in barley against this non-adapted powdery mildew. We assume that the genetic and mechanistic principles of near-nonhost resistance are the same as nonhost resistance. Three mapping populations having SusPtrit as one of the parents were screened for resistance to *Bgt*, revealing a diversity of genomic regions associated with resistance. A major effect QTL, *Rbgnq1*, was mapped to chromosome 5HL in Vada x SusPtrit population. We developed a segregating population to fine-map *Rbgnq1* to a 0.27 cM interval. This interval corresponds to a region of 565.9 Kb in the barley reference genome of cv. Morex, and contains 20 predicted genes. The genomic BAC libraries of Vada and SusPtrit were screened for clones spanning the *Rbgnq1* region using a PCR-based approach. Two contigs were established for the physical map of *Rbgnq1* in cv. Vada, covering a region of approximately 405 Kb containing 17 predicted candidate genes. The findings reported in this study provide an important resource for the necessary additional work on the identification, testing and confirmation of the causal gene(s).

Introduction

Agronomists and breeders face the challenge of finding alternatives to increase food production to meet the demand of an increasing global population under changing climate. It is imperative that the increase in yield be coupled with a reduction of environmental impact. Disease management has played an important role in doubling food production in the few last decades, but it is still estimated that 10-16% of the global crop harvest are lost to pathogens and pests each year (Chakraborty and Newton 2011). Breeding for disease resistance is the most sustainable and effective approach to ensuring crop protection. Over the last century, the development of disease-resistant plant varieties has been mostly based on the introgression of resistance (*R*)-genes. The durability of *R*-gene mediated resistance, however, proved to be an issue due to the potential of rapidly evolving pathogens to overcome this type of resistance (Dangl et al. 2013; Lee et al. 2016).

The most durable and broad-spectrum form of resistance in plants is nonhost resistance, a phenomenon in which all members of a given plant species are immune to all genetic variants of a non-adapted pathogen species (Heath 2000; Mysore and Ryu 2004; Nurnberger and Lipka 2005). The possibility of using nonhost resistance as a source of durable resistance in plants has been listed among the most important questions in plant science research (Grierson et al. 2011). In recent years, there has been increasing interest in the potential practical applications of nonhost resistance in agriculture (Lee et al. 2017; Lee et al. 2016). Three examples of the successful transfer of nonhost resistance genes are barley, in which the barley leaf rust resistance gene *Rph22* was transferred from the nonhost species *Hordeum bulbosum* (Johnston et al. 2013), and the solanaceous plants tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana*, in which the transgenic expression of the *Arabidopsis* EF-Tu receptor conferred enhanced resistance to a number of pathogenic bacteria (Lacombe et al. 2010).

Nonhost resistance relies on the action of receptor proteins that are capable of perceiving potentially harmful microorganisms and responding accordingly. Each plant cell is equipped with surface receptor proteins called PRRs (pattern recognition receptors) that are responsible for recognizing conserved structural components of pathogenic microorganisms known as PAMPs (pathogen-associated molecular patterns or the equivalent "microbe-associated molecular patterns", MAMPs). PAMP recognition activates PRR proteins, which in turn mobilize a complex network of downstream signaling responses culminating in PAMP-triggered immunity (PTI) (Dodds and Rathjen 2010; Jones and Dangl 2006; Macho and Zipfel 2014). Adapted pathogens have evolved ways of suppressing PTI by releasing virulence factors known as effectors, some of which are delivered to the interior of the host cells (Cui et al. 2015). In turn, plants have evolved intracellular receptor proteins, encoded by *R*-genes, that are able to

recognize effectors in a very specific way. R-proteins typically contain a nucleotide-binding (NB) and a leucine-rich repeat (LRR) domain; when activated by effector recognition, these R-proteins trigger ETI (effector-triggered immunity). ETI is typically associated with the programmed death of the attacked cell, a mechanism known as the hypersensitive response (HR), which prevents the pathogen from spreading (Cui et al. 2015; Dodds and Rathjen 2010). A topic of debate is whether both PTI and ETI contribute to nonhost resistance. One model of nonhost resistance postulates that defense responses are mostly based on PTI since non-adapted pathogens do not possess the appropriate effectors to interfere with this first layer of defense (Niks 2014; Niks and Marcel 2009). A second model proposes that nonhost resistance relies on the presence of multiple *R*-gene-encoded NB-LRRs that are responsible for perceiving a number of effector proteins and triggering ETI (Schweizer 2007; Stam et al. 2014).

There are several potential approaches to studying the genetics of nonhost resistance. Studies of *Arabidopsis* mutants, for example, led to the identification of the *PEN* genes, which act by conferring prehaustorial resistance to non-adapted fungi (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). In barley, a number of genes that play a role in resistance to non-adapted fungi were identified using transcriptomics and transient-induced gene silencing (TIGS) (Delventhal et al. 2017; Douchkov et al. 2014; Zellerhoff et al. 2010). The genes discovered through mutagenesis and gene expression are normally involved in general plant defense mechanisms and may be highly conserved among different plant species; for this reason, they are not likely candidates to explain the specificity of the nonhost status (Niks 2014; Niks and Marcel 2009). The differential expression of such genes during nonhost and host interactions is the likely a consequence of nonhost resistance rather than the primary cause. Another approach to investigating the genetics of nonhost resistance involves inheritance and mapping studies. It is argued that inheritance studies are valuable for identifying genes that are determinants of specificity (Niks and Marcel 2009) because in such studies the most upstream gene(s) determining whether the plant can be infected by the non-adapted pathogen will be identified.

To overcome the problems associated with inheritance studies involving two different plant species, it is possible to study the genetics of resistance in plants that display natural variation in the degree of resistance to a non-adapted pathogen (Atienza et al. 2004; Niks and Marcel 2009). Rice (*Oryza sativa*) is immune to fungal rust diseases, although there is genetic variation among rice genotypes that results in different rates of penetration and haustorium formation by cereal rusts (Ayliffe et al. 2011). Variation in nonhost resistance responses were observed for the *Arabidopsis-Pseudomonas syringae* pv. *phaseolicola* pathosystem; these variations allowed quantitative trait loci (QTL) mapping of regions associated with resistance (Forsyth et al. 2010). Barley is considered a near-nonhost to non-adapted rusts of cereals and

grasses (*Puccinia* spp.), meaning that a few (rare) barley accessions, mainly at the seedling stage, are moderately susceptible to rust pathogens that would normally be unable to cause infection. Based on this, Atienza et al. (2004) developed an experimental barley line, SusPtrit, by crossing rare barley accessions that show unusual susceptibility to the wheat leaf rust (*P. triticina*) and selecting for more susceptible phenotypes. The resulting line, SusPtrit, not only shows high susceptibility to the target pathogen *P. triticina* but also exhibits enhanced susceptibility to several heterologous grass rust fungi (Niks 2014) and has become a valuable material for studying the genetic basis of nonhost resistance. Three mapping populations with SusPtrit as one of the parents were developed: Vada x SusPtrit, Cebada Capa x SusPtrit and SusPtrit x Golden Promise, allowing a number of nonhost QTLs for resistance to heterologous rusts to be identified (Dracatos et al. 2016; Jafary et al. 2008; Jafary et al. 2006; Yeo et al. 2014).

In a similar way, Aghnoum and Niks (2010) accumulated genes for rudimentary susceptibility to the wheat powdery mildew *Blumeria graminis* f. sp. *tritici* (*Bgt*) and developed two lines, $SusBgt_{SC}$ and $SusBgt_{DC}$. Both SusBgt lines show increased susceptibility to *Bgt* at seedling stage; they were crossed with cv. Vada to develop two mapping populations, which were used to map resistance factors against non-adapted powdery mildews (*Blumeria graminis* ff. spp.) (Romero et al. 2018). In the present work, we screened SusPtrit mapping populations at the seedling stage for level of susceptibility to *Bgt*. Compared to the majority of barley genotypes, which are immune to *Bgt*, the rate of haustorium formation in SusPtrit is higher (Trujillo et al. 2004). Nevertheless, macroscopic symptoms can only be observed upon high inoculation density (>25 conidia/mm^2). Established infection units (those that succeed in forming haustoria) have limited secondary hyphal growth and form "microcolonies" that appear as tiny white dots on the surfaces of the leaves approximately 7 d after inoculation (Romero et al. 2018). A large-effect QTL, *Rbgnq1*, mapped to the long arm of chromosome 5H in the Vada x SusPtrit population, is the same as a QTL mapped in the SusBgt mapping populations by Romero et al. (2018). We followed a map-based cloning approach to fine-map *Rbgnq1* to a small genetic interval by developing a segregating fine-mapping population. Another objective of this work was to screen genomic BAC libraries for clones covering the QTL region to establish a physical map including all the genes in the interval. Lastly, whole-BAC sequences of clones spanning the *Rbgnq1* interval were tested by transient overexpression.

Materials and Methods

Barley mapping populations and inoculum material

Three barley mapping populations, including two recombinant inbred lines (RIL), Vada x SusPtrit (VxS, 152 lines, (Jafary et al. 2006) and Cebada Capa x SusPtrit (CxS, 113 lines, (Jafary et

al. 2006), and a doubled haploid (DH) mapping population, SusPtrit x Golden Promise (SxG, 122 lines, (Yeo et al. 2014), were evaluated for the level of infection by the non-adapted pathogen, *Blumeria graminis* f. sp. *tritici* (*Bgt*, Swiss field isolate FAL 92315) in three independent inoculation experiments. Two seedlings per RIL were grown in 40 x 60-cm trays containing standard compost soil as substrate. The two parents of each mapping population were included in every tray as controls. The trays were kept in a controlled greenhouse compartment (18-20°C daytime, 15°C nighttime, 40-60% relative humidity, 16 h photoperiod) and watered daily with tap water until the seedlings were 11-14 days old. The first leaf of each seedling was pinned to the substrate with the adaxial side up using metal pins; additional emerging leaves were removed before inoculation. Each entire population was inoculated simultaneously by placing the trays inside a settling box (100 cm x 120 cm x 87 cm height). Fresh sporulating leaves of *Bgt*-infected wheat cv. Vivant were placed on the top of the settling box and blown over the seedlings using a compressed air pump. Inoculation densities were assessed using a hemocytometer and adjusted to 25 to 50 conidia/mm^2. The metal pins were removed the next day so that the seedlings could grow vertically.

Seven to eight days after inoculation with *Bgt*, the seedlings were macroscopically phenotyped for the degree of microcolony formation on a relative scale of 0 (resistant, no microcolony formation) to 5 (susceptible, high degree of microcolony formation), similar to the scale described by Romero et al. (2018). One inoculation experiment of the VxS population was scored microscopically by assessing haustorium formation (HF). In that case, samples of two seedlings per RIL were collected 72 h after inoculation (hai) and prepared for histology as described by Aghnoum and Niks (2010). A total of 100 infection sites were observed per seedling and scored for successful HF and for the formation of elongated secondary hyphae (ESH).

QTL mapping

QTL mapping was performed independently for each of the three replicate experiments per population and also using the average phenotyping data of the three experiments. For the VxS population, the average data were taken from the two inoculation experiments that were assessed macroscopically. Data from the microscopic phenotyping (HF) of VxS were also used for QTL mapping. The marker data set used for QTL analysis consisted of 198 markers for VxS, 242 markers for CxS (both generated by Jafary et al. (2008) and consisting of AFLP and SSR markers), and 698 markers for SxG (generated after genotyping with the Illumina iSelect 9k barley Infinium chip) (Yeo et al. 2014). QTL mapping analyses were performed using MapQTL 6 software (Van Ooijen 2009). After an interval mapping step, the markers with the highest LOD scores were selected to be used as co-factors for multiple-QTL mapping (MQM). The QTL

mapping procedure was followed by a restricted MQM step (Jansen and Stam 1994; Van Ooijen 1999). A LOD score of 3.0 was set as threshold to declare a QTL.

Fine-mapping population and marker development

To generate a suitable segregating population in which to fine-map the *Rbgnq1* QTL on linkage group 5HL, a few VxS lines were selected based on their susceptibility or resistance phenotypes upon *Bgt* inoculation. Lines VxS 57 and VxS 113 showed a resistant phenotype and carried the resistance allele of *Rbgnq1*. These resistant lines were selected for pairwise crossings with one of the most susceptible lines, VxS 143, which carries the susceptibility allele of *Rbgnq1* from SusPtrit. A population of 369 F_2 plants generated from several of these crossing combinations were genotyped (Dr. van Haeringen Laboratorium B.V., Wageningen-NL) using KASPar™ assays with four SNP markers (markers M+33, M+16, M-6 and M-49, Table S1). The Illumina iSelect 9k barley Infinium chip used by Yeo et al. (2014) to genotype SxG was also used to genotype 90 RILs of each of the CxS and VxS populations. The genetic maps of the three populations were then integrated to develop a consensus map (A. Martin-Sanz, unpublished). According to the distances in the VxS genetic map, the KASPar markers span a region of approximately 18.7 cM around the QTL peak marker. The markers were numbered according to their order in the consensus map. Markers apical from the peak marker received the "+" sign, and markers proximal from the peak marker received the "-" sign. SNP markers were selected and KASPar™ probes were developed based on information from the 9k Illumina iSelect barley Infinium chip. F_2 plants showing no recombination between the markers were discarded from the initial population, whereas the heterozygous recombinants were retained so that their progeny could be used in subsequent fine-mapping steps.

Genotyping and phenotyping of recombinants

For each round of fine-mapping, the progeny by selfing of F_2 recombinants were grown and inoculated under the same conditions described for the QTL mapping experiments. In each tray, approximately 3 seedlings from each genotype (Vada, SusPtrit, and the respective parental RILs of the population) were included as controls. Priority was given to plants originating from the crossings VxS 57 x VxS 143 because these two RILs carried the susceptibility allele of the QTL mapped on 2H, thus preventing this QTL from segregating in the background. The seedlings were macroscopically phenotyped at 7-9 dai using the 0 to 5 scale described above. For DNA isolation, a segment of leaf tissue approximately 2 cm in size was sampled and transferred to a tube containing 150 µL of extraction buffer (10 mL of 0.5 M Tris-HCl pH 7.5, 1.76 g NaCl, and 10 g sucrose in distilled water to a final volume of 100 mL). Leaf samples were ground using a TissueLyser II (Qiagen) at 25 Hz for 2 min; the tubes containing the samples were then transferred to a water bath at 80 °C for 5 min. The samples were centrifuged at 4600 rpm for 15

min; 30 µL of each supernatant was transferred to a new tube containing 90 µL of distilled water and used as the DNA template in the genotyping PCR reactions.

The progenies of the F_2 recombinants (F_3 and F_4) were genotyped using the small amplicon genotyping approach (Liew et al. 2004) in the LightScanner® system (Idaho Technology). For each LightScanner marker used in this study, primers were developed around the SNP so that the amplicon size would not be larger than 50 bp. Genotypic information was generated after high-resolution melting analysis of the small amplicons in the presence of the fluorescent double-stranded DNA-binding dye LCGreen. SNP information was obtained either from the 9k Illumina iSelect barley Infinium chip or based on polymorphisms found within the sequence of genes known to be present in the region (according to the barley draft genome sequence (International Barley Genome Sequencing Consortium 2012). The primers were designed using the Primer3Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Table S1 contains information on the primer sequences. The KASPar markers M+33, M+16, M-6 and M-49 were converted to LightScanner markers. The PCR reactions contained 4.5 µL of distilled H_2O, 1.0 µL of LCGreen (Idaho Technology), 0.1 µL of Phire Hot Start II DNA Polymerase (Thermo Scientific), 5X Phire Reaction buffer, 1.0 µL of dNTPs (5 mM), 0.5 µL of each primer (5 µM), and 1 µL of DNA template. PCR was performed under the following conditions: an initial denaturation step at 98 °C for 30 s followed by 35 cycles of 98 °C for 10 s, 60 °C (or 68 °C) for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 1 min. After the PCR was completed, melting analysis data were generated by LightScanner with a start temperature of 67 °C and an end temperature of 98 °C. The LightScanner software was used to analyze the high-resolution melting data, and genotyping results were obtained by comparing the unique melting profile curves of homozygous Vada, homozygous SusPtrit, and heterozygous variants.

Screening of BAC libraries for physical map construction

BAC libraries for the barley genotypes Vada and SusPtrit were constructed (Yeo et al. 2016) using the non-gridded library approach (Isidore et al. 2005; Ma et al. 2000), in which several hundreds of BAC clones are pooled and stored together. This approach allows rapid screening of the genomic library for target clones using a PCR-based approach. To screen the Vada and SusPtrit BAC libraries for clones covering the *Rbgnq1* region, primers were developed (Table S2) based on the sequences of genes in the vicinity of markers M-13, M-15 and M-14 and also on the sequences of genes known to be present in the region according to the barley reference genome of cv. Morex (International Barley Genome Sequencing Consortium 2012). Markers within the *Rbgnq1* locus were used as queries in BLAST searches (http://webblast.ipk-gatersleben.de/barley_ibsc/) against the Morex whole genome shotgun (WGS) sequencing

database to look for positive WGS contigs. Additional BAC clones were later isolated based on PCR screening using primers based on the sequences of the BAC ends or on the sequences of genes present in the BAC monoclones isolated earlier.

Identification of positive pools. The first step in BAC library screening was the identification of BAC pools containing target clones for the QTL region. This was determined by PCR using the plasmid DNA isolated from each pool (20x diluted) as template. PCR reactions were performed in a final volume of 10 µL (the PCR reagents and corresponding volumes are described in Table S3) under the following conditions ("PCR 1"): 98 °C for 30 s followed by 35 cycles at 98 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 1 min. Genomic DNA of cv. Vada and SusPtrit were used as positive controls. Amplification was checked (here and in the next steps) by electrophoresing 5 µL of PCR product on a 1% agarose gel and staining the gel with GelRed™ (Biotium). The presence of a clear bright band with the expected amplicon size indicated that the corresponding pool was positive for the presence of the target BAC clone.

BAC monoclone isolation. For each positive BAC pool, a sample from the stock was diluted 10,000x (in demi water), and 50-µL or 100-µL aliquots of the dilution were added to 20 mL of LB medium and plated in 384-well plates. The 384-well plates were incubated for 14-16 h at 37 °C and then replicated onto square Petri dishes (144 cm^2) containing solid LB medium (LBA) supplemented with 34 µg/mL of the selective antibiotic chloramphenicol (CAM). The colonies that grew from the 384 wells (each well still containing multiple BAC clones) were column-pooled by scraping the colonies from the solid medium using a pipette tip and transferring them to a tube containing 150 µL of distilled water. A total of 24 column pools per plate were sampled in this manner, diluted 10x and used as template in a PCR reaction (1 µL in a final reaction volume of 20 µL; cycling conditions "PCR 2": 94 °C for 5 min followed by 35 cycles at 98 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 1 min). After the identification of a positive column pool, the 16 wells in that column were tested to identify the well(s) containing the target BAC clone; in this step, a 10x dilution of the overnight culture medium was used as template in the PCR reaction (1 µL in a final reaction volume of 20 µL, Table S3; cycling conditions "PCR 2"). The content of the positive well was diluted 100,000x, and a second 384-well plate was prepared, replicated and PCR-screened as described above except for the addition of 34 µg/mL of CAM to the liquid LB medium. Once a positive well was identified for the second 384-well plate, its content was diluted either 10,000x or 100,000x, plated onto selective LBA and incubated for 14-16 h at 37 ºC. Single colonies were picked and individually transferred to tubes containing 100 µL of selective LB. After 14-16 h growth at 37 ºC, the overnight culture was used as template in a PCR reaction (1 µL in a final reaction volume of 20 µL; cycling conditions "PCR 2") to detect positive BAC monoclones.

BAC clone sequencing and gene annotation

Characterization of the BAC clones was performed at the INRA-CNRGV (http://cnrgv.toulouse.inra.fr/) (Castanet Tolosan, France). Sequencing was performed after individual tagging of the BAC clones and pooling on a PacBio RSII sequencer (P6C4 chemistry). After a demultiplexing step, assembly was performed following the HGAP standard PacBio workflow (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/HGAP).

High-confidence predicted barley gene models (IBSC 2012) for the *Rbgnq1* interval in Morex were used to annotate the Vada contig sequences with a match criterion set to greater than 92% identity. To confirm the gene predictions and identify regions containing putative genes not predicted in Morex, we identified the best BAC blast hits received from HarvEST:barley 35 relaxed EST database (http://138.23.178.42/blast/) with >95% similarity threshold.

The sequences of candidate genes in Vada were compared with their SusPtrit alleles when the corresponding SusPtrit gene was represented in the BAC isolated from the SusPtrit library. Predictions of protein sequences and ORFs were performed for the candidate genes based on similarity with transcripts from the high-confidence predictions of Morex transcripts database ("Barley representative Transcripts HC", http://webblast.ipk-gatersleben.de/barley_ibsc/). The results were also analyzed for similarity with barley full-length cDNA sequences deposited in the NCBI database.

Transient transformation of whole-BAC sequences

Transient gene expression assays based on ballistic transformation are commonly used in the study of cereal-powdery mildew interactions (Ihlow et al. 2008; Panstruga 2004; Schweizer et al. 1999). Here, we employed whole BAC sequences in transient transformation assays to check for an effect of the genes represented by the BAC clones on haustorium formation by *Bgt*. The plasmid DNA of BACs V49, V62, V24, V5 and V52 (which together form the minimum tiling path of the two Vada contigs spanning the *Rbgnq1* interval) was isolated using the PureLink® HiPure Plasmid Midiprep Kit (Invitrogen) according to the manufacturer's instructions. Leaf segments of 7-day-old plants of the barley line $SusBgt_{SC}$ and the wheat cv. Kanzler were bombarded as described (Christensen et al. 2004). For the bombardments, gold particles were coated with 7 µg of DNA from individual BACs or with 35 µg of a mixture of DNA from all five BACs. Five bombardment experiments were carried out, each including two samples for the control (empty BAC 632f23). The plasmid pUbiGUS was always co-bombarded to allow the expression of the reporter gene beta-glucuronidase (GUS). The bombarded leaf segments were challenged by inoculation with approximately 200 *Bgt* conidia/mm^2 4 h after bombardment. Two days after the bombardment, the susceptibility index (SI) of GUS-expressing cells was determined by

microscopic examination as described (Douchkov et al. 2005). To test for a decrease in haustorium formation, two-sample T-tests (a = 0.05) were performed for the BACs for which the average SI was lower than on the control.

Results

QTL mapping results

The three mapping populations showed a continuous distribution of macroscopic disease scores (Fig. 1), indicating quantitative inheritance of nonhost resistance to *Bgt* in barley. The majority of lines in the VxS population were immune to *Bgt*, and fewer than 10% showed scores higher than 4. For the SxG and CxS populations, most of the lines showed intermediate scores (scores of 2-4). The regions associated with nonhost resistance to *Bgt* were mapped in the VxS and SxG populations based on the data obtained in individual experiments and also on the average results of the three inoculation experiments (or two inoculation experiments for VxS) (Tables 1, S4). In the CxS population, no QTL with LOD higher than 3.0 was mapped (Table S4). In that population, the correlation between the three replications was less than 0.5. All QTLs mapped in the SxG population had SusPtrit as the parent donor of the resistance allele, and they were located on chromosomes 1H, 2H, 4H and 7H. None of the QTLs detected in the SxG population had a position similar to that of the QTLs detected in VxS.

For VxS, QTL mapping analyses were performed on data originating from two phenotyping approaches: one inoculation experiment was phenotyped microscopically by determining the proportion of infection units that were established, i.e., that formed ESH after haustorium formation, and two inoculation experiments were assessed macroscopically based on the relative 0 to 5 scale. The QTL mapping results obtained with the two phenotyping approaches were very similar, resulting in one large-effect QTL on chromosome 5H and a minor one on 2H, although the LOD score of the latter QTL for HF was 2.7 and hence below the threshold of 3.0. The QTLs on 5H and 2H have the same positions as those found in a previous study in which $SusBgt_{SC}$ and $SusBgt_{DC}$ were used as susceptible parents (Romero et al. 2018); hence, they were named "*Rbgnq1*" and "*Rbgnq2*", respectively. The resistance alleles of *Rbgnq1* and *Rbgnq2* were contributed by Vada. *Rbgnq1* had an LOD score of 19.9 for the average of the three experiments and accounted for more than 40% of the explained phenotypic variance. Due to its large effect, this QTL was considered suitable for fine-mapping.

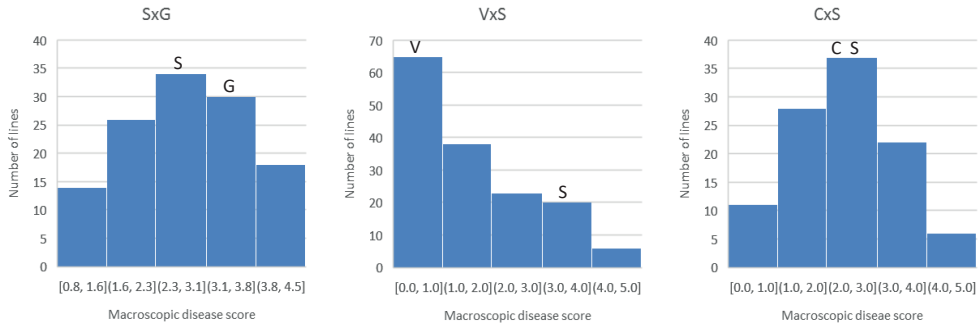

Fig. 1 Histograms showing the frequency distribution of macroscopic disease scores for the barley mapping populations SusPtrit x Golden Promise (SxG), Vada x SusPtrit (VxS) and Cebada Capa x SusPtrit (CxS). The data represent the average of three inoculation experiments for SxG and CxS and the average of two inoculation experiments for VxS.

Fine-mapping of Rbgnq1

Ninety-four recombinants were identified among the initial population of 369 F_2 plants genotyped with markers M+33, M+16, M-6 and M-49. This implies a genetic distance of 12.3 cM between M+33 and M-49. The non-recombinant F_2 plants were discarded. The progeny of four F_2 recombinants, in total 180 F_3 seedlings, were screened in the first round of fine-mapping; the seedlings were genotyped with the same four markers used to genotype the initial F_2 population (M+33, M+16, M-6, M-49) and evaluated based on their phenotypes upon *Bgt* inoculation. Plants that had the SusPtrit marker alleles in the homozygous state for the region between M-6 and M-49 showed a susceptible phenotype corresponding to that of *Bgt*, whereas no such association was found for other marker intervals. Hence, the first fine-mapping screening delimited the resistance gene to an interval of 6.81 cM between markers M-6 and M-49 (Fig. 2a). This interval does not include the original peak marker of the QTL, E42M55-75. Plants that were heterozygous for the region of the QTL were as resistant as the homozygous Vada, demonstrating that the resistance allele of *Rbgnq1* is dominant over the susceptible allele.

After the region between M-6 and M-49 was saturated with additional markers, several fine-mapping screenings were performed on the basis of 494 heterozygous recombinants for the target interval, representing 13 independent recombination events (Table 2). These recombinants were again genotyped and assessed for their level of susceptibility to *Bgt* (Table 2). As a result, *Rbgtq1* was localized to the window between markers M-13 and M-14 (Fig. 2c). The order of markers in Table 2 (M-13, M-15, M-14, etc.) shows that the original order in which the marker numbering was based (consensus map order) was not correct, as the recombinants indicate a different linear order of these sequences. The QTL co-segregates with markers M-15 and $M_{synt980}$. A population of 1489 plants was screened for meiotic recombination events

between the flanking markers M-13 and M14, resulting in an estimated mapping distance of 0.27 cM.

Table 1 Summary of QTL mapping results for the SusPtrit x Golden Promise (SxG) and Vada x SusPtrit (VxS) mapping populations for nonhost resistance to *Blumeria graminis* f.sp. *tritici* (*Bgt*).

Mapping population	Phenotyping[a]	QTL name[b]	Peak marker	Chr[c]	Position (cM)	LOD	% Expl[d]	Additive[e]	Donor[f]
SxG	Macroscopic	*Rbgnq6*	SCRI_RS_152464	1H	19.3	5.4	11.2	-0.31	SusPtrit
	Macroscopic	*Rbgnq8*	SCRI_RS_174063	2H	29.4	3.2	6.3	-0.24	SusPtrit
	Macroscopic	*Rbgnq7*	BOPA1_GBS0288-1	4H	99.1	5.2	10.8	-0.32	SusPtrit
	Macroscopic	*Rbgnq5*	SCRI_RS_104566	7H	106.2	9.3	20.8	-0.42	SusPtrit
VxS	Macroscopic	*Rbgnq2*	P17M54-497	2H	130.5	4.9	8.2	0.34	Vada
	Macroscopic	*Rbgnq1*	E42M55-75	5H	132.3	19.9	43.7	0.79	Vada
	HF	*Rbgnq2*	P17M54-497	2H	130.5	2.7	5.9	2.62	Vada
	HF	*Rbgnq1*	E42M55-75	5H	132.3	13.0	39.1	6.82	Vada

The mapping results for macroscopic infection score are based on the average of three inoculation experiments for SxG and two inoculation experiments for VxS. The third experiment involving the VxS population was assessed microscopically for haustorium formation (HF).

[a] The phenotyping method was either macroscopic using a scale of 0 (resistant) to 5 (susceptible) or microscopic by assessment of haustorium formation (HF)

[b] The QTLs are designated "*Rbgnq*" ("*Resistance to Blumeria graminis nonhost quantitative*") and are followed by numbers that correspond to the order of QTLs mapped by Romero et al. (2018)

[c] The chromosome (linkage group) in which the QTL was mapped

[d] The proportion of phenotypic variance explained by the QTL

[e] The effect of having one allele from SusPtrit on the macroscopic infection score

[f] Parent donor of the resistance allele

Physical mapping of Rbgnq1

Primers developed based on the sequences of genes in the vicinity of markers M-13, M-15 and M-14 were used to identify the initial Vada BAC clones. After the first clones had been isolated for the region, their sequence was used to develop new primers based on the BAC ends or on gene sequences, and these primers were used to identify overlapping clones in the BAC libraries. For Vada, a total of nine clones between markers M-13 and M-14 were identified; these were V18, V57, V49, V62, V24, V5, V56, V52 and V66. For SusPtrit, a single clone harboring marker M-15, S56, was identified. Long-read sequencing by SMRT technology (PacBio) generates reads that are 9 Kb in size on average; these reads are long enough to span complex repetitive regions that are common in the barley genome. As a result, the assembly was successful in generating single contigs for each clone (Table S5).

The distance between markers M-13 and M-14 in the physical map of cv. Morex (Mascher et al. 2017) is 556 Kbp, and the locus contains 20 high-confidence (HC) predicted genes ("HORVU" models, Table S6). Of the 20 genes predicted for Morex, 17 genes are represented in this interval in Vada, although they occur in a different order.

Table 2 Recombinant plants whose progeny (by selfing) was screened to fine-map the *Rbgnq1* interval. Below each marker there is information of the corresponding genotyping, either "H" (heterozygous), "S" (homozygous SusPtrit) or "V" (homozygous Vada).

Recombinants	M -10	M -13	$M_{synt980}$	M -15	M -14	M-24	M -19	Generation	n	Phenotype
18-4	H	S	S	S	S	S	S	F_3	50	Susceptible
2_13	H	H	H	H	H	S	S	F_3	40	Segregates
6_7	H	H	H	H	H	H	S	F_3	79	Segregates
2_15	H	H	H	H	H	H	V	F_3	38	Segregates
1_1	S	H	H	H	H	H	H	F_3	33	Segregates
3_15	V	V	V	V	H	H	H	F_3	75	Resistant
2_3	V	V	V	V	V	H	H	F_3	43	Resistant
2_7.63		H	H	H	V			F_4	22	Segregates
2_7.24		V	V	V	H			F_4	20	Resistant
6_7.31		H	S	S	S			F_4	10	Susceptible
2_7.63-5		H	S	S	V			F_5	30	Susceptible
T1-06(1_6)		H	H	H	S			F_3	28	Segregates
T4-15(4_8)		H	H	H	S			F_3	26	Segregates
							Total number of seedlings:		494	

The QTL region was localized to a shorter interval after stretches of sequences corresponding to the genes "serine/threonine protein kinase" (HORVU5Hr1G104610) and "receptor kinase 2" (HORVU5Hr1G104850) were compared between recombinant plants. Sequencing of the PCR products showed that plants carrying the Vada allele for the serine/threonine kinase locus (Mrx_4610) showed the susceptible phenotype for *Bgt*. Similarly, plants carrying the receptor kinase 2 (Mrx_4850) allele from the susceptible parent (SusPtrit) were resistant to *Bgt* (Table S7).

Five of the nine BAC clones initially isolated for Vada form the two contigs that represent the physical map of *Rbgnq1* (contig 1: 74.5 Kb, contig 2: 330.8 Kb). Additional genes not predicted in the Morex genome are predicted in Vada based on similarity with barley U35 EST database (Table 3). For most of the genes in the *Rbgnq1* interval matching HORVU annotations, there was a corresponding U35 unigene hit, except for HORVU5Hr1G104760 (Bifunctional inhibitor/lipid-transfer protein, gene #4) and HORVU5Hr1G104690 (acetyltransferase (GNAT) domain protein, gene #6) (Table 3).

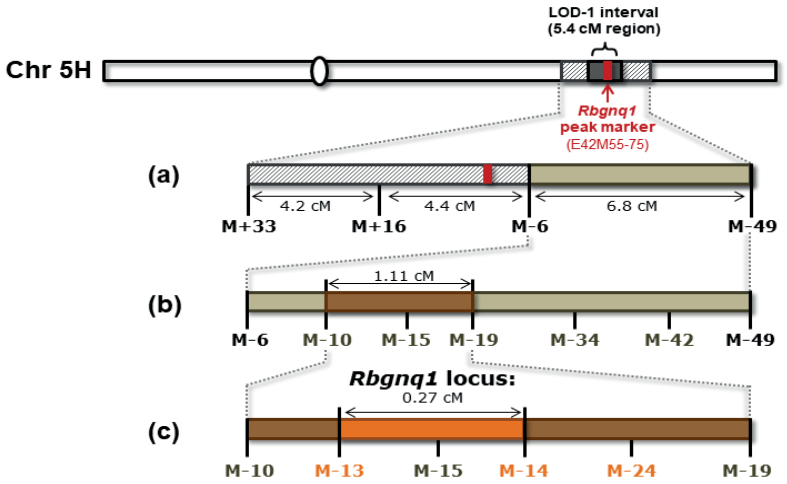

Fig. 2 Fine-mapping of QTL *Rbgnq1*. **(a)** First round of fine-mapping: the progeny of four F_2 recombinants, a total of 180 F_3 seedlings, were screened. The seedlings were genotyped using four markers spanning the QTL in a 12-cM region and evaluated based on their phenotypes upon Bgt inoculation. The QTL region was delimited to the interval between M-6 and M-49. **(b)** Of 360 heterozygous F_2 plants with valid genotyping information between M-6 and M-49, 8 were recombinants between M-10 and M-19, corresponding to a 1.11-cM distance. **(c)** After disease testing was performed on the progeny of several (13) recombinants, the QTL window was delimited to the interval between M-13 and M-14. A population of 1489 segregating plants between M-13 and M-14 were screened, and 8 recombinants were found, corresponding to a 0.27-cM distance.

Sequencing of S56 evidenced the presence of gene sequences in Vada (resistant parent) that are not found in SusPtrit (susceptible parent). Two of these genes appears to be each a duplication of HORVU5Hr1G104800, which encodes an acyl-CoA N-acyltransferases (NAT) superfamily protein, and HORVU5Hr1G104690, which encodes an acetyltransferase (GNAT) domain protein, although their sequences vary slightly. Comparison of the sequences of SusPtrit in S56 and its homologous region in Vada BAC clone V5 shows that the distance between the markers $M_{synt980}$ and M-15 in Vada is larger (126 Kb) than the distance between these markers in SusPtrit (26.2 Kb); however, the order of the genes appears to be the same, with the exception of the duplication of HORVU5Hr1G104800 and HORVU5Hr1G104690 in Vada. Both candidate genes #5 and #9 are annotated as HORVU5Hr1G104800 (acyl-CoA N-acyltransferase), although the sequence of gene #5 is more similar to the sequences of their homologues in Morex (97%) and SusPtrit (99%) than gene #9 (92% identity with Morex and SusPtrit). The same is the case for the two genes annotated as HORVU5Hr1G104690 (acetyltransferase): gene #11 is more similar to their homologues in Morex (97%) and SusPtrit (99%) than gene #6 (96% Morex, 94% SusPtrit) (Table 3).

Other genes present in Vada, but not in the homologous SusPtrit region covered by BAC S56 are gene #7 (B3 domain-containing protein), gene #8 (uncharacterized protein) and gene

#10 (Far1 protein). The homologous region in SusPtrit for gene #16 (Putative polyprotein) is not covered by BAC S56 and therefore it is not possible to determine if this gene is present in SusPtrit or not.

Candidate gene #14 (chitinase) has only a partial match with the sequence of *Hordeum vulgare* subsp. *vulgare* cDNA clone (GenBank: AK249826.1), so it is likely to represent a truncated version of the gene in Vada and SusPtrit. No differences exist in the DNA sequences of candidate gene #15 (uncharacterized protein) between Vada and SusPtrit and no differences at the amino-acid level were found for candidate genes #12 (glutamate dehydrogenase) and #13 (ribosomal protein) between Vada and SusPtrit either.

Transient transformation results

Transient expression of bombarded BAC clones in SusBgt$_{SC}$ (attenuated nonhost for *Bgt*) as well as in wheat cv. Kanzler (host for *Bgt*) were used as the first functional test of the function of the candidate genes contained in the BACs derived from the resistant parent Vada. A causal gene for nonhost resistance would be expected to complement nonhost resistance in SusBgt$_{SC}$ and might also contribute to quantitative resistance in Kanzler. The results for susceptibility index (SI) relative to the empty BAC control are shown in Fig. 3 and correspond to the total amount of transformed cells in the five bombardment experiments. There is a tendency to decreased haustorium formation in SusBgt$_{SC}$ conferred by BAC clone V49 (Fig 3a), and in Kanzler conferred by BAC mix bombardment (Fig. 3b). However, these results were not significant (a= 0.05) due to the large SI variation occurring between experiments.

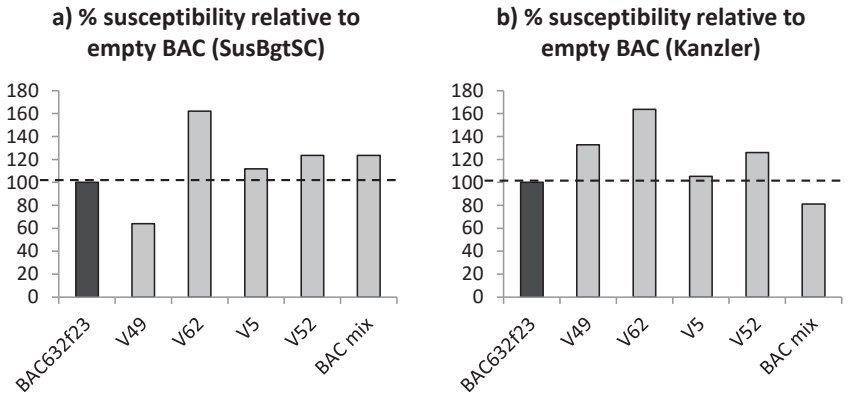

Fig. 3 Susceptibility index results for transient transformation of **a)** barley SusBgt$_{SC}$ line and **b)** wheat cv. Kanzler with Vada BAC clones covering the *Rbgnq1* interval. Results are given in percentage relative to the empty BAC 632f23 control

Table 3 Overview of genes predicted for the *Rbgnq1* interval. Annotation is based on the automatic annotation provided by the HarvEST software (version 2.26) or on the annotation of the corresponding Morex high-confidence gene model.

Candidate gene #	Subject ID	Score	Length	identities (%)	Contig, BAC clone	Start	End	Annotation	U35 seq ID	Remarks
1	U35_39310	1158	757	95%	1, V49	35536	36286	Unknown	U35_39310	Best hits on NCBI database contain repetitive elements
2	HORVU5Hr1G104740	2963	1606	99%	2, V62	52181	53787	UDP-Glycosyltransferase superfamily protein	U35_17121	Vada and SusPtrit sequences not compared
3	HORVU5Hr1G104750	2056	1124	99%	2, V62	73571	74695	Bifunctional inhibitor/lipid-transfer protein (prediction GPI anchor: highly probably)	U35_12218	Polymorphisms between Vada and SusPtrit
4	HORVU5Hr1G104760	1208	668	99%	2, V62	75155	75823	Bifunctional inhibitor/lipid-transfer protein (prediction GPI anchor: not GPI-anchored)	No	Polymorphisms between Vada and SusPtrit
5	HORVU5Hr1G104800	1098	647	97%	2, V62	78798	79445	Acyl-CoA N-acyltransferases (NAT) superfamily protein	U35_22035	Polymorphisms between Vada and SusPtrit
6	HORVU5Hr1G104690	1563	957	96%	2, V62	81862	82819	Acetyltransferase (GNAT) domain protein	No	Polymorphisms with candidate gene #11; polymorphisms between Vada and SusPtrit
7	U35_43338	1005	632	96%	2, V62	89408	90023	B3 domain-containing protein n=1 Tax=Aegilops tauschii	U35_43338	Absent in SusPtrit BAC S56
8	U35_16576, U35_16575	1513	819	100%	2, V62	121911	122729	Uncharacterized protein n=1 Tax=Brachypodium distachyon	U35_16576, U35_16575	Absent in SusPtrit BAC S56
9	HORVU5Hr1G104800	861	614	92%	2, V5	161391	162005	Acyl-CoA N-acyltransferases (NAT) superfamily protein	U35_22035	Polymorphisms with candidate gene #5; polymorphisms between Vada and SusPtrit (truncated protein)
10	U35_26899	1055	675	95%	2, V5	178035	178707	Far1 n=1 Tax=Triticum aestivum RepID=D0QEK9_WHEAT	U35_26899	Absent in SusPtrit BAC S56
11	HORVU5Hr1G104690	1696	995	97%	2, V5	198381	199376	Acetyltransferase (GNAT) domain protein	No	Polymorphisms with candidate gene #6; polymorphisms between Vada and SusPtrit
12	HORVU5Hr1G104700	4750	2664	99%	2, V5	199734	202398	glutamate dehydrogenase 1	U35_2280	No polymorphism between Vada and SusPtrit at amino-acid level
13	HORVU5Hr1G104720	2567	1713	94%	2, V5	214067	215780	30S ribosomal protein S13	U35_14252, U35_42507, U35_42506	No polymorphism between Vada and SusPtrit at amino-acid level
14	HORVU5Hr1G104730	1218	703	98%	2, V5	216803	217506	Chitinase family protein	U35_1882	Only partial sequence; no polymorphisms between Vada and SusPtrit at nucleotide level
15	U35_38051	913	620	95%	2, V5	260527	261118	Uncharacterized protein n=1 Tax=Triticum urartu	U35_38051	No polymorphism between Vada and SusPtrit at nucleotide level
16	U35_44349	835	529	95%	2, V52	315300	315829	Putative polyprotein n=1 Tax=Oryza sativa subsp. japonica	U35_44349	Absent in SusPtrit BAC S56
17	HORVU5Hr1G104840	4501	2502	99%	2, V52	322609	325111	receptor kinase 2	U35_22181, U35_48640	Vada and SusPtrit sequences not compared

Discussion

Powdery mildew fungi of cereals belong to a single species, *Blumeria graminis*, and are classified into *formae speciales* based on host specialization (Inuma et al. 2007). Barley and wheat are both members of the Triticeae tribe, and each of these two crop species is infected by a different *B. graminis forma specialis*. The powdery mildew of barley, *Bgh*, is not able to infect wheat; similarly, the powdery mildew of wheat, *Bgt*, cannot infect barley. This makes barley-*Blumeria* an interesting pathosystem in which to conduct research on host specificity and resistance, since the host and nonhost plants, as well as the adapted and non-adapted pathogens, are closely related. The work of Romero et al. (2018) provided some important clues to the genetics of nonhost resistance in barley to powdery mildew and confirmed previous findings by Aghnoum and Niks (2010) that resistance of barley to *Bgt* is polygenic and mainly based on pre-haustorial mechanisms, with formation of papilla in response to penetration attempts. The specificity of the interaction is not likely to be determined at the PAMP recognition level considering that *Bgh* and *Bgt* are related and probably contain identical PAMPs. The underlying question is why effectors delivered by *Bgt* succeed in suppressing PTI and establishing infection in wheat whereas they fail to do so in barley. Our hypothesis is that the specificity is determined by variation in specific motifs in the effector targets that act as components of the basal defense network (Niks and Marcel 2009). Even slight variations between wheat and barley in the sequences of the defense genes that encode the effector targets would influence the affinity of the targets for the effector and thereby affect the capacity of the would-be pathogen to suppress PTI (Niks 2014; Niks and Marcel 2009). If this hypothesis is correct, the genes causing the QTL effects would encode the targets of effectors. The identification and cloning of nonhost resistance genes in barley may make it possible to transfer this resistance to wheat or to improve quantitative host resistance by allelic optimization of wheat orthologs (Rajaraman et al. 2016).

Susceptibility is a stepwise process. The first step a would-be pathogen must accomplish is to overcome constitutive defense components and induced defense responses at the pre-haustorial level to make it possible to form haustoria. Pre-haustorial defense is supplemented by post-haustorial defense responses that normally activate programmed death of the attacked cells, thereby preventing spread of the pathogen (Lipka et al. 2010; Lipka et al. 2005; Thordal-Christensen 2003). In the barley-*Bgt* pathosystem, the relatively few infection units that succeed in the two initial steps must still overcome a third level of defense to be able to form conidia (Aghnoum and Niks 2010; Romero et al. 2018). In the context of this paper, we use the term “susceptibility” to refer to the fact that although barely genotypes in general are immune to the *Bgt* pathogen, a few barley lines (SusPtrit included) allow a higher rate of penetration success and establishment (i.e. haustorium formation) when challenged by *Bgt*

(Aghnoum and Niks 2010). For those infection units that succeed in establishing haustoria, nutrient uptake from the plant enables secondary elongated hyphae to grow sufficient mycelia to be perceived macroscopically as microcolonies. Microcolony formation occurs only at the seedling stage and is only perceptible at high inoculum density. Even in "susceptible" barley lines, the outcome of *Bgt* infection is quite limited compared to infection of its natural host wheat, where each colony is much larger in size and produces many hundreds of conidia.

The SusPtrit mapping populations segregate for susceptibility to *Bgt*. In the CxS population, there was low correlation between the inoculation experiments, and no QTL was mapped. We have no explanation for this. The QTL mapping results in SxG and VxS indicate that nonhost resistance of barley genotypes to *Bgt* results from a high diversity of genes, as no QTL was shared between the two mapping populations. The genetic basis of nonhost resistance to powdery mildews and rusts show parallels; in both cases, resistance is based on minor genes and a wide diversity of loci are implicated in resistance among barley genotypes (Jafary et al. 2008; Yeo et al. 2014). Despite the fact that Golden Promise and SusPtrit had similar macroscopic scores upon *Bgt* infection, the resistance alleles of SxG QTLs were contributed by SusPtrit, whereas Vada contributed the resistance alleles of the QTLs mapped in the VxS population. Transgressive segregation was observed on SxG and VxS, suggesting that pairs of nonhost barley accessions may complement each other for resistance and susceptibility alleles at different loci. Regarding the phenotyping approaches, macroscopic and microscopic evaluation of microcolonies resulted in the mapping of the same genes; the macroscopic evaluation is more convenient because it avoids the need for histological preparations and thereby saves time. The QTLs mapped in VxS are essentially the same as those mapped in the SusBgt populations (Romero et al. 2018). This is not surprising considering that the resistant parent, Vada, is the same and that SusPtrit is one of the ancestors of both SusBgt lines (Aghnoum and Niks 2010).

Genes determining the specificity of (non)host status

Gene expression studies are important as a means of identifying sets of genes that are differentially expressed during nonhost and host interactions, contributing to the understanding of the molecular basis of nonhost resistance. A recent study analyzed gene expression in wheat and barley during interaction with the adapted and non-adapted forms of three fungal pathogens: powdery mildew, cereal blast and rust (Delventhal et al. 2017). The investigators found that in both plant species a large set of genes that are reprogrammed upon attack by adapted and non-adapted isolates is shared among the three pathosystems and that most of these genes are associated with PTI. The nonhost-specific changes in the transcriptome found for individual pathosystems reflect specific changes in the plant defense response induced by

non-adapted in contrast to adapted pathogens. However, such genes are activated or silenced downstream of the process of primary elicitation of the host/nonhost outcome. A forward genetics approach is necessary to pinpoint the sequence variation underlying the determinant factors of host specificity (Niks 2014).

Map-based cloning of Rbgnq1

Map-based cloning in wheat and barley remained a challenge for a long time due to the size and complexity of the genomes of these species. The highly repetitive nature of the wheat and barley genomes makes marker development a difficult task (Krattinger et al. 2009). The recently available high-quality sequence assembly of barley cv. Morex (Mascher et al. 2017) is an invaluable resource for cereal genetics and genomics and has facilitated map-based cloning. Nearly half of the cloned genes in Triticeae are involved in disease resistance against biotrophic pathogens (Krattinger et al. 2009). Map-based cloning requires that the QTL be fine-mapped to a narrow genetic window so that it is possible to develop the physical map. The QTL with the largest effect mapped in this study, *Rbgnq1*, was also mapped in the SusBgt populations (Romero et al. 2018). In that work, *Rbgnq1* was placed in a rather narrow interval of 1.4 cM that contained 104 predicted genes according to the reference genome of cv. Morex. To more precisely define the QTL interval, a segregating population was developed from VxS RILs. Genotyping of recombinants by high-resolution melting of small amplicons proved to be a rapid and efficient method for the screening of recombinant seedlings, allowing reduction of the genetic window for *Rbgnq1* to 0.27 cM. The genomic BAC libraries available for both parents, Vada and SusPtrit (Yeo et al. 2016), enabled us to advance on the map-based cloning by picking up clones that cover the *Rbgnq1* interval. Because small variations in gene content may occur in different genotypes of the same species, it is important to build a physical map from the same genotypes that originated the population in which the QTL was mapped. In this way, we ensure that the gene(s) responsible for the resistance phenotype is (are) indeed represented.

Haplotype divergence among barley genotypes was observed by Yeo et al. (2016) based on sequencing of both the resistance and the susceptibility loci of *Rphq2* for partial resistance to *P. hordei*. The *Fhb1* locus for *Fusarium* head blight in wheat also showed differences in size and gene content in the susceptible cultivar Chinese Spring and a resistant accession (Schweiger et al. 2016). In the present study, a comparison of the *Rbgnq1* physical map of Vada and the homologous region according to the Morex assembly (Mascher et al. 2017) shows microcolinearity divergence between the two genotypes. We found that gene content and gene order differ between Vada and Morex. The observed discrepancies are not likely due to BAC sequences assembly errors since the long reads produced by PacBio sequencing of the BAC clones enable high-quality assembly even for highly repetitive regions. Sequencing of the single

SusPtrit BAC clone isolated to date for the *Rbgnq1* region also revealed divergences between the Vada and SusPtrit sequences: the lack of homology for the region in between markers M-15 and $M_{synt980}$ in Vada and SusPtrit may explain the lack of recombinants between these markers, which probably limited further fine-mapping of the QTL.

Candidate genes at the Rbgnq1 locus

We present two arguments that the *Rbgnq1* is unlikely to be an NB-LRR gene: first, there is no NB-LRR gene in the list of genes in the Vada interval (Table 3); second, the resistance is pre-haustorial and is not associated with a hypersensitivity reaction. The HORVU gene model annotation is based on cv. Morex, whereas the U35 database contains EST information from several genotypes. In the QTL interval in Vada, we found many regions that match the HORVU annotation but do not contain any corresponding U35 hit, which could indicate that the predicted genes are not generally expressed in barley.

SusPtrit does not contain a full-length copy of U35_26899, encoding a hypothetical protein Far1 of *Triticum aestivum* (gene #10). Also genes #7 and #8 (B3 domain-containing protein and uncharacterized protein, respectively) are not found in the QTL interval in SusPtrit. The B3 domain is a DNA-binding motif unique to plants and characterized in a number of transcription factors that play important roles in developmental processes such as plant growth, embryo development and seed maturation (Waltner et al. 2005). The genes that are absent in SusPtrit may be good candidates for cloning and transient experiments (transient overexpression and TIGS) in barley and wheat. Cultivar Morex is also fully resistant to *Bgt*, although it does not appear to have any of these genes. Nevertheless, nonhost resistance in this genotype may be conferred by different combinations of loci located elsewhere in the genome.

The genes for glutamate dehydrogenase and the ribosomal protein appear to encode identical proteins in Vada and SusPtrit, therefore they can only be candidates if the promoter region differs, resulting in different regulation. Among the candidate genes there is also a UDP-glycosyltransferase, two lipid-transfer proteins (LTP), acetyltransferases, a receptor kinase and hypothetical/uncharacterized proteins. Receptor kinases are well known for their role as pattern recognition receptors (Couto and Zipfel 2016). The UDP-glycosyltransferase BRT1 is a component of *Arabidopsis* nonhost resistance against the soybean rust pathogen *Phakopsora pachyrhizi*, although it is implicated to act at post-invasion resistance (Langenbach et al. 2013). Plants LTPs are known components of plant immunity, and belong to a large family of pathogenesis-related (PR) proteins induced in response to pathogen infection (Finkina et al. 2016). The LTPs are in general synthesized as pre-proteins containing an N-terminal signal peptide that targets the protein to the apoplastic space (Finkina et al. 2016; Salminen et al. 2016). In turn, the GNAT acetyltransferases belong to the histone acetyltransferases (HAT)

family, which are involved in histone binding and acetylation. Modifications in histone proteins shape the chromatin structure and can affect timely and appropriate activation of immune responses in plants (Ding and Wang 2015; Saijo and Reimer-Michalski 2013). Alvarez et al. (2010) proposed that host components involved in epigenetic control of plant inducible defenses may represent attractive targets for microbial effectors.

Functional validation of candidate genes

The final step in map-based cloning consists of the functional validation of candidate genes to demonstrate association between sequences in the target interval and the trait of interest (Krattinger et al. 2009). Transient transformation by particle bombardment is a convenient option for testing the effects of overexpressing or silencing a candidate gene in cereal-powdery mildew interactions (Douchkov et al. 2005; Schweizer et al. 1999). For the transient overexpression assays, we used the barley line $SusBgt_{SC}$ and the wheat cv. Kanzler to test the impact of BAC sequences containing candidate genes on the outcome of the interaction compared to a control transformation (empty BAC). The bombardment results provided some indication (although not significant) that BAC clone V49 has a resistance-enhancing effect against *Bgt*, although this tendency should be confirmed in further transient transformation experiments. This BAC clone contains a single candidate gene in the QTL interval, U35_39310, with unknown annotation. Based on blast results against the NCBI database, the region where this putative gene is located is situated within sequences of several transposable elements. In the same BAC clone also there is also U35_47160, encoding a putative uncharacterized protein (not included in table 3 because it was below the identity significance threshold) and gene HORVU5Hr1G104610, a serine/threonine-protein kinase (although this gene was shown to be located outside of the QTL region, Table S7).

Further experiments are necessary to confirm the effect of genes on V49 on decreasing haustorium formation. If this is confirmed, individual cloning of genes in this BAC may be tested by transient transformation and, ultimately, by stable transformation in Golden Promise barley and in wheat. The physical map of *Rbgnq1* in Vada still contains a gap, which may contain additional genes. Given the comparison with cv. Morex, this gap should not be large. Future BAC clones identified for the *Rbgnq1* region should also be tested by transient transformation. The identification of the gene(s) or region underlying *Rbgnq1* resistance will be a remarkable and unprecedented discovery for the study of nonhost resistance. The findings reported in this study provide an important resource for the necessary additional work of identifying the most likely candidate and confirming its function in conferring nonhost resistance.

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Supplementary material

Table S1 Sequences of KASPar marker probes and primers for small-amplicon genotyping markers

Marker	Locus	Direction	Sequence (5'-3')
M+33 KASPar	SCRI_RS_157238		GATCTCGTCTAGATTCATATGGTTTAGTACCGATTTTGACCCCGTTTAC TACTAGAATCATGCCATGCTT[A/T]GCGTTGGTATGATTGCCAGGGATA ATATCCCCTTCCGCAATGTAAGATTGCCAGGGAAAATGTCCCCTTC
M+16 KASPar	SCRI_RS_9182		CTGCTTCGCAACCGCGGTAGATCTGGACATGTGGTAGTGATTATCGA GTGTTCGTTCTTGCTGTTGCTGG[T/C]GCTCGCTTGCGTAATTAGTGGT TCTCTGTGATGATCAACACTGATTAGATGTCCGATCCAATCCATTCCA
M-6 KASPar	SCRI_RS_220146		TTTTGGTAGATTTTATGTGATATTTTGATAAGTAACATAAAAGTTATGTG TTCATCTGCTTTGACGAAGC[G/A]TGATGTTAAGTTATGTGCATTTCAAA TGTTCTACTGCCCTAGCTCTTGCTATCATCTCGAAGTCTACGCG
M-49 KASPar	SCRI_RS_238426		CCCGTTTTCCTCATGTTTCCTTTTCTTTTTTGAATGGACATTTACTTCAT TCACATTTCCCTTGCTGCTC[A/G]CTATAGCTGGCTTTTTACTCTGTTCT GATTTCAAACCAAGACGTTTAATTTCTTCTGCTGTCGAAGGATA
M +33	SCRI_RS_157238	Fwd	TGGCTCTAGTTCGAATTGTGTC
		Rev	GCAATAAGCTGTACATATCCGG
M +32	SCRI_RS_197532	Fwd	CGGATAATCTCCTGTGGTTAACTG
		Rev	ATTCTTCTGCAGCATTCCTACCA
M +18	BOPA2_12_31234	Fwd	GCGTGTGGTCTACTATTTGCCAGT
		Rev	AAGTTGTACACGGTACACATCCACAC
M -1	BOPA1_4686-1281	Fwd	CAGACCAAAGCGATTGCGAC
		Rev	GAAGATGCTCCCAGTTCTCAGTC
M -6	SCRI_RS_220146	Fwd	GTGTTCATCTGCTTTGACGAA
		Rev	TAGGGCAGTAGAACATTTGAA
M -10 (A)	SCRI_RS_199904	Fwd	CGAGGGGTGTAACGGCAC
		Rev	GCATTATCCGTCCATGCAA
M -11	SCRI_RS_204797	Fwd	TGCACATCTGGTGATCCTGT
		Rev	TTGACGACAACTCAGAGAAGG
M -13 (B)	BOPA2_12_31165	Fwd	TCAATAAACATGTCGGTATCCAT
		Rev	CCTTGTGGCTTATATTTAAATTGC
$MSynt_{980}$	HORVU5Hr1G104750	Fwd	TGAAGCTGATTAACACTGAACCA
		Rev	GCAATTGGCCCTGCAAGT
M -14 (D)	SCRI_RS_125258	Fwd	AACGGCCAAACAATCGAA
		Rev	AGTTTTGACATCCCTGAATGG
M -15 (C)	BOPA2_12_30062	Fwd	TCTGCACCACGAAGAACAT
		Rev	AGAATTCACACACTGACTGACA
M -19	SCRI_RS_193063	Fwd	AAATTGCCAGTTTCTCACTATG
		Rev	AAATGGGTGAGGAGGAGAAG
M -24	SCRI_RS_3287	Fwd	CGTTAAGTAGACGATGGTGCAT
		Rev	TCACAAAGCACTGGGTATACAT
M -42	SCRI_RS_204809	Fwd	GTAATTAACCCGCAGCTCCT
		Rev	AGTGCAGTGGTTGACTCG
M -47	SCRI_RS_222314-F	Fwd	CGCCGGCAGTGAAGCAAG
		Rev	ATCAGTTCAATCTCATGCAGATAGTCC
M -49	SCRI_RS_238426	Fwd	CAGAACAGAGTAAAAAGCCAGCTA
		Rev	ATTCACATTTCCCTTGCTGCTC

Table S2 List of primer pairs used to pick up BAC clones for the *Rbgnq1* region

Primer	Direction	Sequence (5'--> 3')	Product size	Remarks on primers development
Synt57970	Fwd Rev	GCACTTACGACAGCCCATTTAG GAAGATCACCGCAACGAGAT	549 bp	Based on morex_contig_41924; Amplifies a region in Vada clone V18
Synt57980	Fwd Rev	CAGGGTCTGCAAATCCATCT ACCAGAAGAACGGAGGGAGT	530 bp	Based on morex_contig_2550379; Amplifies a region in Vada clone V24
Synt57990	Fwd Rev	TGGCACGGACAGTTCTCTTA TGAAGGAGCAAATTGGACTG	461 bp	Based on morex_contig_2550379; Amplifies a region in Vada clone V24
Synt58040	Fwd Rev	AAGCCCAGCACCTACTTTAGAC CTTTCAGACCCGTGGATGAT	288 bp	Amplifies a region of Vada clone V56
Synt58050	Fwd Rev	TTTTCATGTCTACGATCCTTCTG CATGCTCTTGCCAAACATTG	528 bp	Amplifies a region of Vada clone V56
Synt58060	Fwd Rev	TAGGGAAACCAACAGAGAGAGG GTAAGGCAGACAAGCCAAGG	748 bp	Based on sequence of morex_contig_136690
Mo2550	Fwd Rev	CCGATGCCTGACTAGAGACC TTAGAAACGGAGGGAGTATGAA	805 bp	Based on sequence of morex_contig_2550379
Mo5576	Fwd Rev	GGACTATGGTGTCTGGAACAA CGGATGCGAGTTAGGAAGAC	944 bp	Based on sequence of morex_contig_55763
Mo1366	Fwd Rev	GGTCTTTGCTGGGTTATGAG ACGGTGAATTTGGGTCAGTC	1003 bp	Based on sequence of morex_contig_136690
V18.2_end	Fwd Rev	GCTCCCTGTGTGGATTTCAT GATCCTCCCTGGTTCTCTCC	846 bp	BAC end of Vada clone V18
V18_end	Fwd Rev	CAAGCTCATACCGCAAACAA TCTTCCCACCCCTTTAGCC	288 bp	BAC end of Vada clone V18
V49_end2	Fwd Rev	ACCTCGCCTGCTTTCTTTATTC ACTCTCTCATCCCCTCTCATTCTC	1083 bp	BAC end of Vada clone V49
V24_end1	Fwd Rev	AAGCATGGCTCTCGTTGTCT TCCGAACCATTCAGATTGA	293 bp	BAC end of Vada clone V24
V24_end2	Fwd Rev	GAGCCCTACGAGTCCACAGA CACGGTGACACGAATCAGTC	437 bp	BAC end of Vada clone V24
V24.2_end2	Fwd Rev	GGACCGCCGTAGTTGCTCT TAAAGCCCAGAAACGCCAGT	883 bp	BAC end of Vada clone V24
V56_end1	Fwd Rev	TCTGGGTCAAGGGCAGTATC AAGGTTGAGAGAGCACAGAAGG	151 bp	BAC end of Vada clone V56
V56.2_end1	Fwd Rev	ATGCTTAGAGTGCCCTGCTC ATTCATGTTTTCGCCTGGTG	726 bp	BAC sequence of Vada clone V56 (end region was too repetitive)
V56_end2	Fwd Rev	GAAAATCGTCAAATAGGGAATG CGGCTCCATCAATTTCTTGT	367 bp	BAC end of Vada clone V56
V66_end2	Fwd Rev	TTTGTTGTGTTGCCTTGGAA GAAGAGGAAATGGGGAGACC	183 bp	BAC end of Vada clone V66
Mrx_4670	Fwd Rev	CGTGCCGTTTTGATTATTACC CCCCATGAAGTAATGCGTCT	1287 bp	Based on HORVU5Hr1G104670 (Glutathione S-transferase family protein); amplifies sequence of V18
Mrx_4630	Fwd Rev	CCGATCAGTGTACTCGTCAGC AATCAGTGCCGATGGTCAG	807 bp	Based on HORVU5Hr1G104630 (Vicilin); amplifies sequence of V57
Mrx_4620	Fwd Rev	CTGCCATCTCCTTCCCTTC GCCCTTGATGTTGAACCACT	1039 bp	Based on HORVU5Hr1G104620 (Type IV inositol polyphosphate 5-phosphatase 7); amplifies sequence of V57
Mrx_4610	Fwd Rev	ATTCAAGCGGTTTTGTTTGC TCATAGTGTTCCTTCATAGCAGCA	987 bp	Based on HORVU5Hr1G104610 (Serine/threonine-protein kinase); amplifies a sequence of V49
Mrx_4740	Fwd Rev	GAGACATGCCGACAGTGATG CCTCACGAAGAAACCCTGAA	913 bp	Based on HORVU5Hr1G104740 (UDP-Glycosyltransferase superfamily protein); amplifies a sequence of V62
Mrx_4840	Fwd Rev	TGTCGCTCTACTCCTGCTGA GATATTCCCCATTACGATCAACC	1704 bp	Based on HORVU5Hr1G104840 (receptor kinase 2); amplifies sequence of V52
Mrx_4840-50	Fwd Rev	TCTTGTATAGTTCACCTGCGTACC GACTGAAGGCAAGGCTCATC	1063 bp	Based on sequence between kinases HORVU5Hr1G104840 and HORVU5Hr1G104850
Mrx_4850-1	Fwd Rev	TATTTGCTTTCGGGGTGTTC TGAGAGGCTAGTTGCGTTGG	394 bp	Based HORVU5Hr1G104850 (receptor kinase 2); amplifies sequence of V52
Mrx_4850-2	Fwd Rev	TTATAGAAACAAGGACGAGTGAGTC TTGGTAGCTTGCTGATGTCG	1217 bp	Based HORVU5Hr1G104850 (receptor kinase 2); amplifies sequence of V52
Mrx_4850-3	Fwd Rev	GGCATTGGGGATACTGAACC ATCGACCCTTCATCTCGTTG	1806 bp	Based HORVU5Hr1G104850 (receptor kinase 2); amplifies sequence of V52

Table S3 PCR reaction components for 10-µL and 20-µL final volume reactions using Thermo Scientific™ Phire™ Hot Start II DNA Polymerase

Component	10-µL reaction	20-µL reaction
H_2O	6.0 µL	13.0 µL
5X Phire Reaction buffer	2.0 µL	4.0 µL
dNTPs (5 mM)	0.4 µL	0.8 µL
Primer F (5 µM)	0.25 µL	0.5 µL
Primer R (5 µM)	0.25 µL	0.5 µL
Phire Hot Start II DNA Polymerase	0.1 µL	0.2 µL
Template*	1.0 µL	1.0 µL

*The template is either plasmid DNA or a dilution of bacterial culture, as indicated in the text.

Table S4 QTL mapping results for the SusPtrit x Golden Promise (SxG), Vada x SusPtrit (VxS) and Cebada Capa x SusPtrit mapping populations for nonhost resistance to *Blumeria graminis* f.sp. *tritici* (*Bgt*). The mapping results are based on macroscopic phenotyping, except the third experiment involving the VxS population, which was assessed microscopically for haustorium formation (HF).

Mapping population	Rep	QTL name[a]	Peak marker	Chr[b]	Position (cM)	LOD	% Expl[e]	Additive[f]	Donor[g]
SxG	Rep1	*Rbgnq6*	SCRI_RS_152464	1H	19.3	6.9	16.8	-0.52	GP
	Rep1	*Rbgnq7*	BOPA2_12_30158	4H	97.4	4.9	11.5	-0.44	GP
	Rep1	*Rbgnq5*	SCRI_RS_104566	7H	106.2	5.2	12.2	-0.44	GP
	Rep2		SCRI_RS_159331	4H	64.1	3.1	10	-0.43	GP
	Rep2	*Rbgnq5*	SCRI_RS_194291	7H	103.7	5.0	16.5	-0.52	GP
	Rep3		BOPA1_4407-1344	4H	65.7	3.1	10.3	-0.32	GP
	Rep3	*Rbgnq5*	SCRI_RS_104566	7H	106.2	4.6	15.6	-0.37	GP
	Average	*Rbgnq6*	SCRI_RS_152464	1H	19.3	5.4	11.2	-0.31	GP
	Average	*Rbgnq8*	SCRI_RS_174063	2H	29.4	3.2	6.3	-0.24	GP
	Average	*Rbgnq7*	BOPA1_GBS0288-1	4H	99.1	5.2	10.8	-0.32	GP
	Average	*Rbgnq5*	SCRI_RS_104566	7H	106.2	9.3	20.8	-0.42	GP
VxS	HF	*Rbgnq2*	P17M54-497	2H	130.5	2.7	5.9	2.62	Vada
	HF	*Rbgnq1*	E42M55-75	5H	132.3	13.0	39.1	6.82	Vada
	Rep1	*Rbgnq2*	P17M54-497	2H	130.5	4.5	9.0	0.44	Vada
	Rep1	*Rbgnq1*	E42M55-75	5H	132.3	14.9	36.6	0.88	Vada
	Rep2	*Rbgnq2*	P17M54-497	2H	130.5	2.7	5.8	0.29	Vada
	Rep2	*Rbgnq1*	E42M55-75	5H	132.3	14.0	36	0.73	Vada
	Average	*Rbgnq2*	P17M54-497	2H	130.5	4.9	8.2	0.34	Vada
	Reps 1 and 2	*Rbgnq1*	E42M55-75	5H	132.3	19.9	43.7	0.79	Vada
CxS	Rep1		E45M55-439	3H	4.9	2.7	12.8	0.37	CC

[a] The QTLs are designated "*Rbgnq*" ("*Resistance to Blumeria graminis nonhost quantitative*") and are followed by numbers that correspond to the order of QTLs mapped by Romero et al. (2018)

[b] The chromosome (linkage group) in which the QTL was mapped

[d] The proportion of phenotypic variance explained by the QTL

[e] The effect of having one allele from SusPtrit on the macroscopic infection score

[f] Parent donor of the resistance allele

Table S5. Assembly results for Vada and SusPtrit BACs after PacBio sequencing

BAC clone	Genotype	Mean coverage (X)	Mean QV*	Size (Kb)
V18	Vada	399	48.56	99
V57	Vada	119	48.84	101
V49	Vada	64	48.73	266
V62	Vada	178	48.78	150
V24	Vada	579	48.56	113
V5	Vada	179	48.78	117
V56	Vada	463	48.55	114
V52	Vada	339	48.81	119
V66	Vada	571	48.54	136
S56	SusPtrit	273	48.53	139

*QV: Quality Value (Probability of incorrect base call: QV40=99,99%, QV50=99,999%)

Table S6 List of Morex high-confidence gene models predicted for the *Rbgnq1* locus in the interval between markers M-13 and M-14. Information of whether the candidate gene is included in the current *Rbgnq1* interval is provided in the column "Present in BACs".

Gene #	Mrx HC gene model	Annotation	Present in BACs
1	HORVU5Hr1G104600	Non-specific lipid-transfer protein-like protein	outside new interval
2	HORVU5Hr1G104610	Serine/threonine-protein kinase	outside new interval
3	HORVU5Hr1G104620	Type IV inositol polyphosphate 5-phosphatase 7	outside new interval
4	HORVU5Hr1G104630	Vicilin	outside new interval
5	HORVU5Hr1G104640	ATP-dependent RNA helicase dhh1	outside new interval
6	HORVU5Hr1G104660	Ripening related protein family	outside new interval
7	HORVU5Hr1G104670	Glutathione S-transferase family protein	outside new interval
8	HORVU5Hr1G104680	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	not present
9	HORVU5Hr1G104690	Acetyltransferase (GNAT) domain protein	yes
10	HORVU5Hr1G104700	glutamate dehydrogenase 1	yes
11	HORVU5Hr1G104720	30S ribosomal protein S13	yes
12	HORVU5Hr1G104730	Chitinase family protein	yes
13	HORVU5Hr1G104740	UDP-Glycosyltransferase superfamily protein	yes
14	HORVU5Hr1G104750	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	yes
15	HORVU5Hr1G104760	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	yes
16	HORVU5Hr1G104790	Protein kinase	not present
17	HORVU5Hr1G104800	Acyl-CoA N-acyltransferases (NAT) superfamily protein	yes
18	HORVU5Hr1G104810	unknown protein; BEST Arabidopsis thaliana protein match is: unknown protein .	not present
19	HORVU5Hr1G104840	receptor kinase 2	yes
20	HORVU5Hr1G104850	receptor kinase 2	outside new interval

Table S7 Genotyping results for recombinant plants in the *Rbgnq1* QTL interval. H = heterozygous; S = homozygous SusPtrit allele; V = homozygous Vada allele

Recombinant plant code	M -13	**Mrx 4610**	M -15	M Synt980	**Mrx 4850**	M -14	Phenotype	Remarks
6_7.31-2-3	H	H	S	S		S	Susceptible	Susceptible plant; carries the Vada allele of the serine/threonine kinase gene
2_7.63-5.4-1	V	V	S	S	S	V	Susceptible	Susceptible plant; carries the Vada allele of the serine/threonine kinase gene
3_15.1-2	V	V	V	V	S	S	Resistant	Resistant plant; carries the SusPtrit allele of the receptor kinase gene
2_7.24-1-1	V		V	V	S	S	Resistant	Resistant plant; carries the SusPtrit allele of the receptor kinase gene
1_6(T1-06).7-1	V		V	V	S	S	Resistant	Resistant plant; carries the SusPtrit allele of the receptor kinase gene
4_8(T4-15).19-2	V		V	V	S	S	Resistant	Resistant plant; carries the SusPtrit allele of the receptor kinase gene

Chapter 4

Identification of a large-effect QTL associated with grain quality problems in barley

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Abstract

Three barley mapping populations sharing the parental line SusPtrit suffered from an unknown condition affecting grain quality and germination. Grains showed dark blotches or even whole-grain discoloration and shrivelling. Symptoms were similar to kernel discoloration as reported in wheat and barley. Some covered-seed lines affected by this quality condition produced grains that were either not covered at all or in which partial hull loss was observed, a phenotype that resembles that of grain skinning. No fungal or bacterial agent was identified as causal organism, and we demonstrate that the poor quality phenotype is not transmitted to the next generation – therefore unlikely due to a seed borne pathogen. A major effect QTL on chromosome 6H was mapped independently in all three populations, with the poor quality allele contributed by SusPtrit. A second, minor effect QTL, was mapped on chromosome 2H in the SusPtrit x Golden Promise population, with Golden Promise as the parent donor of the allele conferring poor quality. The grain quality disorder is not linked to the *nud* gene on chromosome 7H for the naked/covered seed trait.

Introduction

Barley (*Hordeum vulgare* L.) was one of the first plant species to be domesticated, enabling the establishment of early agricultural societies during the Neolithic period (Pankin and von Korff 2017). The importance of barley was maintained in the course of agriculture, and in 2014 it was the fourth most produced cereal crop worldwide, with 144.5 million tonnes, after maize, rice and wheat (http://www.fao.org/faostat/). Barley grains were presumably first used as food, but nowadays the production is mainly aimed for livestock feed and for malting and brewing (Ullrich 2010). As a model research organism, barley has contributed to important progress in several areas, such as plant physiology, plant pathology, agronomy, genetics and breeding. Genetic studies using barley as a model organism benefit from its diploid nature ($2n = 2x = 14$), autogamous mode of reproduction and diversity of phenotypes. Research on barley genetics has focused initially on trait inheritance and conventional genetic mapping, and more recently on molecular and physical mapping (Ullrich 2010). Aspects for which barley is a very useful model crop include quantitative host resistance (e.g. Parlevliet 1979) and nonhost resistance to rust and powdery mildew fungi (Aghnoum et al. 2010; Atienza et al. 2004; Jafary et al. 2008). A high-quality reference sequence of the barley genome has been made publicly available recently (Mascher et al. 2017), with important implications for cereal breeding and genomics. Due to their close phylogenetic relationship, research outcomes in barley are very likely applicable to tetraploid and hexaploid wheat as well.

Many agronomic traits of interest are quantitatively inherited. The identification and characterization of quantitative trait loci (QTLs) associated with traits of interest have important applications in crop breeding programs. Exotic germplasm from landraces and the wild barley *H. vulgare* ssp. *spontaneum* are valuable sources of genetic diversity, and can provide favourable alleles for the improvement of several agronomic traits, particularly for disease resistance (Yun et al. 2005). Extensive data are available on molecular linkage maps for barley (e.g. GrainGenes database https://wheat.pw.usda.gov/GG3/), and are useful not only for breeding but also for genomics studies. Linkage map construction relies on the development of mapping populations, usually derived from parents that are highly homozygous and phenotypically contrasting for the trait of interest. The development of recombinant inbred lines (RIL) and doubled haploid (DH) 'immortal' mapping populations is advantageous because each line can be propagated indefinitely through seeds without any genetic change, allowing recurrent genotyping and phenotyping for different traits.

Three barley mapping populations were developed by Plant Breeding, Wageningen University and Research (NL) to study the inheritance of basal and nonhost resistance to rust fungi (Jafary et al. 2008; Yeo et al. 2014) and powdery mildew (Aghnoum et al. 2010). The three

mapping populations have one parental line in common: SusPtrit, a special barley line developed by crosses of barley landrace accessions and selected for susceptibility to non-adapted rust (*Puccinia*) species (Atienza et al. 2004). SusPtrit has naked grains (i.e. not covered by hulls) of amber color. Initial multiplication of seed material from these mapping populations was carried out both under greenhouse and field conditions, resulting in healthy grains. After a few rounds of multiplication, however, in each mapping population some of the lines produced very poor quality grains, that looked dark-discolored and shrivelled, and showed poor germination rate. Both naked- (hulless) and covered- (hulled) seed lines seemed to be affected by this condition. Growing the plants in the greenhouse, under controlled temperature and humidity and sulphur fumigation to control pathogens, did not solve the problem. The poor quality condition of the grains was initially presumed to be due to infection by a seed borne pathogen, but no fungal or bacterial agent was identified as causal organism after samples were analysed by a seed laboratory certified by ISTA (International Seed Testing Association) standards ('NAK', Nederlandse Algemene Keuringsdienst voor zaaizaad en pootgoed van landbouwgewassen http://www.nak.nl/organisatie/nak-services-).

Grain discoloration in barley and wheat is reported in literature under several terms: kernel discoloration (KD, term that will be mostly used in this study), weather staining, kernel blight, dark smudge, black stain and more (Jacobs and Rabie 1987; Li et al. 2003; Mathre 1997). Dark discoloration at the embryo end of the grain is particularly referred to as black point, and is considered as a type of kernel discoloration by some authors (de la Pena et al. 1999; Li et al. 2003) but as a distinct phenomenon by others (Walker et al. 2008). Kernel discoloration in general is a problem for the barley grain industry because it negatively affects the quality of malting barley, consequently downgrading it as feed and leading to significant economic losses (Li et al. 2003; Miles et al. 1987). Also seed germination rate and seedling emergence and vigour are negatively affected (Li et al. 2003; Li et al. 2014). There is no consensus on the causes of kernel discoloration. Early reports linked it to a number of fungal pathogens, mainly *Alternaria alternata*, *Bipolaris sorokiniana*, and *Fusarium graminearum* (Li et al. 2003; Mathre 1997). Other studies, however, provided no evidence of association between discoloration and fungal infection, with no differences in the amount of mycelium observed in black-pointed and normal barley or wheat grains (Jacobs and Rabie 1987; Williamson 1997). A general agreement is that environmental conditions play a key role in grain discoloration. QTL mapping studies have been performed for black point (BP) and KD in barley (Canci et al. 2003; de la Pena et al. 1999; Li et al. 2003; Tah et al. 2010; Walker et al. 2008), with several genomic regions identified. KD, therefore, is a complex and poorly understood trait, governed by multiple genes and under strong influence of environmental factors.

In the present study, we set out to investigate whether the poor grain quality character in the three SusPtrit-derived mapping populations had a genetic basis. The identification of QTLs mapped in this study allowed comparison with regions mapped for KD and BP formation in other studies. We also tested the viability of grains affected by this unknown condition, and investigated whether the poor quality phenotype was transmitted to the next generation - and therefore might be due to a seed borne pathogen. Finally, we tested whether extra fertilizer application could have an effect on improving grain quality.

Materials and methods

Plant material and QTL mapping for grain quality

The mapping populations used for QTL mapping analysis belong to two recombinant inbred line (RIL) mapping populations: Vada x SusPtrit (VxS, 152 lines) and Cebada Capa x SusPtrit (CxS, 113 lines); and one doubled-haploid (DH) mapping population: SusPtrit x Golden Promise (SxG, 122 lines). Sowing was initially intended for seed multiplication, and took place at the beginning of 2013. One seed per line was sown in 13 cm diameter pots containing standard substrate ("Lentse potgrond B.V.", Horticoop, Bleiswijk, The Netherlands). Plants were grown under greenhouse conditions (adjusted to 18-20°C day time, 15-16°C night time, 40-60% relative humidity, 16h light). At the time of flowering, the approximate amount of 2 g of commercial granular fertilizer (NPK, 12-10-18) was applied to each pot. Plants were treated against thrips (order *Thysanoptera*) infestation with ~250 mL of imidacloprid insecticide (1 g/L solution, commercial name: 'Admire', Bayer CropScience Inc.). To protect plants against fungal infection, sulphur vapour was applied daily in the greenhouse compartment, for about one hour per day.

After ripening, grains from individual plants were manually harvested and threshed. The phenotyping for grain quality aspect was made by visual assessment, and scores were given on a scale of 1 to 5: lines with grain batches containing shrivelled, dark discolored grains got score 1 ('poor quality'; Fig. 1a) whereas grain batches containing only healthy-looking grains (symptomless, no discoloration) score 5 ('good quality'; Fig. 1b). Intermediate scores were given to batches containing a mix of poor and good quality grains (Fig. 1c), and also to batches where most grains were of intermediate quality.

The marker data set used for QTL analysis consisted of 198 markers for VxS, 242 markers for CxS (both generated by Jafary et al. 2008), consisting of AFLP and SSR markers, and 698 markers for SxG (generated by Yeo et al. 2014 after genotyping with the Illumina iSelect 9k barley infinium chip). Markers were homogeneously distributed along the chromosomes. MapQTL 6 software (Van Ooijen 2009) was used for QTL mapping: first, an interval mapping step was performed, and markers with the highest LOD scores were selected to be used as co-

factors for multiple-QTL mapping (MQM). The QTL mapping procedure was then followed by a restricted MQM step. A LOD score of 3.0 was set as threshold to declare a QTL.

Fig. 1 Grains of lines from the Vada x SusPtrit (VxS) barley mapping population showing different phenotypes for grain quality. **(a)** VxS 149, poor quality (score = 1.0); **(b)** VxS 58, good quality (score = 5.0); **(c)** VxS 90, good and poor quality grains in the same batch (score = 3.0)

Comparison of QTL positions

A total of 88 RILs from each of the VxS and CxS mapping populations had been previously re-genotyped using the Illumina iSelect 9k barley infinium chip – the same used for genotyping the entire SxG population. This enabled comparison, by correlation analysis, between the peak markers of QTLs in the VxS and CxS populations and the SNP markers in the chip. After the most correlated markers ($r \geq 0.75$) were determined, their sequences (as spotted in the SNP array) were used as queries to blast against the database of barley cv Morex whole-genome shotgun sequencing assembly (http://webblast.ipk-gatersleben.de/barley_ibsc/), and their position on the POPSEQ map of cv Morex (Mascher et al. 2013) was determined. The same was done for the peak markers of SxG QTLs.

Transmission of poor quality phenotype to the next generation

For several lines of the three populations, a mix of 'good quality' and 'poor quality' grains was observed in the same batch (Fig. 1c). We sampled grains from five of those lines (CxS 109, CxS 68, VxS 112, VxS 39 and VxS 4), that we call here the first generation, to investigate whether the poor quality character is transmitted to the next (second) generation and hence may be caused by a seed borne pathogen. The trial followed a complete randomized design (CRD), with ten plants per genotype: five originated from good quality grains and five from poor quality grains; extra grains were sown to make sure that five plants were available from each quality category. Plants were grown in individual pots in a greenhouse compartment (same as previously described: conditions adjusted to 18-20°C day time, 15-16°C night time, 40-60% relative

humidity, 16h light) and watered daily with tap water. Each plant was given a code to hide its identity during phenotyping. The following traits were assessed when first generation plants were around two months old: plant length, yellowing (on a scale of 1 to 10) and leaf necrosis (on a scale of 1 to 10). The quality of the second generation grains (produced by those first generation plants) was assessed after harvesting, using the 1 to 5 scale previously described.

Statistical analyses were performed using Genstat 18th edition (VSN International 2015). A two-way ANOVA was performed to test for significant differences in the phenotypic characters of plants of the first generation, as well as on the quality scores of second generation grains produced by them.

Germination assessment

The effect of discoloration on the seed germination was tested. Nine lines of the CxS population were selected from the 2013 annual multiplication material based on their quality score: either 'high' (score 5.0: CxS 37, CxS 89), 'medium' (scores around 3.0: CxS 56, CxS 23, CxS 41, CxS 81) or 'low' (scores around 1.5: CxS 101, CxS 72, CxS 68). Forty grains from 'high' and 'low' quality lines were tested, while lines belonging to the 'medium' category had their grains separated into 'high' and 'low' quality, with 25 grains from each of these categories tested. Grains were immersed in sterile distilled water for about 10 h, followed by two rinsing steps, and next transferred to 9 cm Petri dishes containing two layers of filter paper damped with sterile distilled water. The Petri dishes were kept at 25 ºC and in darkness for two days, after which the plates were kept open and submitted to a 12 h photoperiod. Plates were daily moistened with sterile distilled water. The germination was assessed 7 days after the grains had been immersed in water, by counting the number of emerged seedlings (shoot longer than 1 cm).

Influence of different levels of fertilizer on grain quality character

We observed an improvement on the general quality of grains produced during the subsequent annual multiplication (2014) in comparison to the 2013 multiplication previously scored for grain quality (Fig. S1). The only factor that we identified as differing between the 2013 and 2014 multiplication was that in the latter one, extra fertilizer was applied to the plant pots. Two trials were then set up to investigate the influence of fertilizer input on the quality of grains.

Fertilizer Trial I: We set out to test whether extra fertilizer input would improve grain quality. Grains produced in the previously described experiment ('second generation' grains) were selected to test the effect of adding different levels of fertilizer (NPK, 12-10-18) on the quality of the produced grain. The genotypes CxS 109, CxS 68, VxS112, VxS 39 and VxS 4 were sown in 13 cm diameter pots containing standard substrate. Three grains were sown in each pot but only one seedling was kept. The experiment was set up in a randomized complete block design

(RCBD) with three blocks and four treatments: 'no application', no addition of fertilizer to the pots; '1X sowing', application of 1 g of fertilizer per pot at sowing; '1X flowering', application of 1 g per pot of fertilizer at flowering; and '3X application', application of three times 2 g of fertilizer per pot: at sowing, four weeks after sowing and at flowering. Plants were grown in a greenhouse compartment under controlled conditions (18-20°C day time, 15-16°C night time, 40-60% relative humidity, 16h light) and watered daily with tap water.

Plants from the VxS population (VxS 4, 39 and 112) had poor germination and therefore had to be sown again. The grains used for the re-sowing were not originated from the previous experiment described in this work, but from the 2014 multiplication. Therefore, all plants from the VxS 112, VxS 39 and VxS 4 genotypes had a two-weeks delay in comparison to CxS 109 and CxS 68. Grains from VxS 112 genotype did not germinate for the second sowing as well, so this genotype was discarded from the experiment.

Fertilizer Trial II: A simultaneous trial was set up to test whether the quality of the grains would fall back without extra fertilizer input to the substrate. In addition to lines CxS 109, CxS 68, VxS 39 and VxS 4, that had been previously used in the first fertilizer trial, lines VxS 112, Vada and SusPtrit were sown in 13 cm pots containing standard substrate. A RCBD with four blocks was set up, with two treatments: 'no fertilizer', no addition of fertilizer; and 'fertilizer', application of 2 g per pot of fertilizer (NPK, 12-10-18) one week after sowing, 4 weeks after sowing and at flowering. Plants were grown in a greenhouse compartment under the same condition as previous experiments.

For both fertilizer trials, grains harvested from single plants were scored on the 1 to 5 scale previously used (5 being the healthy-looking grains, with no shrivelling or discoloration). The weight of 1000 grains was estimated based on the weight of 100 grains (or less, when less than 100 grains were available). Data were analysed using Genstat 18th edition (VSN International 2015). A two-way ANOVA followed by a Fisher's unprotected LSD was performed to check the effect of the genotype and fertilizer input on the quality of the grains and on the 1000 grain weight.

Results

Grain appearance and QTLs controlling grain quality character

Within each SusPtrit mapping population there were lines representing extreme cases of discoloration and shrivelling, with either the whole surface of the grains dark-stained or showing dark blotches. In such extreme cases, all grains are affected (Fig. S2a). In lines that are not so seriously affected, the proportion of discolored grains varies, as well as the extension of

dark blotches. In partially blotched grains, the blotching did not concentrate around the germ. SusPtrit is a hulless line and this trait segregates in the three SusPtrit populations. The condition observed in our mapping populations affects hulless as well as hulled lines. On severely affected grains of hulled lines the hull does not seem to be properly adhered to the grain, resembling the 'grain skinning' phenotype (Hoad et al. 2016) (Fig. S2b).

A few lines from VxS and CxS mapping population either did not germinate or set too few grains to be scored for grain quality. The total number of lines assessed was VxS = 146, CxS = 101 and SxG = 121. The grain quality scores showed a continuous distribution in the three mapping populations, with over 40% of lines scoring at least 4 (Fig. S3). A large-effect QTL was mapped on chromosome 6H in the three populations, accounting for 47-57% of the phenotypic variation (Table 1). The donor parent of the 'poor quality' allele of this QTL is SusPtrit, and the corresponding positions of the QTL peak markers on the POPSEQ map of the reference barley cultivar Morex (Mascher et al. 2013) range from 112 to 118.4 cM on chromosome 6H (Table S1). A second QTL was identified on 2H for the SxG population, with a minor effect. For this QTL, SusPtrit is the parent contributing the 'good quality' allele. Neither of the QTLs mapped in this study is linked to the *nud* locus controlling the naked/covered seed character, located on chromosome 7HL (Taketa et al. 2008).

Table 1 Summary of QTLs mapped for grain quality in three barley mapping populations, Vada x SusPtrit (VxS), Cebada Capa x SusPtrit (CxS) and SusPtrit x Golden Promise (SxG).

Mapping population	Chr[a]	Peak marker	Position (cM)	Position Morex map (cM)[b]	LOD	% Expl[c]	Additive[d]	Donor 'poor quality'
VxS	6H	E33M54-123	119.9	116 - 116.2	20.2	47.0	-0.91	SusPtrit
CxS	6H	E32M55-102	141.4	105.1 - 113.2	18.8	57.6	-0.87	SusPtrit
SxG	6H	BOPA2_12_30025	131.2	116.8	24.2	56.7	-0.87	SusPtrit
SxG	2H	BOPA2_12_30657	45.6	~38.1 - 41.9	4.4	6.8	0.31	Golden Promise

[a] The chromosome (linkage group) in which the QTL was mapped.
[b] The position of the peak marker of the QTL on the POPSEQ map of barley cv Morex (Mascher et al. 2013); (map position of E33M54-123 and E32M55-102 is based on the position of the most correlated markers included in the Illumina iSelect 9k barley infinium chip). Position of QTL on 2H is approximate and based on the position of markers SCRI_RS_182408 and BOPA1_4410-284, that are neighbors to the peak marker BOPA2_12_30657.
[c] The proportion of phenotypic variance explained by the QTL.
[d] The effect of having one allele from SusPtrit on the grain quality score.

Good and poor quality grains trial

The quality scores of the grains did not significantly influence the plant length (P = 0.79), degree of yellowing (P = 0.87), occurrence of necrotic spots on the leaves (P = 0.55) or the quality of second generation grains (P = 0.250) (Fig. 2). The scores for the second generation grains ranged for individual plant progenies from 1 (very poor quality) to 4, with 5 being the ideal (= very good quality) (Fig. 2d). Line VxS 39 showed the lowest average score (1.1) among the lines tested, while CxS 109 had the highest average score (3.3). Plants originating from 'good grains'

produced a second generation of grains with an average quality score of 2.2, while plants originating from 'poor grains' produced a second generation of grains with an average quality score of 2.3. There was a significant effect of the genotype on the traits assessed, but the quality of the grain within each line did not affect the plant quality or the quality of the second generation of grains.

Effect of grain quality on germination

We compared the germination rates between high and low quality grains by assessing the rate of seedling emergence one week after imbibition of the grain in water. The results show that discoloration symptoms clearly reduced the viability of grains and affected seedling emergence (Fig. 3). Good quality grains did not consistently show a higher seedling emergence rate compared to poor quality grains of the same line, although poor quality grains always showed low seedling emergence rates, with a maximum of 32% and a minimum of 0% for lines VxS 41 and CxS 101, respectively.

Fertilizer trials

The effect of fertilizer input on the quality of grains was evaluated in two experiments. For both experiments, the flowering times differed for the genotypes used: plants of CxS 68 and VxS 39 were 6 weeks old at the time of flowering, while VxS 4 and CxS 109 were about 7 weeks old. This implied different fertilizer application dates for different genotypes. For the first experiment, 'Fertilizer Trial I', scores for grain quality varied among genotypes ($P < 0.001$), and fertilizer application also had a significant effect on grain quality ($P < 0.001$). There was no significant interaction effect for the genotype and the level of fertilizer added ($P = 0.209$). The '3X application' was the only treatment that significantly differed from the others, with the lowest mean of quality scores for all the genotypes (Fig. 4a, Table S2)

The 1000 grain weight was also significantly affected by fertilizer treatment ($P = 0.008$) and genotype ($P < 0.001$). There was no significant effect of the interaction between genotype and fertilizer application ($P = 0.203$). The lowest average weight was found for the "3X application" and "no application" treatments (Table S2). The "1X at sowing" treatment statistically did not differ from "no application" (Fig.4b, Table S2).

The second fertilizer experiment ('Fertilizer trial II') was carried out almost simultaneously with Fertilizer trial I, to investigate the effect of extra fertilizer on grain quality. There was a significant effect of the genotype ($P < 0.001$) as well as of the treatment ($P < 0.001$) on the quality of the grains. The highest scores (better grain quality) for all genotypes were obtained when no fertilizer was added ('no fertilizer' treatment), meaning that the application of fertilizer had an adverse effect on grain quality (Fig. 4c). The means for 1000 grain weight were, in general, also higher for the 'no application' treatment, with exception of lines CxS 68 and

SusPtrit, for which the average value for both treatments was very similar (Fig. 4d). The two-way ANOVA showed a significant effect of genotype ($P < 0.001$) and of the fertilizer application ($P = 0.008$) on the weight, but no interaction effect.

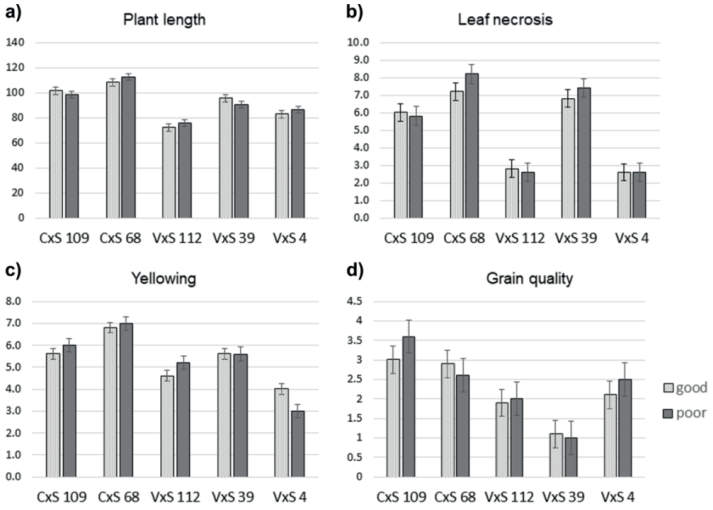

Fig. 2 Average scores for **(a)** plant length (cm), **(b**) yellowing (1 to 10 scale), **(c)** leaf necrosis (1 to 10 scale) and **(d)** grain quality measured from plants originating from good and poor quality barley grains. Error bars represent the standard error of the mean. One-tailed T-test was performed (α = .05, assuming plants from poor grains produce poorer plants and poorer grains than plants from good grains) and no significant differences were found

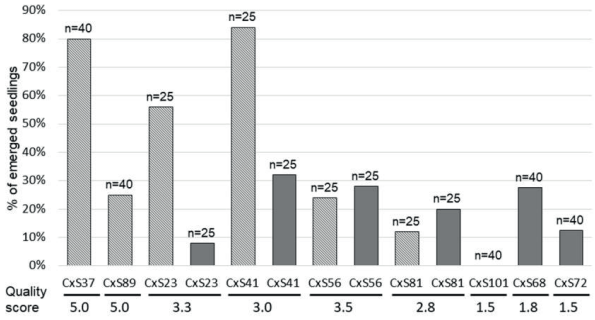

Fig. 3 Effect of grain quality score on seedling emergence rates in lines of the Cebada Capa x SusPtrit barley mapping population. Shaded bars represent seed lots with good quality grains and grey bars represent lines with poor quality grains. The quality score attributed to each line is specified below each bar

Line VxS 39 showed the highest average grain quality score for all the treatments, in the two fertilizer experiments (Figs 4, S3b). This was unexpected because in previous experiments the quality of grains for this line was in general much lower (Figs. 2d, S3a). Line VxS 39 had also the highest averages for the 1000 grain weight for all fertilizer treatments.

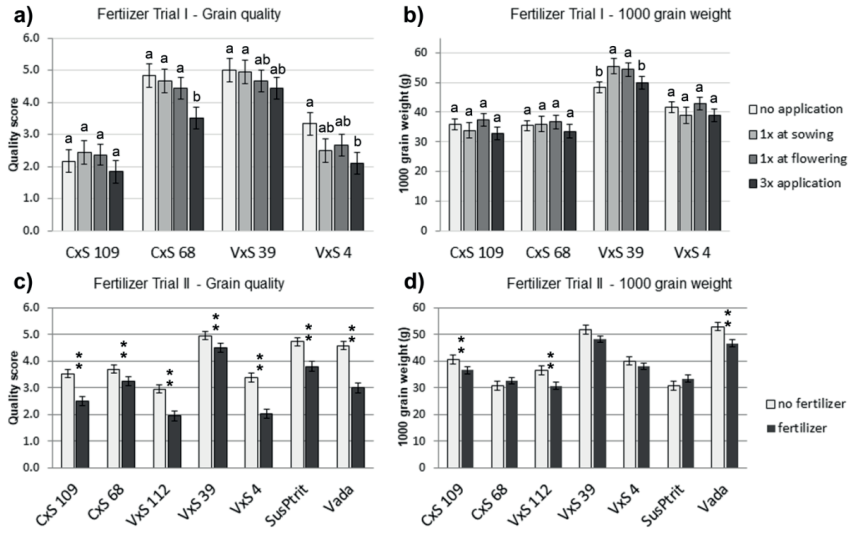

Fig. 4 Means for barley genotypes at different levels of fertilizer application for **(a)** grain quality, at Fertilizer trial I; **(b)** 1000 grain weight for Fertilizer trial I; **(c)** grain quality for Fertilizer trial II and **(d)** 1000 grain weight for Fertilizer trial II. Grain quality scores are on a scale of 1 (poor) to 5 (good); 1000 grain weight (in g) is estimated based on the weight of 100 grains or less, when not enough grains were available. Error bars represent the standard error of the mean. For (a) and (b) - fertilizer trial I – per tested barley genotype, bars under the same letter do not statistically differ. For (c) and (d) – fertilizer trial II - asterisks indicate significant differences at $\alpha = 0.01$

Discussion

The motivation for this work arose from the need to protect the integrity of the three SusPtrit mapping populations, VxS, CxS and SxG, for which an unknown condition caused grains to have poor quality (dark discoloration and shrivelling) and low germination. The SusPtrit-derived mapping populations are a valuable asset for studying the genetic basis of nonhost resistance to heterologous rusts and powdery mildews in barley. The same QTL, on chromosome 6H, was mapped independently in the three populations, demonstrating that this condition is genetically determined. The allele conferring the poor quality trait is from SusPtrit, which has the barley accessions Menelik, L100, Trigo Biasa and Nigrinudum as ancestors. Trigo Biasa is an Indonesian accession, while the others have Ethiopian origin; it is not known which of them has contributed the allele associated with the grain quality disorder. Established cultivars like Vada, Cebada Capa and Golden Promise have likely been selected in their ancestry against this gene, contrasting with SusPtrit, for which the only selection criterion was its susceptibility to rust fungi (Atienza et al. 2004).

Comparison of grain appearance with literature descriptions

The condition affecting our mapping populations is more dramatic than what literature for kernel discoloration (KD) in cereals usually reports. Table 2 summarizes several descriptions of the phenomenon in literature. There is no consensus about the terms used to refer to grain discoloration, nor about the causes, nor whether discoloration restricted to the embryo end (BP) should be categorized as KD. The description of our material does not fit the typical definition of BP, therefore it is not likely to be the same disease. However, we do not rule out the possibility that our grain condition is under the influence of at least partially the same network of genes and/or environmental factors, as already discussed by Walker et al. (2008) comparing BP and KD.

In barley, discoloration seems to be in most of the cases confined to the hull tissue (lemma and palea), while the germ aleurone is only to a lesser extent affected (March et al. 2008; Walker et al. 2008). In wheat, mature grains are not covered by hulls and discoloration occurs in the pericarp and testa layers (Fernandez et al. 2011). SusPtrit is a hulless line, and therefore our populations segregate for being hulled or not. Discoloration affects both hulled and hulless grains, with the outer seed layers dark-stained and also shrivelled in severe cases. For barley no reports were found describing shrivelling or any sort of grain shape abnormality among the symptoms associated with discoloration, like observed in our populations. A few reports in wheat mention the occurrence of grain shrivelling in severe cases, though (Conner and Davidson 1988; Li et al. 2014).

Causes of kernel discoloration

There is probably not a single factor causing discoloration symptoms in cereals, as evidenced by the lack of agreement among authors when reporting on the causes of KD or BP (Table 2). Much of the research on the topic has attributed the symptoms to fungal infection (de la Pena et al. 1999; Mathre 1997; Miles et al. 1987), under strong influence of genotype and environmental conditions (Canci et al. 2003; de la Pena et al. 1999). Other authors proposed that the main cause of KD are environmental factors triggering physiological changes (Li et al. 2003; March et al. 2008; Walker et al. 2008). In the latter case, their claim is supported by studies that found no correlation between fungal colonization and disease symptoms (Ellis et al. 1996; Jacobs and Rabie 1987; Williamson 1997). A proteomic analysis found no protein of fungal or bacterial origin in tissues of black-pointed grains, suggesting again that biotic factors are not the primary cause of discoloration (Mak et al. 2006). The results of our study also do not support the hypothesis that the symptoms were caused by a seed borne pathogen, since healthy and affected grains did not produce a second generation of grains with significantly different quality scores, and the certified seed quality testing lab did not find any causal organism.

Table 2. Description of terms, symptoms and presumed causes of kernel discoloration (KD) and black point (BP) found in the literature for barley (B, *Hordeum vulgare*), wheat (W, *Triticum aestivum*), and durum wheat (DW, *Triticum durum*) grains.

Reference	Species	Terms to describe discoloration	Symptoms description	Cause presumed by authors
Jacobs and Rabie, 1987	B	BP	Dark-brown to purple discoloration, extends from embryo end to middle of the grain	No definite fungal association established from this study
Miles et al., 1987	B	KD (black stain or carameling)	Carameling: straw color to brown. Black stain: black/dark brown discoloration of palea and lemma; most severe cases: >50% of grain surface stained black	Primarily fungi, under strong influence of genotype and environment
Ellis et al., 1996	W	BP	Black/dark brown discoloration. In severe cases extends from the embryo end along the crease and over the shoulders	Fungal infection, although no significant difference was found for fungal isolation between BP and symptomless grains
Mathre, 1997	B	BP syn. kernel blight	Black/dark brown discoloration. In severe infections, discoloration extends from the embryo to the base of the lemma	Fungal infection
de la Pena et al., 1999	B	KD syn. BP and kernel blight	Black/dark brown discoloration. Most severe cases: >50% of grain surface stained black	Fungal infection
Canci et al., 2003	B	KD	Black/brown discoloration of lemma and palea. Most severe cases: >50% of grain surface stained black	Fungal infection; severity varies depending on genotype and environmental conditions
Li et al., 2003	B	KD syn. weather staining	Three forms: caramelising (yellow/brown), BP (black-brown discoloration at the embryo end), and an extreme form where spots of grey mould is formed.	Environmental conditions (high humidity before harvest)
Mak et al., 2006	W	BP	Dark discoloration at the embryo end. In severe cases, discoloration may extend along the groove on the ventral side.	Not a single cause for BP, might be due to fungal infection or abiotic stresses; proteomic analyses showed no fungal proteins though.
March et al., 2008; Walker et al., 2008	B	BP ≠ KD	BP: black/brown discoloration at the embryo end, usually confined to the lemma and palea, but also to the germ aleurone tissue	Physiological changes influenced by environmental conditions
Tah et al., 2010	B	BP ≠ KD	Black/brown discoloration at embryo end of the grain	In Australia discolorations is mostly associated with biochemical responses rather than fungal infection
Fernandez et al., 2011	W, DW	BP, dark smudge	Black/brown discoloration of the pericarp and testa, restricted to the embryo end (BP) or occurring in other parts of the grain (dark smudge)	Fungal infection (main factor), although abiotic factors also contribute
Li et al., 2014	W	BP	Symptoms range from dark discoloration at the embryo end, to severe shrivelling and dark discoloration extending to other areas of the grain	Fungal infection
Present study	B	KD (≠ BP)	Dark discoloration not confined to the embryo end. In severe cases the whole grain is dark stained and shrivelled.	Not caused by biotic agents; enhanced at higher fertilizer application. Under strong genetic influence

Because our experiments were carried out in greenhouse compartments, in the presence of a routinely used sulphur burner, it is also unlikely that any pathogen typically associated with KD, such as *F. graminearum*, *B. sorokiniana* and *A. alternata*, would be present in the environment. We assume, therefore, that the disorder affecting our mapping populations is not primarily caused by fungal infection, but rather by stress-induced physiological changes during grain development. High humidity during grain filling and ripening is one of the main abiotic factors associated with KD in barley and wheat (Fernandez et al. 2011; Li et al. 2003). Walker et al. (2008) observed that high humidity and low temperatures favours BP in susceptible barley varieties. In our case, it is still unclear what the triggering abiotic conditions are, since our experiments were carried out under controlled temperature, humidity and light (see Materials and methods).

Effect of fertilizer application

We tested the effect of extra fertilizer application on the quality of grains, since fertilizer input varied slightly between the annual seed multiplication of 2013 (in which the QTL mapping results of this study were performed) and that of 2014 (which showed a better overall grain quality). The pot soil used for these experiments and seed multiplications had the same composition, containing a substantial amount of NPK fertilizer (0,810 kg/m^3). The objective of the fertilizer trials was not to assess how lack of nutrients would affect the grain quality, but rather to check whether extra fertilizer input could improve it. We observed that extra addition of fertilizer did not have a beneficial effect on the quality of the grains, but to some degree reduced the quality scores and grain weight at the 3x application treatment. The two fertilizer trials were run simultaneously, and regardless of application timing (at earlier or later growth stages of the plant), no quality improvement was detected. Conner and Davidson (1988) reported that BP incidence in soft white spring wheat increased with higher levels of nitrogen application, and attributed this to the delay in maturity of grains caused by elevated N inputs, that would favour fungal infection. Even though the effect of high levels of fertilizer application was also negative in our grains, our findings do not support the idea that the grain condition is caused by a pathogen, as already discussed above. Nevertheless, we could not point a possible explanation for this negative association between fertilizer inputs and grain quality.

The negative impact on seed germination was confirmed in this study, even though some seed lots with a high quality score also showed a reduced seedling emergence rate. In our experiment, out of four lines containing a mix of good and poor quality grains in the same batch, for two the germination rate of the good quality was slightly lower than the grains classified as poor quality and for two considerably higher. This suggests that in these particular cases, even

the grains in the good quality category were not unaffected, but rather less affected than the poor quality category, and therefore their germination was compromised as well.

QTLs for KD

Studies mapping QTLs for BP in barley (March et al. 2008; Walker et al. 2008) do not report involvement of any chromosomal region located on chromosome 6H. For KD, however, de la Pena et al. (1999) and later Canci et al. (2003) have mapped two major QTL*s* on 6H in the Chevron x M69 population. Chevron, a landrace from Switzerland, is the donor of KD resistance. In both studies, KD was assessed on grains harvested from plants inoculated with *F. graminearum* or *B. sorokiniana*. The two QTL*s* are about 50 cM apart, situated around markers MWG916 and Amy1, respectively. Comparison of these two markers with position of the 6H QTL mapped in our study was possible with data from the barley integrated map of (Aghnoum et al. 2010). On the integrated map, MWG916 and Amy1 are located at 47.8 cM and 86.4 cM, respectively, while the CxS peak marker (E32M55-102) is located at 109.2 cM and the VxS peak marker (E33M54-123) is at 122.1 cM. Li et al. (2003) assessed KD and detected a QTL on 6H in the Chebec x Harrington population. This QTL, however, does not seem to co-localize with the QTL mapped in our study, since the closest linked marker (AWBMA36) is located close to the telomere of 6HS.

The second, weak effect, QTL mapped in our study, on chromosome 2H, has Golden Promise as the parent donor of the poor quality character. Several QTLs have been identified on 2H for KD and BP (de la Pena et al. 1999; Li et al. 2003; March et al. 2008; Walker et al. 2008) and it is possible that some of them locate at the same region as our SxG QTL, although lack of common markers between maps did not allow an accurate comparison between map positions. A rough comparison was made based on the position of the markers on the POPSEQ map of cv. Morex using sequence information of primers. A QTL mapped for BP by Walker et al. (2008) on the Alexis x Sloop DH mapping population, as well as several QTLs for grain brightness mapped by Li et al. (2003) in different populations located around the centromere region of 2H, close to marker EBmac684; this marker is located at position 58.05 cM, while the peak marker of the SxG QTL (BOPA2_12_30657) is approximately at 38.1 - 41.9 cM. Due to the proximity of the positions and the lack of further markers in common for the mapping populations, we do not discard the possibility that these are the same QTLs.

Our data demonstrate that the grain quality disorder is not associated with the naked/covered seed trait, which is controlled by a single gene situated on chromosome 7HL. The allele for covered-seed (*Nud*) is dominant over the allele for naked-seed (*nud*) (Taketa et al. 2008). In covered barley a lipid-rich cementing substance is produced by the pericarp epidermis that keeps the lemma and palea firmly attached to the grains (Hoad et al. 2016). In naked barley

the cementing substance is not produced. We noticed that, in addition to discoloration (and occasional shrivelling) symptoms, some covered-seed lines affected by the grain quality disorder produced grains that were either not covered at all or in which partial hull loss was observed (Fig. S2). This phenotype resembles that of grain skinning, also known as 'hull peeling', characterized by poor adhesion of the hull to the grain. Grain skinning is attributed to compromised quality of the cementing material due to changes in its structure or composition, and is for several reasons a problem for malting industry (Brennan et al. 2017; Hoad et al. 2016). According to Mak et al. (2006) BP discoloration in wheat may be due to degradation of the seed coat, caused by increased levels of active enzymes (particularly peroxidases) and phenolic compounds. If the outer layers of the barley grain are damaged, it is possible that the production of the cementing material is impaired, thus explaining the skinned phenotype in our populations. Additional research should be undertaken to further investigate it. Grain skinning is a heritable trait highly influenced by environmental conditions, although the fundamental causes are unknown (Brennan et al. 2017). Not much is known about the genetics governing grain skinning, due to the difficulty in setting up a reliable screening method. The SusPtrit populations might provide suitable material to carry out further research on the topic and help identify genomic regions associated with it.

Conclusions

This study has identified a major QTL on chromosome 6H associated with a grain disorder in barley. This genomic region does not seem to coincide with QTLs mapped previously for KD, and the symptoms in our mapping populations are seemingly more serious than the usual phenotype of grain discoloration. It remains to be elucidated whether our mapping populations suffer from a severe manifestation of KD or whether this is a non-related (newly described) condition governed by a rare allele introgressed from exotic germplasm. The observation that hulled grains show a skinned phenotype when affected suggests that the SusPtrit mapping populations might be useful to the study of grain skinning. Further research, including microscopy and biochemical analyses are required to determine which seed layers are affected and which enzymes are possibly playing a role.

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Supplementary material

Table S1. Markers with highest correlation with peak marker of QTL for grain quality on chromosome 6H and their corresponding map positions on the POPSEQ map of the reference barley cultivar Morex

Mapping population	Locus	Morex POPSEQ (cM)[a]
	BOPA2_12_20448	105.1 (morex_contig_45177)
	SCRI_RS_204188	? (morex_contig_1559139)
	BOPA2_12_30734	107.65 (morex_contig_266856)
	SCRI_RS_169022	105.1 (morex_contig_141325)
CxS (markers with highest	SCRI_RS_135063	105.1 (morex_contig_6221)
correlation with peak marker	SCRI_RS_213956	105.1 (morex_contig_134979)
E32M55-102 (r>0.75)	SCRI_RS_206207	105.52 (morex_contig_177673)
	SCRI_RS_143018	? (morex_contig_1563915)
	SCRI_RS_157323	113.24 (morex_contig_49997)
	SCRI_RS_111434	113.24 (morex_contig_101143)
	SCRI_RS_102455	113.21 (morex_contig_47728)
SxG (peak marker)	BOPA2_12_30025	116.79 (morex_contig_53592)
	SCRI_RS_179580	116.15 (morex_contig_49316)
VxS	SCRI_RS_205578	116.15 (morex_contig_2548850)
(markers with highest	BOPA1_5225-703	116.15 (morex_contig_50052)
correlation with peak marker	SCRI_RS_126069	116.15 (morex_contig_49316)
E33M54-123 (r>0.85)	SCRI_RS_138188	116.00 (morex_contig_58189)
	SCRI_RS_206827	116.22 (morex_contig_41348)

[a] POPSEQ map of the reference barley cultivar Morex (Mascher et al., 2013)

Table S2. Average scores for grain quality and 1000 grain weight among different genotypes according to fertilizer treatment for the 'Fertilizer Trial I'. Grain quality scores are on a scale of 1 (poor) to 5 (good). The weight of 1000 grains was estimated based on the weight of 100 grains or less, when not enough grains were available. Means under the same letter do not statistically differ

			Genotype			
Trait	Treatment	Treatment mean	CxS 109	CxS 68	VxS 39	VxS 4
grain quality score	no application	3.83 b	2.2	4.8	5.0	3.3
	1x at sowing	3.63 b	2.4	4.7	4.9	2.5
	1x at flowering	3.53 b	2.4	4.4	4.7	2.7
	3x application	2.97 a	1.8	3.5	4.4	2.1
1000 grain weight (g)	no application	40.31 ab	35.8	35.4	48.4	41.7
	1X at sowing	41.06 bc	33.8	36.0	55.5	38.9
	1X at flowering	42.86 c	37.5	36.7	54.4	42.9
	3X application	38.81 a	32.8	33.6	49.9	39.0

Fig. S1 Seeds from line VxS 48 originated from 2013 (left) and 2014 (right) seed multiplications. Seeds from the 2014 multiplication were, in general, of better quality compared to the 2013 multiplication.

Fig. S2 Grains of barley (*Hordeum vulgare*) lines belonging to the Cebada Capa x SusPtrit (CxS) mapping population showing severe discoloration symptoms. **(a)** Line CxS 23 carries the naked-seed allele (*nud*); **(b)** Line CxS 72 carries the covered-seed allele (*Nud*), although the phenotype resembles that of 'skinning', a condition observed when hulls are not properly adhered to the grains.

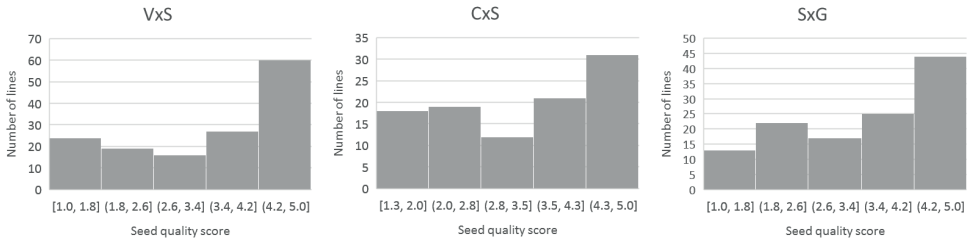

Fig. S3 Histograms of the frequency distributions for grain quality scores in the three barley SusPtrit mapping populations (VxS: Vada x SusPtrit; CxS: Cebada Capa x SusPtrit; and SxG: SusPtrit x Golden Promise) harvested in 2013 seed multiplication

Fig. S4 Seeds of barley (Hordeum vulgare) line VxS 39 (a) originating from the 'good and poor quality seeds' trial and (b) originating from the 'fertilizers trial I'

Chapter 5

General discussion

A given plant species may be susceptible to a small percentage of the microorganisms in the environment. Against the vast majority of attackers, plants will mount nonhost resistance, a phenomenon that is the result of an interplay of constitutive barriers and inducible defense mechanisms (Nurnberger and Lipka 2005). Adapted pathogens have evolved the ability of infecting particular plant species, and it is the effector repertoire that seems to determine the pathogen host range (Schulze-Lefert and Panstruga 2011). According to their host range, pathogens can be specialists (narrow host range, normally infecting a single host species), generalists (broad host range), or anything in between these two extremes. Powdery mildews of the *Blumeria graminis* species belong to the first group, and therefore show a high degree of specialization to their grass and cereal hosts. Based on this, Marchal (1902) classified the *B. graminis* into seven *formae speciales* (ff.spp., singular: *forma specialis*, f.sp.), according to the host they were able to colonize. In the present thesis we aimed at investigating the inheritance of genetic factors determining the specificity of nonhost interactions between barley and non-adapted *B. graminis* ff.spp.

Formae speciales of *Blumeria graminis*

Oku et al. (1985) extended the *B. graminis* classification to eight ff.spp; four infecting cultivated cereals: f.sp. *hordei* (from barley), f.sp. *tritici* (from wheat), f.sp. *secalis* (from rye), f.sp. *avenae* (oat); and another four infecting wild grasses: f.sp. *poae* (genus *Poa*), f.sp. *agropyri* (*Elymus*), f.sp. *dactylidis* (*Dactylis*) and f.sp. *bromi* (*Bromus*) (Troch et al. 2014). Two of the isolates used in chapter 2 of this thesis do not have an official classification, and infect *H. murinum* and *H. secalinum*. Nevertheless, we decided to refer to them as f.sp *hordei-murini* (*Bghm*) and f.sp. *hordei-secalin*i (*Bghs*), respectively, since cross-infectivity tests showed they do not infect any of the other cereals/grasses tested in this study (data not shown).

The evolutionary relationship between the different *ff.spp.* is also controversial, with some phylogenetic studies suggesting co-evolution between *B. graminis* and its hosts (Inuma et al. 2007) whereas others do not support this hypothesis (Troch et al. 2014; Wyand and Brown 2003). A recent study applied phylogenomic methods to whole genome sequence data in order to infer the phylogenetic topology and divergence time between different *ff.spp*. (Menardo et al. 2017). The phylogenomic analysis suggests different processes shaped the diversification of *B. graminis*, including co-evolution between some of the *ff.spp*. and their hosts, but also host jumps and fast radiation (Menardo et al. 2017). *B. graminis* f.sp. *hordei* is estimated to have diverged from the *tritici* clade about 7.1 to 8 Myr, which is in accordance with the estimation obtained by Wicker et al. (2013).

The *B. graminis* genomes contain a high amount of repetitive DNA, due to retrotransposon activity (Spanu 2014). Comparison between *B. graminis* f.sp. *tritici* (*Bgt*) and

Bgh genomes revealed that protein coding genes are conserved and syntenic, whereas the majority of retro-transposons are extremely diversified (Oberhaensli et al. 2011). In *Bgh*, genes encoding candidate effector genes are usually linked to transposable elements (Pedersen et al. 2012). Due to the dynamic nature of transposons, it has been suggested that the physical proximity would contribute to diversification of effectors and thus promote pathogen evolution in response to the evolution of host resistance (Spanu 2014). Candidate effectors correspond to at least 7% of the protein coding genes in the *Bgh* genome and 9.2% of the total gene complement on *Bgt* (Spanu 2014; Wicker et al. 2013).

Studying inheritance on near-nonhosts

There are no records of any *H. vulgare* s.l. (the s.l. sensu lato, so, including *spontaneum*) genotype that is (nearly) as susceptible to *B. graminis* f.sp. *tritici* (*Bgt*) as wheat, therefore the ideal scenario to perform inheritance studies of resistance to *Bgt* in barley would be to make crosses with wheat (the natural host). Nevertheless, interspecific hybridization is rarely possible in nature, due to reproductive barriers. A proposed alternative is to explore the natural variation existing among genotypes of a plant species in the degree of resistance to non-adapted pathogens (Atienza et al. 2004; Ayliffe et al. 2011; Niks and Marcel 2009; Shafiei et al. 2007). The categorization of plants into hosts or nonhosts is not always straightforward, since all kinds of intermediate situations may occur (Niks 1987). Barley is an interesting model in which to study the genetics and molecular basis of nonhost resistance, due to natural variation for efficacy of nonhost resistance to non-adapted rusts and powdery mildews. A small percentage of barley accessions are moderately susceptible to several non-adapted rusts of the *Puccinia* spp., thus barley is considered a "near-nonhost" to such pathogens (Atienza et al. 2004; Niks 2014). Barley accessions with rudimentary susceptibility at seedling stage to the non-adapted *Bgt* were also observed and allowed the development of the two SusBgt lines, $SusBgt_{DC}$ and $SusBgt_{SC}$ (Aghnoum and Niks 2010).

The results presented in chapter 2 (Romero et al. 2018) represent the first time in which the inheritance of nonhost resistance against a powdery mildew pathogen was studied in barley (or in any crop, to our knowledge). This was possible thanks to the development of the SusBgt mapping populations. The maximum susceptibility achieved for *Bgt* in barley is probably represented by the $SusBgt_{SC}$ line. In this line, the largest microcolonies cannot produce more than a few conidia, and are not comparable in size to colonies formed on the natural host, wheat. This suggests that many genes contributing to nonhost resistance of barley to *Bgt* may be fixed for the resistance allele, and therefore there is uniformity on great part of the genetic loci controlling this trait in the germplasm screened by Aghnoum and Niks (2010) to develop $SusBgt_{SC}$. Even though a large complement of nonhost resistance for this pathosystem remains

unknown due to lack of variability for this trait among barley accessions, the increased susceptibility shown by the SusBgt, SusPtrit and Golden Promise parents of the mapping populations were sufficient to enable the mapping of part of the genetic factors playing a role in nonhost resistance. Similarly to what was found for non-adapted rusts (Jafary et al. 2008; Jafary et al. 2006), the immunity of regular barley accessions may be due to different sets of genes, so that many combinations of genes within the barley species will result in the same phenomenon: immunity to *Bgt*.

Diversity of QTLs

In total, five barley mapping populations were screened in this thesis: Vada x SusBgt$_{DC}$ (VxS$_{DC}$) and Vada x SusBgt$_{SC}$ (VxS$_{SC}$) in chapter 2, and Vada x SusPtrit (VxS), SusPtrit x Golden Promise (SxG) and Cebada Capa x SusPtrit (CxS) in chapter 3. The two QTLs mapped in VxS for resistance to *Bgt*, *Rbgnq1* and *Rbgnq2*, were also mapped in the SusBgt populations, although the high-density genetic maps constructed for the SusBgt populations using genotyping-by-sequencing (GBS) enabled QTL mapping to a much smaller interval.

The first molecular linkage maps in barley were from RFLP markers (Graner et al. 1991). Later, with the development of PCR-based markers, AFLP markers became the most used marker type, followed by microsatellites or simple sequence repeats (SSRs) (Varshney et al. 2007). In our research group, genotyping with a SNP array chip provided 2943 polymorphic SNP markers between SusPtrit and Golden Promise, part of which were used to construct the linkage map for this population (Yeo et al. 2014). The linkage maps constructed for the SusBgt populations using the GBS approach resulted in 6966 (VxS$_{SC}$) and 7422 (VxS$_{DC}$) SNP markers (chapter 2). GBS technology has become a cost-effective approach for genotyping mapping populations due to the rapid advances in next-generation sequencing (Poland and Rife 2012). Besides providing a high-density of markers, another strength of the GBS approach is that marker discovery and genotyping are completed simultaneously (Poland and Rife 2012).

Rbgnq1 is the major determinant of the nonhost status against *Bgt*/*Bghm* found in this study. The resistance allele of *Rbgnq1* is contributed by the resistant parent Vada, and it seems to act at pre-haustorial resistance stage, as indicated by the numerous infection units that fail to produce any haustorium in segregants that carry the Vada allele. In the context of this thesis, *Rbgnq1* is a major-effect QTL sufficient to confer immunity to *Bgt* independently of the background QTLs. *Rbgnq1* was not mapped for basal host resistance (chapter 2), suggesting that *Bgh* might have evolutionarily adapted to it. The major effect of *Rbgnq1* on nonhost resistance to *Bgt*, explaining around 40% of the phenotypic variation, facilitated the fine-mapping (chapter 3). A high-density of markers was developed to saturate the region and fine-map the QTL to a 0.27 cM interval (further discussed below).

The susceptible parents $SusBgt_{SC}$ and $SusBgt_{DC}$ contributed a minor-effect QTL, *Rbgnq3*, mapped on chromosome 4H for resistance against the non-adapted *Bgt* and *Bghm*. In chapter 2 we speculated that this could be the same QTL mapped by Jafary et al. (2008) for resistance against four non-adapted rusts, with the resistance allele contributed by SusPtrit. *Rbgnq3* was not mapped in the Vada x SusPtrit population (VxS), though. Further research should be carried out to determine whether this gene represents a case of wide-spectrum resistance against different classes of fungi or if there are several linked genes conferring pathogen specific resistance. In wheat, at least three wide-spectrum resistance genes against biotrophic pathogens, viz. powdery mildew and various rust fungi, were identified (Ellis et al. 2014). Two of these genes, *Lr34* (synonyms: *Yr18*/*Sr57*/*Pm38*) and *Lr67* (*Yr46*/*Sr55*/*Pm46*) have been cloned and encode for membrane-localized transporter proteins (Krattinger and Keller 2016; Krattinger et al. 2009b; Moore et al. 2015).

The diversity of QTLs involved in nonhost resistance becomes more apparent when we analyze the QTL mapping results for SxG (chapter 3). The QTLs mapped for SxG, from *Rbgnq5* to *Rbgnq8*, were not mapped in any other population tested in this thesis. In this mapping population, all QTLs had SusPtrit as the parent contributing the resistance allele, implying that Golden Promise has some susceptibility factors to *Bgt* or lacks some resistance factors. It is possible that the resistance QTLs contributed by SusPtrit in this population were not mapped in VxS because Vada carries the same alleles and hence no segregation occurs in the population. The SxG population has not been assessed for resistance to *Bgh* by us or in any other study to date. Screening SxG for resistance to the adapted pathogen *Bgh* will reveal whether any of the QTLs for nonhost resistance mapped here also have an effect on basal host resistance, as is the case for *Rbgnq2*.

The minor-effect QTL *Rbgnq2* was mapped in the SusBgt populations as well as in VxS, and it seems to play a role in basal host and nonhost resistance. *Rbgnq2* is located on 2HL and co-localizes with the powdery mildew resistance gene *MlLa*. *MlLa* has been transferred to European barley cultivars from '*Hordeum laevigatum*', and is known to confer an intermediate reaction type associated with a hypersensitive response (HR) (Giese et al. 1993; Marcel et al. 2007). A mild necrotic phenotype was observed on some RILs of the two SusBgt and the VxS populations after inoculation with non-adapted *B. graminis* forms. Even though no scoring was performed for this trait, the co-localization of *Rbgnq2* and *MlLa* led us to speculate that these might correspond to the same gene. Further investigation is needed to find out whether *Rbgnq2* indeed corresponds to *MlLa*, and if this is confirmed it will be a not so common example of an *R*-gene with a partial effect not only against adapted (*Bgh*) but also non-adapted (*Bgt*, *Bghm*) mildew forms. Nonhost resistance of barley to powdery mildews is known to be mostly based on pre-invasive (pre-haustorial) mechanisms (Aghnoum and Niks 2010; Trujillo et al. 2004) but it

might be that *R*-genes are expressed following post-invasive stages of infection, causing the HR phenotype observed in some RILs of our populations. Nonhost resistance to *Puccinia* rust fungi in barley is also based on multiple QTLs, although rare involvement of *R*-genes has been reported (Jafary et al. 2008; Niks 2014). However, the *Rph7* gene in Cebada Capa, for race-specific resistance against *P. hordei* appeared not to affect infection by non-adapted rusts (Jafary et al. 2008).

Distinct stages of defense at penetration, haustorium formation and conidiation

As highlighted in the discussion sections of chapters 2 and 3, the microorganisms must overcome several layers of defense in order to infect the host cell. Due to the phylogenetic proximity between the pathogens *Bgt*/*Bgh* as well as the hosts wheat and barley, constitutive defenses are not likely to impose major obstacles for the non-adapted pathogens in these pathosystems. In the nonhost barley-*Bgt* interaction, pre-invasion defense mechanisms prevent almost all penetration attempts to succeed. Entry failure correlates with a broad range of defense responses such as with papilla formation and HR of attacked cells (even those resisting penetration) and expression of pathogenesis-related genes (Huckelhoven et al. 2001; Trujillo et al. 2004). In Arabidopsis interaction with the non-adapted *Bgh*, only 5% of the penetration attempts succeed, and are stopped by post-invasive (post-haustorial) resistance mechanisms that culminates with HR-like cell death (Lipka et al. 2008); the same seems to be true in barley against *Bgt* (Aghnoum and Niks 2010; Trujillo et al. 2004).

Our work demonstrates that some minor genes for nonhost resistance may hamper haustorium formation while others prevent conidiophore formation, indicating that the two stages in pathogen development depend on presence or absence of different plant factors (Chapter 2). Aghnoum and Niks (2010) observed that conidiation rate varied for barley accessions showing similar rates of haustorium formation by *Bgt*. This is well illustrated by the two SusBgt lines: in both $SusBgt_{SC}$ and $SusBgt_{DC}$, a similar proportion of germlings succeeded in penetration and haustorium formation, 51% and 59%, respectively. However, there was a great difference in the extent to which they allowed established germlings to form conidia: 34% in $SusBgt_{SC}$, compared to only 6% in $SusBgt_{DC}$. In chapter 2, $SusBgt_{DC}$ inoculated with *Bgt* showed a conidiation rate close to zero, but still the RILs from the Vada x $SusBgt_{DC}$ population segregated for the level of conidiophore formation. This suggests that Vada, even though immune to *Bgt* due to effective pre-invasion papilla-based resistance, somehow contributes factors that enable pathogen development at later stages of infection. Aghnoum and Niks (2010) and Romero et al. (2018) (chapter 2) were the first publications to show that conidiophore formation require presence or absence of particular factors in the plant. QTL mapping for conidiation was not possible due to the limited number of RILs in the two SusBgt populations allowing *Bgt* to form

haustoria. In future investigations, it might be possible to perform QTL mapping for conidiation by selecting RILs that do not carry the major-effect nonhost resistance QTL, *Rbgnq1* (since this QTL alone will confer immunity for *Bgt*). In this case, phenotyping should be done on the basis of microscopic observations, for visualization (and possibly quantification) of conidiophores. After phenotyping, graphical genotyping may be employed to look for candidate chromosomal regions implicated in stimulating or hampering conidiophore formation. This approach can be done for RILs in the VxS_{SC}, VxS_{DC} and VxS populations, for which results can be compared.

In this thesis we showed that *Bgt* is, to a limited extent, able to overcome nonhost resistance of barley and manipulate the host cell metabolism to complete its life cycle by forming viable conidia (chapter 2). However, barley cannot be considered a suitable host for *Bgt*, since the pathogen cannot sustain an epidemic. This indicates that even though *Bgt* manages to partially evade or suppress PTI on barley, it does not seem to have the necessary effectors to support the infection and allow a proper amount of conidia to be formed. In the conidia viability test in chapter 2 we ruled out the possibility that microcolonies were resulting from contaminants by *Bgh* because 1) elongating hyphae never grow to a size equivalent to a *Bgh* colony on barley, even weeks after inoculation; 2) *Bgh* contaminants would not develop on wheat plants.

PTI x ETI

Plant innate immunity relies on the action of receptors at the cell surface or in the cytoplasm capable of recognizing invading microorganisms and triggering an array of defense responses. Cell surface receptors known as pattern-recognition receptors (PRRs) act at the front line, detecting either conserved pathogen-associated molecular patterns (PAMPs, also known as "microbe-associated molecular patterns", MAMPs) or damage-associated molecular patters (DAMPs), which are endogenous plant molecules released as consequence of pathogen attack. PRRs activate PAMP-triggered immunity (PTI), which confers broad-spectrum resistance and represents the first phase of the zig-zag model of plant immunity (Jones and Dangl 2006). Pathogens may deliver effector proteins into host cells in order to suppress PTI and render the plant susceptible. To counter this, plants have intracellular receptors encoded by resistance (*R*) genes that may recognize effectors in a very specific way, leading to effector-triggered immunity (ETI), typically associated with HR (Jones and Dangl 2006; Stam et al. 2014).

According to the evolutionary model for nonhost resistance proposed by Schulze-Lefert and Panstruga (2011), the relative contribution of PTI or ETI to nonhost resistance changes as a function of the evolutionary distance between the nonhost and the natural host plant species. In cases where the host and nonhost plant species are phylogenetically closely related, like barley and wheat, ETI is proposed to play a major role compared to PTI. This hypothesis does not find

support in our work, since our overall results suggest that resistance to the non-adapted mildew forms relies mostly on nonhypersensitive prehaustorial mechanisms – therefore implying a predominant role for PTI rather than ETI. Also the nonhost resistance in barley to non-adapted rust fungi it was argued to depend almost entirely on PTI, even when the rust fungus was pathogenic on a plant species closely related to barley (Niks et al. 2015). The results presented in previous chapters are in agreement with the hypothesis that nonhost resistance relies on the same defense mechanisms as quantitative (or basal) host resistance. Nonhost resistance of barley to *Blumeria* and rusts is likely due to complete failure of the non-adapted pathogen to overcome PTI, whereas basal resistance represents a partial failure of an adapted pathogen to overcome PTI (Niks et al. 2015).

Effectors are presumed to manipulate one or more host target proteins or processes in order to enhance pathogen virulence (Stam et al. 2014) by interfering with plant defense pathways, and also by manipulating host metabolic pathways and transporters, redirecting nutrients for the pathogen's benefit (Stassen and Van den Ackerveken 2011). Niks and Marcel (2009) hypothesized that QTLs represent effector targets that, once manipulated would confer enhanced pathogen fitness. The failure of non-adapted *B. graminis* ff.spp. in infecting barley might be due to lack of appropriate effector molecules in the pathogen and/or due to lack of matching effector targets in barley. As proposed by Antonovics et al. (2013), failure of infection of a nonhost plant is due to an incidental by-product of pathogen specialization to its "source" host, rather than the consequence of evolved resistance in the plant.

Rbgnq1 - fine-mapping and physical map construction

In chapter 3 we report on the fine-mapping and important steps towards the map-based cloning of *Rbgnq1*. A commonly used approach for fine-mapping requires the introgression of the QTL into near-isogenic lines (NILs), which is a very effective strategy because no additional QTL will be segregating in the background and thus influence the phenotype of the trait of interest (Yeo et al. 2017). The development of NILs is, however, very time-consuming. To speed-up the process we generated a fine-mapping population from the crossing of recombinant inbred lines (RILs) selected for the susceptibility allele of the non-target QTL and contrasting for the allele of the target QTL, which was a successful approach due to the great effect of *Rbgnq1*.

Fine-mapping requires the QTL region to be saturated with markers; therefore the high-density genetic maps available in the SusBgt populations would provide a good starting point for fine-mapping of *Rbgnq1*. It would have been convenient to develop the fine-mapping population using RILs from any of the two SusBgt mapping populations. Nevertheless, we used VxS RILs instead, for two reasons. The first reason was that SusBgt populations were not yet available when *Rbgnq1* was first mapped in VxS – therefore when we obtained the QTL mapping results

from the SusBgt populations, *Rbgnq1* had already been narrowed down to a < 1.0 cM region on the basis of the VxS material. The second reason the fine-mapping was pursued on VxS RILs is the availability of genomic BAC libraries from Vada and SusPtrit (Yeo et al. 2016). As explained in chapter 3, this is relevant because gene content may vary even between genotypes of the same species, therefore the presence of the target gene can only be assured in a BAC library from its specific genotype.

The fine-mapping efforts delimited the QTL to a 0.27-cM region, which, according to the assembly of the barley reference genome of cv. Morex (Mascher et al. 2017), corresponds to a physical region of 556 Kb. Primer pairs were developed based on the sequences of genes predicted for Morex for the QTL interval, and used to screen the Vada and SusPtrit BAC libraries using a PCR-based approach. Because of time constraints, after having isolated a single BAC clone for SusPtrit, S56, we decided to focus on screening the Vada BAC library for positive clones; Vada carries the resistance allele of *Rbgnq1*. The comparison of S56 with its homologous region in Vada was of ultimate importance, since it revealed lack of homology in this area. The distance between markers M-15 and $M_{synt980}$ was much larger in Vada (126 Kb) compared to SusPtrit (26.2 Kb), and the suppressed recombination in this area explains the lack of recombinants that limited the fine-mapping. Despite the lack of homology, the gene content and ordering was in agreement between Vada and SusPtrit, with the exception of two possible gene duplications in Vada. By comparing Vada and Morex, we observed that the gene content is similar; however the order of genes differs. It is not possible for us to assess to what extent this is due to real differences or due to possible inaccuracies in the Morex assembly. As discussed in chapter 3, BAC clones from Vada and SusPtrit were sequenced using the PacBio technology, which generates reads that are long enough to span complex repetitive regions that are common in the barley genome, thus providing an accurate assembly.

The physical map of *Rbgnq1* in Vada is incomplete, and the gap between contigs 1 and 2 may still contain additional genes. The two contigs cover a 405 kb region in between markers Mrx_4610 and Mrx_4850 flanking the resistance QTL. Further work is necessary in order to establish a single contig spanning the QTL interval, which would have all the genes represented. A search for new Vada BAC clones to close the gap requires screening of the Vada BAC library with primer pairs that are based on the sequences located at the ends of contigs 1 and 2. It would also be important to isolate clones from the SusPtrit BAC library, so it will be possible to compare the sequences of resistance and susceptibility alleles. So far, the list of genes predicted for the *Rbgnq1* interval in Vada (Table 3 of chapter 3) contain several genes that are implicated in disease resistance responses, such as receptor kinase, lipid transfer proteins (LTPs), glutamate dehydrogenase, chitinase and UDP-glycosyltransferase. It is possible, however, that the causal gene(s) has (have) functions that have not been previously associated with disease

resistance. The list of candidate genes includes also some uncharacterized proteins. None of the genes in the candidates list identified is of the NB-LRR type, indicating that the resistance conferred by *Rbgnq1* is not *R*-gene mediated.

The final step of map-based cloning is a careful analysis of all the genes present in the physical target interval in order to determine the nature of the genes (coding or regulatory) underlying the trait (Krattinger et al. 2009a). The BAC clones isolated for the QTL region were tested by transient gene expression through ballistic transformation of epidermal leaf cells. After transformation, samples were challenge-inoculated with *Bgt* to test whether any BAC clone would have an impact on the outcome of the interaction. This was done by assessing the susceptibility index, i.e., the number of transformed cells in which a haustorium was formed divided by the total number of transformed cells, and comparing this index to the index obtained by an empty BAC control.

The results of the transient transformation experiments did not provide significant evidence that the tested BACs contain the causal gene. However, the overall results for V49 indicate a trend of this BAC to decrease haustorium formation by *Bgt* in the transformed cells in $SusBgt_{SC}$. Additional bombardments with V49 and other BACs isolated in the future for the gap region must be performed. It is also possible that the resistance phenotype is conferred by a cooperation of several genes in the interval, so it is necessary that a combination of BACs are also tested by bombardment. In case positive results are achieved for a given BAC(s), all the genes present in that clone should be individually cloned and further validated by stable transformation.

Grain quality problems

Recombinant inbred lines (RIL) and doubled haploids (DH) are permanent mapping populations in which each line can be multiplied perpetually without any genetic change. This is beneficial for genetic mapping studies because such material enables recurrent genotyping for additional markers and phenotyping for different traits, allowing several studies to be performed in genetically identical material (Boopathi 2013). An unknown condition affected the quality and germination of seeds from the three SusPtrit mapping populations, which constitute invaluable material for studies of inheritance of nonhost resistance in barley. Affected grains showed dark discoloration and a hideous shriveled appearance. The severity of symptoms varied among lines, ranging from a few dark blotches to whole-grain discoloration associated or not with shriveling. A certified laboratory analyzed the grain samples, but could not find any biotic agent causing the grain quality problem. Investigation of the possible factors causing or influencing this grain quality disorder and mapping of barley genomic regions associated with it resulted in chapter 4 of this thesis.

An extensive literature search was made with the aim of comparing the symptoms of our seeds with reports of grain discoloration in cereals. There is no consensus about the terminology used to refer to grain discoloration, but the mostly used term is probably kernel discoloration (KD). Dark discoloration restricted to the embryo end of the grain is referred to as black point (BP). Some authors consider BP as a type of KD (e.g. (de la Pena et al. 1999), while others refer to BP and KD as distinct phenomena (e.g. (Walker et al. 2008). The condition we observed in our mapping populations is unlikely to be black point, since the observed discoloration is not restricted to the germ end – and it can be confined to other parts of the grain than the germ end. Although some of the symptoms are similar to reports of kernel discoloration in cereals, we could not find any report describing such severe manifestation as in our seed material. As far as we are concerned, this could be a newly described condition governed by a rare allele introgressed from exotic germplasm, since SusPtrit has four landraces in its parentage (Atienza et al. 2004).

The SusPtrit mapping populations were scored for grain quality and a large-effect QTL on chromosome 6H was mapped in common for the three populations. Our suspicions were confirmed that SusPtrit, the common parent of the three populations, was contributing the allele conferring the poor quality character. This QTL for grain quality was taken into account when we selected the RILs from VxS to develop the fine-mapping population for *Rbgnq1*, in chapter 3. To avoid that germination problems would interfere with the fine-mapping screenings, the selected parental RILs do not have the SusPtrit allele of the QTL on 6H.

Barley genotypes may have hulled (covered) or hulless (naked) seeds, which is a trait determined by the *nud* locus on chromosome 7HL (Taketa et al. 2008), and therefore not linked to the major QTL mapped in chapter 4. SusPtrit is a hulless line, while the other parents of the three mapping populations have hulled seeds – therefore this character segregates in the three mapping populations. The grain quality problem affects both hulless and well as hulled lines, and an interesting aspect observed is that some affected covered-seed lines produced grains that were either not covered at all or in which partial hull loss was observed. The phenotype of such lines resembles that of grain skinning, characterized by poor adhesion of the hull to the grain due to compromised quality of the cementing material (Brennan et al. 2017). Grain skinning was shown to be a heritable trait, although largely influenced by environmental factors. Further research should determine whether these two conditions are associated, for example by assessing the SusPtrit mapping populations for grain skinning. In chapter 4 we suggest that the SusPtrit populations might be suitable material to conduct research on grain skinning in order to help mapping genomic regions associated with it.

Initially, multiplication of the seed material from the SusPtrit populations occurred without any grain quality problem. After a few years of annual multiplication under field and

greenhouse conditions, however, the seed quality problems started (Rients Niks and Anton Vels, personal communication). Both SusBgt lines have SusPtrit in their parentage (Aghnoum and Niks 2010), so there is a chance that the allele for poor quality condition on 6H is segregating in the two populations. So far, no problems were observed in these populations regarding grain quality, but it can also happen that somehow this condition will be triggered in the future.

Concluding remarks

The idea of using nonhost plant species as donors of durable resistance is not recent. The influential plant pathologist J. E. Vanderplank wrote, in 1976, a "science fiction essay" in which DNA was transferred from maize to oat plants, in order to produce an oat plant resistant to rust pathogens of oat and maize. Progress in molecular genetics led to the identification of several important components of nonhost resistance, mainly in model plant species. Advances in genetic engineering enabled multiple examples of successful transfer of nonhost resistance factors across plant species (reviewed in Lee et al. 2016).

The main goal of this PhD thesis was to identify genetic factors involved in nonhost resistance of barley to non-adapted powdery mildews. The relevance of this research relies on two main reasons. First, because the inheritance studies carried out in the barley-*Blumeria* pathosystem can contribute to the understanding of the evolutionary and genetic basis of host-pathogen interactions (Aghnoum and Niks 2010). And second, because the acquired knowledge on the genetic basis of nonhost resistance may lead to interesting practical applications. The cloning of nonhost resistance genes against powdery mildews in barley will enable future research to determine the effectiveness of transferring these nonhost resistance factors to related crop species. The recently developed gene editing technology of clustered, regularly interspaced, short palindromic repeats (CRISPR) may be an effective tool for the transferring of target genes for nonhost resistance to other plant species (Lee et al. 2016). As more nonhost resistance genes become known, the ones that represent susceptibility alleles in different cereal crops can be edited to function as resistance alleles, therefore creating new specificities (Dracatos et al. 2018).

In order to suppress PTI in wheat, *Bgt* must have the necessary (adapted) effectors that will target defense factors. The identification of nonhost resistance genes in barley against *Bgt* may provide the opportunity of replacing effector-suppressed defense factors in wheat by their (nonhost) barley orthologues (Douchkov et al. 2014). Nonhost resistance must be understood as the result of a multitude of genetically determined factors, as there is no single mechanism that is responsible for its durability and effectiveness (Fan and Doerner 2012). Therefore, I speculate that, if a gene such as *Rbgnq1* can be individually transferred to wheat in the future, it most likely will contribute to quantitative resistance against *Bgt* instead of conferring immunity. The

effect of *Rbgnq1* is "strong" enough to confer immunity in barley against *Bgt*, but this may be only true because barley already has in its background several nonhost resistance genes. *Rbgnq1* supposedly represents an operative target of a particular *Bgt* effector that is not efficiently suppressed, and therefore can normally perform its role in basal defense. The defense response triggered (or contributed) by *Rbgnq1* will not be properly suppressed by *Bgt* because this pathogen does not have adapted effectors to barley. In the wheat context, however, it might be that the role played by *Rbgnq1* on PTI will not be enough to confer immunity, because many other effectors are adapted to wheat targets. Whether *Rbgnq1* will indeed contribute to resistance against *Bgt* in wheat remains to be further examined in the future.

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Summary

The durability and effectiveness of nonhost resistance suggests promising practical applications for crop breeding, relying upon elucidation of key aspects of this type of resistance. Inheritance and mapping studies are valuable for identifying genes that are determinants of specificity of the host or nonhost status. To overcome the problems associated with inheritance studies involving two different plant species, it is possible to study the genetics of resistance in plants that display natural variation in the degree of resistance to a non-adapted pathogen, the so-called "near-nonhosts". Barley can be considered a near nonhost to non-adapted powdery mildews of cereals and grasses (*Blumeria graminis* ff. spp.), because a few accessions are somewhat susceptible at seedling stage. In the present thesis, the main goal was to investigate the genetic factors responsible for the specificity of the (non)host status in barley to *B. graminis* ff. spp. We also investigated the causes of an unknown condition affecting the grain quality in three mapping populations having the barley line SusPtrit as a parent.

Chapter 1 presents an introduction on the molecular mechanisms of plant immunity and nonhost resistance and an overview of barley's importance as a crop and model organism, as well as the relevance of barley-*Blumeria* as a model pathosystem to study quantitative basal and nonhost resistance. In **chapter 2**, two recombinant inbred lines (RIL) mapping populations were developed from the crossing of each of the SusBgt lines (experimental lines with increased susceptibility to the powdery mildew of wheat, *B. graminis* f. sp. *tritici* - *Bgt*) with barley cultivar Vada. High-density genetic maps were constructed for each of the SusBgt populations using the genotyping-by-sequencing technology. The SusBgt populations were screened, at seedling stage, for resistance to one adapted and two non-adapted forms of *B. graminis*. Results confirmed that nonhost resistance of barley to powdery mildew relies mostly on non-hypersensitive mechanisms and showed that some QTLs seem effective only to non-adapted mildews, while others also play a role in defense against the adapted form. Histological analyses of nonhost interaction showed that most penetration attempts are stopped in association with papillae, and also suggested independent layers of defense at haustorium establishment and conidiophore formation. In **chapter 3** the SusPtrit mapping populations Vada x SusPtrit (VxS), Cebada Capa x SusPtrit (CxS) and SusPtrit x Golden Promise (SxG) were also screened at seedling stage for susceptibility to *Bgt*. QTLs were mapped in the VxS and SxG populations, but no QTL was found in CxS. None of the QTLs detected in the SxG population had a position similar to that of the QTLs detected in VxS, indicating that a wide diversity of loci are implicated in resistance among barley genotypes. QTL mapping results for VxS were similar to what was found for the SusBgt populations in chapter 2, including a large-effect QTL on chromosome 5HL. This QTL, *Rbgnq1*,

has Vada as the parent donor of the resistance allele, and was fine-mapped to a 0.27 cM interval. The genomic BAC libraries of Vada and SusPtrit were screened for clones spanning the *Rbgnq1* region using a PCR-based approach. Two contigs were established for the physical map of *Rbgnq1* in cv. Vada, covering a region of approximately 405 Kb containing 17 predicted candidate genes. Candidate genes in the interval were tested by transient transformation, using the sequences of whole BAC clones. **Chapter 4** describes the investigations made regarding an unknown quality problem affecting the grains of the three SusPtrit populations. Affected grains showed a dark discolored and shrivelled appearance, and germination was impaired – therefore compromising the multiplication and maintenance of the populations. No biotic agent was recognized as the fundamental cause of the problem. QTL mapping was performed based on quality scores attributed to the seed material of the three populations. A major effect QTL on chromosome 6H was mapped independently in all three populations, with the poor quality allele contributed by SusPtrit. A second, minor effect QTL, was mapped on chromosome 2H in SxG, with Golden Promise as the parent donor of the allele conferring poor quality. We compared the symptoms of our seeds with symptoms described in the relevant literature for cereal grain discoloration. The grain quality problem affects both hulless (naked grains) and well as hulled (covered grains) lines, and an interesting aspect observed is that some affected covered-seed lines produced grains that were either not covered at all or in which partial hull loss was observed. The phenotype of such lines resembles that of grain skinning, characterized by poor adhesion of the hull to the grain. The general discussion of the thesis is presented in **chapter 5**. Inheritance studies carried out in the barley-*Blumeria* pathosystem can contribute to the understanding of the evolutionary and genetic basis of host-pathogen interactions and the acquired knowledge on the genetic basis of nonhost resistance may lead to interesting practical applications.

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Finally, my deepest gratitude goes to my family, for their endless love and support. My late grandmother **Jacira** was an extraordinary woman who helped raise me and taught me the importance of studying. I thank my mom, **Roseli**, for her unlimited love and care and for always encouraging me. Mãe, obrigada pelo seu amor e apoio incondicional. I thank my little sister **Sara** for always being there for me and for our family when I was not around.

I'm grateful to my beloved husband **Rafael** for being my life partner and for staying by my side during this journey. Thank you for your patience, love and always encouraging words. Te amo!

Group picture: Niks *et al*., 2017

Thank you!

Cynara

About the author

Cynara Cassandri Teixeira Romero was born on March 19th 1986 in Campo Mourão (PR), Brazil. In 2004 she started her BSc in Biology at State University of Maringá (UEM), mainly driven by her interest in Genetics. After concluding the BSc in 2008, she moved to Londrina in 2009 to start the MSc in Genetics and Molecular Biology at State University of Londrina (UEL). During the MSc she worked at the Laboratory of Plant Biotechnology and Bioinformatics at EMBRAPA-Soybean studying the nonhost resistance of soybean to the common bean rust fungus (*Uromyces appendiculatus*) at microscopic and molecular levels. After obtaining her MSc degree she was granted with a 'Science without Borders' scholarship by the Brazilian National Council for Scientific and Technological Development (CNPq) to study her PhD abroad. Due to her growing interest plant nonhost resistance, she moved to The Netherlands to work with Dr Rients Niks at the Laboratory of Plant Breeding of Wageningen University & Research. The main focus of her PhD was to investigate the genetic factors responsible for the specificity of the (non)host status in barley to powdery mildews of cereals and grasses. This work was performed under the supervision of Dr Rients Niks and Dr Yajun Wang and the results are present in this thesis.

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Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Cynara Cassandri Teixeira Romero
Date: 4 July 2018
Group: Laboratory of Plant Breeding
University: Wageningen University & Research

1) Start-up phase

	date
► **First presentation of your project**	
Title: Genes determining the nonhost resistance of barley to the powdery mildew of cereals and grasses	07 Nov 2013
► **Writing or rewriting a project proposal**	
Title: Genes determining the nonhost resistance of barley to the powdery mildew of cereals and grasses	Sep 2013
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure

	date
► **EPS PhD student days**	
EPS PhD students day, Leiden, NL	29 Nov 2013
EPS PhD Student day ' Get2Gether', Soest, NL	28-29 Jan 2016
► **EPS theme symposia**	
EPS Theme 2 Symposium I Interactions between Plants and Biotic Agents, together with Willie Commelin Scholten Day, Amsterdam, NL	25 Feb 2014
EPS Theme 3 Symposium "Metabolism and Adaptation', Wageningen, NL	11 Mar 2014
EPS Theme 4 Symposium 'Genome Biology', Wageningen, NL	03 Dec 2014
EPS Theme 3 Symposium 'Metabolism and Adaptation, Wageningen, NL	10 Feb 2015
EPS Theme 2 Symposium I Interactions between Plants and Biotic Agents, together with Willie Commelin Scholten Day, Utrecht, NL	20 Feb 2015
EPS Theme 2 Symposium I Interactions between Plants and Biotic Agents, together with Willie Commelin Scholten Day, Leiden, NL	22 Jan 2016
EPS Theme 2 Symposium I Interactions between Plants and Biotic Agents, together with Willie Commelin Scholten Day, Wageningen, NL	23 Jan 2017
► **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016
Annual Meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017
► **Seminars (series), workshops and symposia**	
Wageningen PhD Symposium	10 Dec 2013
Symposium 'All-inclusive Breeding: Integrating high-throughput science', Wageningen, NL	16 Oct 2014
Symposium 'Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding', Wageningen, NL	11 Dec 2014
Seminar Professor Monica Höfte	06 Feb 2015
Crop Pathology and Plant-Microbe Interactions Symposium	08 May 2015
EPS Flying Seminar Gero Steinberg	05 Jun 2015
EPS Flying Seminar Prof. Dr. Jane Parker	21 Jan 2016
Lectures "Rewriting our genes?" - Prof. Dr. Jennifer Doudna and Dr. Edze Westra	30 Sep 2016
EPS Flying Seminar Prof. Dr. Hans Thordal-Christensen	12 Dec 2016
1st Symposium 'WURomics: Technology- Driven Innovation for Plant Breeding'	15 Dec 2016
Mini-Symposium applied Phytopathology, Wageningen	01 Mar 2017
► **Seminar plus**	
► **International symposia and congresses**	
15th International Cereal Rusts and Powdery Mildews Conference - Denmark	05-08 Jul 2015
5th Plant Genomics & Gene Editing Congress - Amsterdam, NL	16-17 Mar 2017
► **Presentations**	
Talk: PBR monday seminars	31 Mar 2014
Poster: Annual EPS meeting Lunteren	14-15 Apr 2014
Talk: PBR monday seminars	29 Jun 2015

	Talk and poster: 15th International Cereal Rusts and Powdery Mildews Conference	07 Jul 2015
	Talk: Guest speaker DURESTrit (ERA-CAPS) meeting - Wageningen	07 Apr 2016
	Talk: Annual EPS meeting Lunteren	12 Apr 2016
	Talk: PBR Monday Seminars	27 Jun 2016
	Talk: Guest speaker DURESTrit (ERA-CAPS) meeting - Gatersleben, Germany	20 Oct 2016
	Poster: 5th Plant Genomics & Gene Editing Congress - Amsterdam	17 Mar 2017
	Talk: Guest speaker DURESTrit (ERA-CAPS) meeting - Aachen, Germany	06 Apr 2017
	Talk: PBR monday seminars	03 Jun 2017
►	**IAB interview**	
►	**Excursions**	
Subtotal Scientific Exposure		*19.9 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	'Genome Assembly', Wageningen, NL	28-29 Apr 2015
	'Basic Statistics', Wageningen, NL	22, 24, 29, 30 Jun-01 Jul 2016
	'JoinMap', Wageningen, NL	18 May 2016
►	**Journal club**	
►	**Individual research training**	
	Stay at "The Leibniz Institute of Plant Genetics and Crop Plant Research" in Gatersleben - Germany	04-20 Oct 2016
Subtotal In-Depth Studies		*5.4 credits**

4) Personal development		*date*
►	**Skill training courses**	
	'Information Literacy including EndNote Introduction', Wageningen, NL	26-27 Aug 2014
	'Project and Time Management', Wageningen, NL	17 Sep, 01, 29 Oct 2014
	'PhD Workshop Carousel', Wageningen, NL	17 Apr 2015
	'Techniques for Writing and Presenting a Scientific Paper', Wageningen, NL	21-24 Apr 2015
	'Reviewing a Scientific Paper', Wageningen, NL	17 Mar 2016
►	**Organisation of PhD students day, course or conference**	
	Organization of meetings "Insect and Nonhost Resistance" group	Aug 2015-Sep 2016
►	**Membership of Board, Committee or PhD council**	
Subtotal Personal Development		*5.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	**33.5**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

The research described in this thesis was performed at Plant Breeding of Wageningen University & Research. The doctoral study program was supported by the Brazilian National Council for Scientific and Technological Development (CNPq), an organization of the Brazilian Government for the development of Science and Technology. Research was also supported by the ERA-CAPs DURESTrit project (13.006).

Thesis cover: young leaf of barley line SusBgt$_{SC}$ infected with the non-adapted fungus *Blumeria graminis* f.sp. *tritici*

Cover design: Lorena Lenara Batista

Thesis layout by the author

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