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Locating QTL conferring resistance against net blotch, leaf rust, and stripe rust in the wild barley nested association mapping (NAM) population HEB-25

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AFLP, amplified fragment length polymorphism

AM, association mapping

ANOVA, analysis of variance

AO, average ordinate

AUDPC, area under the disease progress curve

BC_1S_3, backcross one, selfing three generation

BIC, Bayesian information criterion

CAPS, cleaved amplified polymorphic sequence

CV, cross-validation

DH, doubled haploid

DNA, deoxyribonucleic acid

DR, detection rate

FAO, Food and Agriculture Organization of the United Nations

GO, gene ontology

GWAS, genome-wide association study

HEB, Halle exotic barley

HIF, heterogeneous inbred family

IBS, identity-by-state

JLMA, joint linkage association mapping

LD, linkage disequilibrium

LM, linkage mapping

LSMEANS, least squares means

MAGIC, multi-parent advanced generation inter-cross

MAS, marker assisted selection

MNI, mean imputation

MTA, marker trait association

NAM, nested association mapping

PCR, polymerase chain reaction

PILA, percentage of infected leaf area

QTL, quantitative trait locus/loci

RFLP, restriction fragment length polymorphism

RGA, resistance gene analog

RIL, recombinant inbred line

RT, reaction type

SNP, single nucleotide polymorphism

SSR, simple sequence repeat

Barley

Cultivated barley (*Hordeum vulgare* ssp*. vulgare*) is a self-pollinating, diploid cereal with 7 chromosomes (2n=4x=14) and a genome size of 5.1 gigabases (Graner et al. 2010; IBGS 2012). Following wheat, maize, and rice it is the fourth most grown cereal worldwide, with an area harvested of 46.9 million ha and a production totaling 141.3 million tons in 2016 (FAO 2018). Barley has a high adaptability and stress tolerance, allowing production in a wider range of environments than any other cereal and making it a very important crop in dry, marginal areas (Newton et al. 2011). Growing area stretches from 70°N in Norway to 46°S in Chile, including extreme environments like arid regions of Africa and the Middle East, and the highlands of Central Asia (Akar et al. 2004; Grando and Macpherson 2005). The majority of harvested barley is used as animal feed and in the brewing industry, whereas only a small fraction is used directly for human nutrition (Baik and Ullrich 2008). Rising health awareness, combined with studies highlighting that its high β-glucan and phytochemicals content can help to reduce the risk of developing cardiovascular disease and type 2 diabetes (Baik et al. 2010; Collins et al. 2010; Idehen et al. 2017), increased interest in the nutritional properties of barley in recent times (Baik and Ullrich 2008; Newton et al. 2011). Besides its use as a crop, barley is of high importance for genetic studies, serving as genetic model for the Triticeae (Muñoz-Amatriaín et al. 2014). This results from barley exhibiting several essential features, namely a diploid nature, a low chromosome number with large chromosomes, ease of hybridization and cultivation, and a diverse set of phenotypes being readily available (Graner et al. 2010; Saisho and Takeda 2011). A further benefit is the availability of extensive sequence data (IBGS 2012; Mascher et al. 2013), with the highly contiguous reference genome sequence recently published by Mascher et al. (2017) as newest contribution.

Barley is one of the earliest crops to be domesticated, with initial domestication from its wild progenitor *Hordeum vulgare* subsp. *spontaneum* occurring at least 10,000 years ago (Zohary et al. 2012). The Fertile Crescent is commonly recognized as the region in which initial domestication of barley occurred (Badr and El-Shazly 2012; Preece et al. 2017), however there is an ongoing debate among researchers, as several studies suggest that domestication might have occurred independently in

other regions (Molina-Cano et al. 1999; Morrell and Clegg 2007; Orabi et al. 2007; Dai et al. 2012; Ren et al. 2013; Wang et al. 2015). The loss of seed shattering and the appearance of a six-rowed spike represent the two major domestication events in barley, decreasing harvesting loss and resulting theoretically in a 3-fold increase in yield potential, respectively (Sakuma et al. 2011). Loss of seed shattering is caused by an independently occurring microdeletion in the complementary dominant genes *Btr1* and *Btr2* (Pourkheirandish et al. 2015). The six-rowed spike is the result of a single mutation of the wild-type *Vrs1* allele (Komatsuda et al. 2007). Cultivated Barley exists as winter type, requiring vernalization to initiate the reproductive phase, and spring type that lacks the vernalization requirement. This lack of vernalization requirement results out of the natural deletion of the flowering repressor gene *Vrn-H2* (Yan et al. 2004; von Zitzewitz et al. 2005).

Net blotch

The causal agent of net blotch of barley, *Pyrenophora teres*, exists in two forms, *P. teres* f. *teres* and *P. teres* f. *maculata*, which induce net form net blotch and spot form net blotch, respectively (Liu et al. 2011b). The net form of net blotch caused by the necrotrophic fungus *Pyrenophora teres* f. *teres* (anamorph: *Drechslera teres* (Sacc.) Shoemaker) is a major disease of barley occurring in barley growing areas worldwide (Liu et al. 2011b). *P. teres* f. *teres* is classified as stubble-born disease as it generally produces pseudothecia as an over-seasoning structure that can survive on kernels and barley debris in the field (Liu et al. 2011b). The implementation of reduced or zero tillage practices therefore resulted in a significant increase in the incidence of *P. teres* f. *teres* (Lehmensiek et al. 2007). Primary infection occurs in spring-time with ascospores being actively discharged from pseudothecia (Fig 1, Jordan 1981). Following successful host colonization, the fungus produces a high number of conidia which are wind dispersed (Jordan 1981). Once they land on barley leaves, infection takes place by penetration of the leaf, mostly between epidermal cells (Lightfoot and Able 2010). Accumulation of toxins produced by *P. teres* f. *teres* and fungal growth ultimately results in the typical disease symptoms of transverse and longitudinal streaks, forming a net-like pattern of necrosis on barley

leaves often accompanied by chlorosis (Mathre 1997; Sarpeleh et al. 2007; Lightfoot and Able 2010).

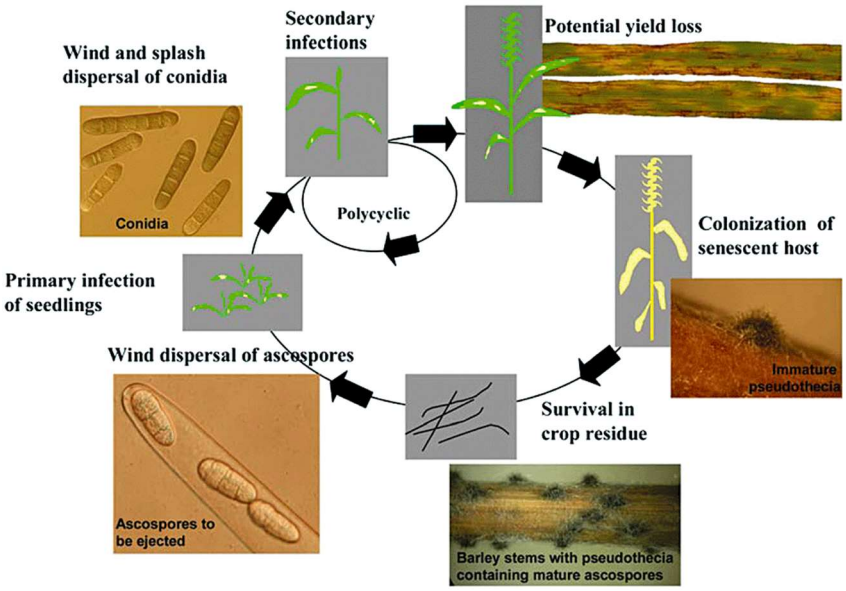

Fig 1. Life cycle of *Pyrenophora teres* f. *teres* (Liu et al. 2011b)

Yield losses, caused by infections, typically range from 10 to 40% with the potential to result in total yield loss if susceptible cultivars are grown (Mathre 1997; Murray and Brennan 2010). Reduction of kernel size, plumpness, and bulk density as a result of infection are of high economic impact, as feed and malting quality is reduced (Grewal et al. 2008). Disease severity depends strongly on the environmental conditions, with temperatures between 20 to 25°C combined with leaf wetness representing optimal growth conditions (Jordan 1981; van den Berg and Rossnagel 1990). *P. teres* f. *teres* can be managed to a certain degree by agricultural practice like the implementation of crop rotation and ploughing of straw and stubble to reduce the source of initial infection (Ma et al. 2004; Liu et al. 2011b). In addition, disease control can be achieved via foliar fungicide application and seed dressing (Ma et al. 2004; Liu et al. 2011b). However, both approaches are time and cost intensive, and only allow for temporary control. Therefore, the use of resistant cultivars represents the most economical, eco-friendly and consumer-friendly approach for long-term disease control (Ma et al. 2004; Grewal et al. 2008). The development of cultivars with improved resistance to *P. teres* f. *teres* however is complicated by the highly variable nature of the fungus and the highly complex host-pathogen interaction (Liu et al. 2011b). Cultivars showing a low infection or

slow disease development compared to susceptible cultivars are known (Steffenson and Webster 1992b), but up to now no cultivars showing complete resistance to *P. teres* f. *teres* exist in Germany (Anonymous 2016). Numerous genetic studies focusing on resistance of barley to *P. teres* f. *teres* revealed net blotch resistance to be inherited mostly in a quantitative manner, but resulted also in the identification of several dominant and recessive major genes (see Chapter 1).

Leaf rust and stripe rust

The two rust fungi *Puccinia hordei (Ph)*, causing leaf rust, and *Puccinia striiformis* f. sp. *hordei (Psh)*, the causal agent of stripe rust, are important barley pathogens in temperate barley-growing areas worldwide (Chen et al. 1995; Mathre 1997). Both fungi are biotrophic, requiring living plant tissue to survive and complete their life cycle (Koeck et al. 2011). The disease cycle was reviewed in detail by Kolmer et al. (2009). At the beginning of the growing season teliospores formed as a resting structure at the end of the last growing season germinate and produce basidiospores which are forcibly ejected into the air. Basidiospores are then spread by wind and infect the host by penetrating the cell walls. Subsequently the fungus forms aeciospores, which are spread by wind and once landed on host leaves invade through stomata, resulting in the formation of pustules that break through the epidermis and contain urediniospores. This stage can result in an epidemic spread of the disease, as the urediniospores can be long-range dispersed by wind and are able to reinfect their host repeatedly (Fig 2). The fungus does not depend on its sexual stage but can survive in this asexual stage on volunteers or winter barley, allowing for a direct infection of barley at the start of the growing period (Marcel et al. 2007). Selected species of the genera *Ornithogalum*, *Leopoldia*, and *Dipcadi* represent alternate hosts of *Ph*, but are not important in Europe (Park et al. 2015). *Berberis* spp. were identified to be alternate hosts of wheat stripe rust (Jin et al. 2010), but up to now no information exists on alternate hosts of *Psh*.

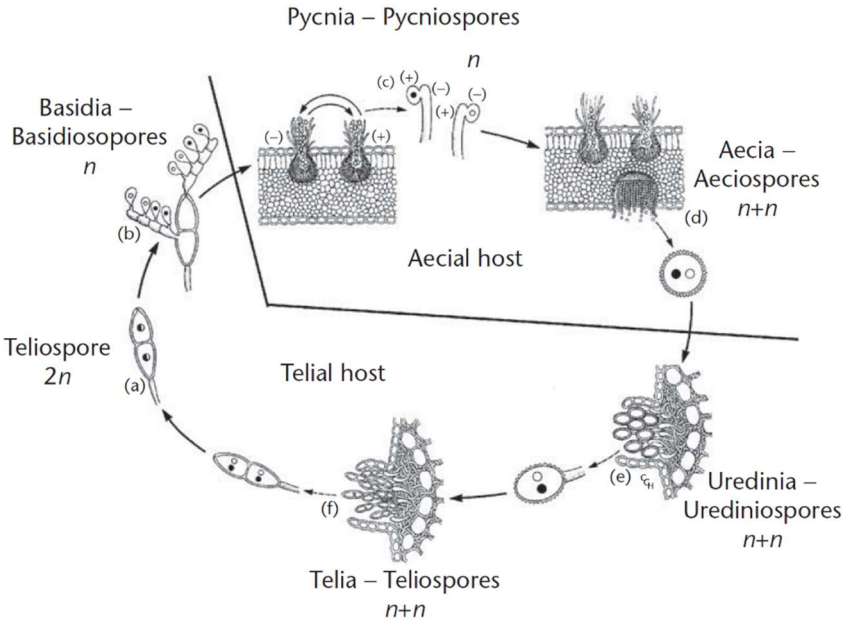

Fig 2. Life cycle of a macrocyclic-heteroecious rust. (a)mature, diploid teliospore, (b) basidia with basidiospores, (c) pycnial (sprermogonial) stage, (d) aecial stage, (e) uredinial stage and (f) telial stage (Alexopoulos et al. 1996).

The pustules formed at this stage allow for a clear differentiation of the two rust fungi (Fig 3). In case of *Ph* the pustules are orange-brown, scattered across the leaf, and can be surrounded by chlorotic halos or green islands (Park et al. 2015), whereas, in case of *Psh* pustules are yellow-orange and appear linearly along the leaf veins (Line 2002).

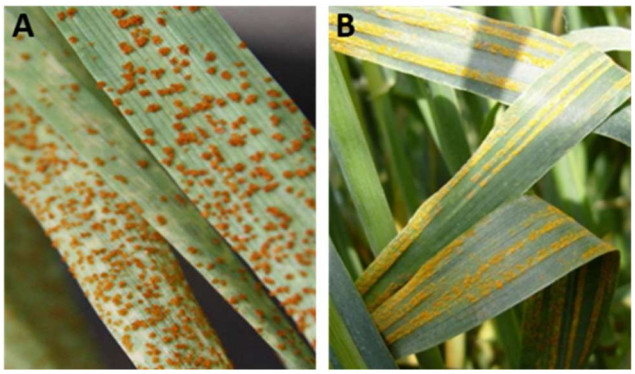

Fig 3. Infection symptoms. (A) leaf rust (*Puccinia hordei*) and **(B)** stripe rust (*Puccinia striiformis* f. sp. *hordei*).

Successful infection and disease spread depends strongly on environmental conditions, resulting in a high variability in disease severity between years. Leaf wetness is required and optimal germination temperatures are 15 to 22°C and 10 to

12°C for *Ph* and *Psh*, respectively (Newton and Johnson 1936; Mathre 1997). Infection can cause yield losses of up to 62% in case of *Ph* and up to 70% in case of *Psh* and a reduced grain quality, by reducing the photosynthetic area and diverting assimilates (Cotterill et al. 1992; Line 2002; Das et al. 2007; Helfer 2014). *Ph* is probably the most common and distributed rust disease of barley (Park et al. 2015), whereas *Psh* currently is of minor economic importance, but caused severe epidemics in the past (Stubbs 1985; Münnich et al. 2000; Line 2002; Esvelt Klos et al. 2016). Most likely the biggest economic threat face Australian growing areas where *Psh* did not occur yet, as studies showed that 70% of Australian barley varieties are susceptible (Wellings et al. 2000; Wellings 2007). Both fungi can be controlled by timely fungicide application, however the most economical, environmental, and consumer-friendly approach represents the deployment of cultivars exhibiting a combination of quantitative and qualitative resistance (Rubiales and Niks 2000; Chen 2007; González González et al. 2013). Up to now 25 major genes (*Rph1*-*Rph24*) and *Rph*$_{\text{MBR1012}}$ conferring resistance to *Ph* have been reported (König et al. 2012; Park et al. 2015; Ziems et al. 2017), but out of these only *Rph15*, *Rph16*, and *Rph*$_{\text{MBR1012}}$ are known to be still effective in Europe (Niks et al. 2000; Perovic et al. 2004; König et al. 2012). For *Psh* 26 major genes conferring resistance have been reported up to now (reviewed in Chen and Line 2001). However, given the potential of the two fungi to mutate rapidly, it is most likely that still effective resistance genes will be overcome in the future (Stubbs 1985; Park et al. 2015). For both pathogenes, but especially for *Ph*, numerous quantitative trait loci (QTL) have been identified (see Chapter 2), that generally represent a more durable source of resistance.

Broadening the genetic base of resistance

Over the course of the domestication of crop plants, a considerable loss in genetic diversity occurred. Strong selection pressure exerted on wild progenitors of modern crop plants during early domestication, and subsequently the application of modern plant breeding strategies represent the two major bottlenecks responsible for the loss of genetic diversity in modern cultivars (Tanksley and McCouch 1997), resulting in a strong reduction of allelic richness. As in case of other crops, many

modern barley varieties are genotypically quite similar, usually caused by their origin from closely related parental lines (Tanksley and McCouch 1997; Wang et al. 2010; Muñoz-Amatriaín et al. 2014). Studies showed genetic diversity in elite germplasm to be around half of that found in wild barley (Russell et al. 2004; Pankin et al. 2016). This narrow genetic base of modern cultivars increases the genetic vulnerability to diseases and fosters the occurrence of severe epidemics, especially in cases in which a few cultivars are grown on a large acreage (Adugna 2004). Therefore, there is an urgent need to broaden the genetic base of resistance of modern cultivars. In this regard, wild crop progenitors like *Hordeum vulgare* subsp. *spontaneum* play a key role, as they maintained a high genetic diversity and thus represent a valuable source of exotic resistance alleles no longer present in the current breeding gene pool (Nevo et al. 2012; Zhang et al. 2017). The risk of occurrence of epidemics is especially high in case resistance is caused by the presence of a single resistance gene. Given the high variability of most pathogens this form of resistance can be overcome rapidly, resulting in the cultivar being fully susceptible (Lo Iacono et al. 2013). One option to increase the durability of resistance is breeding of cultivars exhibiting race-non-specific resistance, i.e. quantitative resistance that is based on several genes with minor effects (Parlevliet 2002; Miedaner and Korzun 2012). However, the identification and introgression of genomic regions harboring respective QTL is much more difficult to achieve than that of qualitative resistance (McDonald 2010). Especially promising for the development of cultivars with durable resistance is the combined introgression of several resistance genes and QTL into a cultivar, i.e. gene pyramiding (Pilet-Nayel et al. 2017). The introduction of DNA markers and their use for marker assisted selection (MAS) considerably increased the efficiency of breeding for disease resistance, allowing for reduced phenotyping expenditures, and a targeted identification and introgression of resistance genes and QTL into modern cultivars (Torres 2009; Miedaner and Korzun 2012).

DNA-based markers and QTL mapping techniques

The development of DNA-based markers represents the foundation of a new era of plant breeding and plant research in general (Moose and Mumm 2008). The

successful application of DNA-based markers depends on the presence of polymorphisms in the nucleotide sequence between genotypes (Nadeem et al. 2017). The first DNA-based markers, namely restriction fragment length polymorphism (RFLP), were developed by Botstein et al. (1980), allowing for a differentiation of genotypes based on differences in the length of DNA fragments after digestion with sequence-specific restriction endonucleases. However, first wide application of DNA-based markers resulted out of the invention of the polymerase chain reaction (PCR) by Mullis in 1983 (Mullis 1990). Many prominent DNA-based markers, like amplified fragment length polymorphism (AFLP, Vos et al. 1995), simple sequence repeats (SSR, Weber and May 1989), or cleaved amplified polymorphic sequence (CAPS, Konieczny and Ausubel 1993) depend on this technique. In the late 1990s focus changed to the use of single nucleotide polymorphism (SNP), which are highly abundant and relatively even distributed in the genome (Gray et al. 2000; Ganal et al. 2009). Due to these features, SNPs were chosen as marker type for the development of DNA arrays, that allow for genotyping with thousands of markers simultaneously using high-throughput techniques (Gupta et al. 2008; Close et al. 2009). In case of barley, the currently largest SNP array is the 50k Illumina Infinium iSelect genotyping array, recently developed by Bayer et al. (2017). Furthermore, genotyping by sequencing and exome-capture are important tools for generating new markers (He et al. 2014; Warr et al. 2015). DNA-based markers are a prerequisite for the identification and localization of QTL conferring resistance. In case of barley, the BARLEYMAP pipeline (Cantalapiedra et al. 2015) that makes use of the reference genome sequence information (IBGS 2012; Mascher et al. 2017) and the POPSEQ map (Mascher et al. 2013) may then be used to identify candidate genes in the QTL interval. Several methods have been developed that can be used to achieve this goal, with linkage mapping (LM) in bi-parental populations and association mapping (AM) being the two most widely applied methods (Sehgal et al. 2016), and the use of multi-parental mapping populations as a more recently developed method (Yu et al. 2008; Kover et al. 2009). All methods are based on the principle that QTL can be identified via their genetic linkage to one or more markers (Mackay et al. 2009).

Traditional LM makes use of segregating populations, constructed by crossing two genotypes differing substantially in the characteristic of the target trait (Ingvarsson

and Street 2011). To identify QTL linked to disease resistance, these biparental populations are constructed by crossing a resistant with a susceptible genotype followed by selfing of the obtained F_1. Genotyping and phenotyping of the F_2 population allows for the identification of predictive markers that segregate together with the causal loci and thus, the resistance QTL (Mackay et al. 2009). This can be achieved by various statistical methods. The simplest method is based on single-factor ANOVA, evaluating marker by marker, with the drawback that in case of low marker density large genome regions are not considered (Lynch and Walsh 1998). Improved LM methods are interval mapping, developed by Lander and Botstein (1989) and composite interval mapping, described independently by Zeng (1994) and Jansen and Stam (1994). Interval mapping enables to scan for QTL between markers. Composite interval mapping is a modification of the interval mapping method, that implements the use of markers as cofactors to reduce the genetic background noise. This results in a significant increase of the QTL detection power (Jansen and Stam 1994). In general, the power to detect and the precise localization of QTL are restricted by their effect size and allele frequency, and the degree of recombination present in the F_2 population (Mackay et al. 2009; Xu et al. 2017). These factors can necessitate a large population size. Although LM can be performed based on a F_2 population, the use of stable mapping populations that allow for repeated analysis is preferred (Keurentjes et al. 2007). Methods to achieve stable mapping populations are the development of recombinant inbred lines (RILs) populations by repeated selfing of the F_2 plants, or by development of doubled haploids (DH) out of F_1 gametes via anther culture (Keurentjes et al. 2007; Germanà 2011). The two methods differ in the fact that DH are fully homozygous, whereas in case of RILs a certain amount of heterozygosity remains. Development of DH includes only one recombination event, whereas in the process of developing RILs recombination can occur at each selfing step. Therefore, RILs exhibit smaller linkage blocks, which allows for a higher mapping resolution.

AM, also referred to as linkage disequilibrium (LD) mapping, makes use of a natural population that is constructed by collection of a genetically diverse set of individuals like landraces, wild relatives, or cultivars (Xu et al. 2017). Contrary to LM mapping the characteristics of the individuals in regard of the target trait are generally unknown. Benefits compared to traditional LM are the exploitation of historic

recombination, the potential to evaluate a high number of alternative alleles, and time savings as the construction of an artificial mapping population is not needed (Flint-Garcia et al. 2003; Rafalski 2010; Korte and Farlow 2013). The accumulation of historic recombination events results in much smaller LD blocks than that generally observed in bi-parental populations, thereby, enabling a higher mapping resolution (Nordborg and Weigel 2008; Zhu et al. 2008; Myles et al. 2009). This property allows for a precise localization of QTL, however, results in the need for a higher number of markers to detect marker trait associations (MTAs) defining QTL. However, this drawback is alleviated by the availability of large SNP arrays (Comadran et al. 2012; Bayer et al. 2017) that allow genome-wide association studies (GWAS). A stronger restriction is, that the high number of alternative alleles at limited frequency results in a reduced QTL detection power compared to that obtained using bi-parental mapping populations (Myles et al. 2009; Korte and Farlow 2013). In addition, the presence of hidden genetic relatedness in the mapping population can result in the detection of spurious MTAs, so-called false-positives (Pritchard et al. 2000; Lewis 2002; Rafalski 2010). Considering the genetic relatedness and genetic background in the applied AM model can significantly reduce the number of false-positives (Yu et al. 2006; Kang et al. 2008; Segura et al. 2012).

Multi-parental mapping populations were developed to overcome some of the limitations of LM and AM populations and combine their positive attributes (Huang et al. 2011; Dell'Acqua et al. 2015; Ladejobi et al. 2016). Nested association mapping (NAM) populations and multi-parent advanced generation inter-cross (MAGIC) populations represent the most commonly developed multi-parental mapping populations (Ladejobi et al. 2016). Recently, Liller et al (2017) developed a mapping population using a mixture of both approaches and Giraud et al. (2017) performed QTL mapping based on a hybrid population derived by crossing two MAGIC populations. The NAM design to identify QTL was introduced by Yu et al. (2008) in maize. Crossing of 25 diverse founders with a common parent, followed by selfing resulted in 25 F_2 populations. Out of each F_2 population 200 RILs were derived and combined to one mapping population comprising 5000 RILs. Finally, analysis was performed across the 25 biparental populations to detect QTL. This results in a QTL detection method that combines the advantages of conventional LM and AM

strategies, namely the high detection power per SNP offered by LM, and the high allelic richness offered by AM (Yu et al. 2008; McMullen et al. 2009; Lu et al. 2010). In addition, exploiting historic and recent recombination events allows for a high mapping resolution and due to the biparental subpopulations allele effects can be traced back to the parents (Yu et al. 2008). Furthermore, genome shuffling of common parent and founder in the process of developing stable subpopulations and combined analysis minimize the occurrence of false-positives (Yu et al. 2008). Currently NAM populations are available in maize (Yu et al. 2008; Li et al. 2013), sorghum (Jordan et al. 2011), wheat (Bajgain et al. 2016; Li et al. 2016), rice (Fragoso et al. 2017), and barley (Maurer et al. 2015; Nice et al. 2016), highlighting the power of this method.

Originally developed in mice (The Complex Trait Consortium 2004), the MAGIC approach to detect QTL was applied to plants by Kover et al. (2009). The concept of MAGIC is the development of a mapping population by inter-crossing several selected founders until all founders are combined with equal proportions in the inter-crosses, followed by development of RILs (Cavanagh et al. 2008). Therefore, as in case of NAM, the MAGIC approach enables to obtain RILs exhibiting small linkage blocks by taking advantage of historic and recent recombination events, and to correct for bias caused by population stratification (Dell'Acqua et al. 2015). Major difference compared to NAM is that due to the absence of subpopulations, allele effects can not directly be traced back to the parents. Furthermore, it could be shown that the MAGIC approach allows the use of smaller populations than NAM to achieve a high haplotype diversity and effective QTL detection (Dell'Acqua et al. 2015; Ladejobi et al. 2016). In case of cereals, MAGIC populations were developed and successfully applied in maize (Dell'Acqua et al. 2015), wheat (Huang et al. 2012; Mackay et al. 2014; Thépot et al. 2015; Milner et al. 2016), rice (Bandillo et al. 2013; Meng et al. 2016), sorghum (Ongom and Ejeta 2017), and barley (Sannemann et al. 2015).

Halle exotic barley 25

This thesis is based on the Halle exotic barley 25 (HEB-25) population, a barley NAM population introduced by Maurer et al. (2015). HEB-25 was developed by crossing

25 divergent wild barley accessions (*Hordeum vulgare* ssp. *spontaneum* and *H. agriocrithon*) with the spring barley cultivar Barke. The wild barley accessions, serving as donors, were selected to cover the high genetic diversity present in wild barley originating from Afghanistan, Iran, Iraq, Israel, Lebanon, Syria, Turkey, and China. To allow for easier phenotyping, F_1 plants were backcrossed to Barke, resulting in each line carrying approx. 25% of the wild barley genome. Three selfing steps were performed to obtain the final population of 1420 BC_1S_3 lines subdivided into 25 families.

Objectives

This thesis presents the first time use of the NAM population HEB-25 to detect QTL conferring resistance to important barley pathogens, i.e. *Pyrenophora teres* f. *teres*, the causal agent of net blotch, *Puccinia hordei*, the causal agent of leaf rust, and *Puccinia striiformis* f. sp. *hordei*, the causal agent of stripe rust. Two-year field trials were conducted at the Julius Kuehn-Institute, Federal Research Centre for Cultivated Plants, in Quedlinburg, Germany, to achieve the following objectives:

I) Screen the HEB-25 population for resistance against net blotch, leaf rust, and stripe rust.
II) Identify QTL conferring resistance against net blotch, leaf rust, and stripe rust.
III) Compare QTL positions detected in this study with those previously reported in literature.
IV) Identify HEB-25 lines with strong resistance, suitable to be introduced in pre-breeding programs.
V) Identify putative candidate genes underlying the identified resistance QTL.

Abstract

The net form of net blotch caused by the necrotrophic fungus *Pyrenophora teres* f. *teres* is a major disease of barley, causing high yield losses and reduced malting and feed quality. Exploiting the allelic richness of wild barley proved to be a valuable tool to broaden the genetic base of resistance of modern elite cultivars. In this study, a SNP-based nested association mapping (NAM) study was conducted to map QTL for *P. teres* resistance in the barley population HEB-25 comprising 1,420 lines derived from BC_1S_3 generation. By scoring the percentage of infected leaf area followed by calculation of the average ordinate (AO) and scoring of the reaction type (RT) in two-year field trials a large variability of net blotch resistance across and within families of HEB-25 was observed. Genotype response to net blotch infection showed a range of 48.2% for AO (0.9-49.1%) and 6.4 for RT (2.2-8.6). NAM based on 5,715 informative SNPs resulted in the identification of 24 QTL for resistance against net blotch. Out of these, six QTL are considered novel showing no correspondence to previously reported QTL for net blotch resistance. Overall, variation of net blotch resistance in HEB-25 turned out to be controlled by small effect QTL. Results indicate the presence of alleles in HEB-25 differing in their effect on net blotch resistance. Results provide valuable information regarding the genetic architecture of the complex barley-*P. teres* f. *teres* interaction as well as for the improvement of net blotch resistance of elite barley cultivars.

Introduction

The net form of net blotch caused by the necrotrophic fungus *Pyrenophora teres* f. *teres* is a major disease of barley worldwide. Infections can cause high yield losses typically ranging from 10 to 40% with the potential to result in total yield loss if susceptible cultivars are grown (Mathre 1997; Murray and Brennan 2010). Furthermore, infection results in a reduction of kernel size, plumpness, and bulk density, negatively affecting malting and feed quality (Grewal et al. 2008).

Typical disease symptoms are transverse and longitudinal streaks, forming a net-like pattern of necrosis on barley leaves often accompanied by chlorosis (Mathre 1997). Severe infection ultimately results in death of leaves in case of susceptible cultivars. *P*. *teres* f. *teres* can survive on kernels and barley debris in the field (Liu et al. 2011b). As a consequence, reduced or zero tillage has significantly increased the incidence of *P*. *teres* f. *teres* (Lehmensiek et al. 2007). Although *P*. *teres* f. *teres* can be controlled by agricultural practice, e.g. wide crop rotation and ploughing or via fungicide application (Ma et al. 2004; Liu et al. 2011b), focus should be placed on breeding for durable resistance as a cost effective, environmental, and consumer-friendly approach.

No cultivars with complete resistance to *P. teres* f. *teres* have been identified up to now in Germany (Anonymous 2016), but cultivars showing a low infection or slow disease development compared to susceptible cultivars are known (Steffenson and Webster 1992b). The highly variable nature of *P. teres* f. *teres* and the influence of the developmental stage (Tekauz 1990; Steffenson and Webster 1992a; Douiyssi et al. 1998; Manninen et al. 2000; Cakir et al. 2003a; Afanasenko et al. 2007; Gupta et al. 2010) turn the development of cultivars with improved resistance to *P. teres* f. *teres* into a challenging task. Numerous studies focusing on resistance of barley to *P. teres* f. *teres* resulted in the identification of a high number of QTL located on all barley chromosomes (Cakir et al. 2003a; Gupta et al. 2010; Liu et al. 2011b; Grewal et al. 2012; König et al. 2013; König et al. 2014; Liu et al. 2015; Islamovic et al. 2017; Richards et al. 2017). These studies revealed net blotch resistance to be inherited mostly in a quantitative manner, especially in the adult plant stage. However, several dominant and recessive major genes were identified as well (Bockelman et al. 1977; Graner et al. 1996; Ho et al. 1996; Manninen et al. 2000; Ma et al. 2004; Manninen et

al. 2006; Abu Qamar et al. 2008; O'Boyle et al. 2014; Yaniv et al. 2014; Liu et al. 2015; Richards et al. 2016). Especially chromosome 6H turned out to harbor a high number of QTL and most of the major genes inducing resistance against a wide range of *P. teres* f. *teres* isolates (Steffenson et al. 1996; Richter et al. 1998; Manninen et al. 2000; Cakir et al. 2003a; Ma et al. 2004; Friesen et al. 2006; Manninen et al. 2006; Abu Qamar et al. 2008; Liu et al. 2010; St. Pierre et al. 2010; O'Boyle et al. 2014; Yaniv et al. 2014; Liu et al. 2015; Richards et al. 2016). However, despite numerous studies conducted, the exact relationship among the various *P. teres* f. *teres* QTL and resistance genes remains uncertain as studies used different populations, isolates, and marker types (Liu et al. 2015).

The majority of QTL and genes conferring resistance to *P. teres* f. *teres* have been identified by bi-parental LM (reviewed in Liu et al. 2011b). AM to detect *P. teres* f. *teres* resistance QTL was applied only in the study of Richards et al. (2017). Up to now, no NAM study has been performed to identify QTL linked to resistance to *P. teres* f. *teres*. The NAM concept is based on a multi-parental mapping design and was introduced as a genome-wide complex trait dissection strategy by Yu et al. (2008). NAM combines the advantages of conventional LM and AM strategies, namely the increased power of QTL detection and the increased allelic variation compared to bi-parental populations, allowing for an exceptional high mapping resolution (Yu et al. 2008; McMullen et al. 2009; Lu et al. 2010). Next to several studies based on the initial maize NAM population (Yu et al. 2008; Buckler et al. 2009; Lu et al. 2010; Brown et al. 2011; Kump et al. 2011; Poland et al. 2011; Tian et al. 2011; Cook et al. 2012; Peiffer et al. 2013; Peiffer et al. 2014), NAM studies focusing on sorghum (Jordan et al. 2011), wheat (Bajgain et al. 2016; Li et al. 2016), barley (Maurer et al. 2015; Maurer et al. 2016a; Maurer et al. 2016b; Nice et al. 2016; Saade et al. 2016), and maize (Li et al. 2013) highlight the power of this mapping approach.

Up to now, the world's first barley NAM population introduced by Maurer et al. (2015) named HEB-25 has not been used to identify QTL linked to biotic stress resistance. Thus, in this study the high genetic diversity present in HEB-25 and the high mapping power offered by NAM was used to achieve the five main objectives: I) to screen the HEB-25 population for resistance against *P. teres* f. *teres*; II) to identify HEB-25 lines showing high resistance suitable to be introduced in pre-breeding programs; III) to identify net blotch resistance QTL by NAM based on two resistance measures; IV) to compare QTL positions found in this study with those

previously reported in literature, and V) to identify putative candidate genes underlying the identified resistance QTL.

Material and methods

Plant material

This study is based on the HEB-25 NAM population (Maurer et al. 2015). HEB-25 comprises 1,420 BC_1S_3 lines in 25 families originating from a cross of 25 highly diverse wild barley accessions (*Hordeum vulgare* ssp. *spontaneum* and *H. agriocrithon*) with the modern spring barley cultivar Barke (*Hordeum vulgare* ssp. *vulgare*). For more detailed information on population development see Maurer et al. (2015). Due to a loss of genotypes during field trials the analysis is based on 1,403 genotypes of the HEB-25 population.

Field trials

Field trials were conducted at the Julius Kuehn-Institute, Federal Research Centre for Cultivated Plants, in Quedlinburg, Germany, in 2014 and 2015 using a special experimental design called summer hill trial design developed by König et al. (2013). Genotypes were sown in rows of so called hill-plots comprising 25 seeds each, with a spacing of 0.5 m between hills. Spreader strips of susceptible varieties (Candesse and Stamm 4046) were sown between hill-plot rows with a row to row spacing of 1.0 m (S1 File). Trials were laid out in a randomized incomplete block design with two replicates of 18 incomplete blocks each. A resistant standard (gene bank accession HHOR 10860) was integrated three times in each of the incomplete blocks. Net blotch (*P. teres* f. *teres*) infected barley straw was incorporated in the topsoil before sawing to serve as a source of infection and to ensure homogenous disease pressure. Infected barley plants were harvested at the end of the first year and the straw was used as infection material for the second year. Field trials were sown in the first half of August as König et al. (2013) had shown that at that time growing conditions in Germany are more favorable for *P. teres* f. *teres* in comparison to *Rhynchosporium secalis*, which is often opposite in spring, thereby preventing reliable scoring of *P. teres* f. *teres* resistance.

Phenotypic data

The percentage of infected leaf area (PILA), according to Moll et al. (2010), and the reaction type (RT), applying the disease scale of Tekauz (1985), were recorded at three consecutive dates, starting when disease symptoms were clearly visible in the susceptible spreader strips. A time period of two weeks between phenotyping dates was chosen to allow for a sufficient disease development. PILA data was used to calculate the area under the disease progress curve (AUDPC). AUDPC data was then used to calculate the average ordinate (AO) as a measure of infection severity:

$$AO = \frac{\sum_{i=1}^{N_{i-1}} \frac{(y_i + y_{i+1})}{2} * (t_{i+1} - t_i)}{tp}$$

where (N) is the total number of observations, disease level at the ith observation is coded by (y_i), time at the ith observation is coded by (t_i), and the trial period in days is coded by (tp).

Statistical analysis

Phenotypic data analysis was performed using the software package SAS 9.4 (SAS Institute Inc., Cary, NC, USA) using *proc mixed*. Genotype, year, and genotype *x* year interaction were set as fixed. Design effects were set as random statement. Separate covariances were set for years to account for the difference in disease pressure between years. AO least squares means (lsmeans) as well as RT lsmeans were used for subsequent NAM.

To estimate variance components to be used for the calculation of broad sense heritability, all model parameters were set as random. Broad sense heritability across years was calculated as:

$$h^2 = \frac{V_G}{V_G + \frac{V_{GY}}{y} + \frac{V_R}{yr}}$$

where genotypic variance is coded by (V_G), genotype *x* year variance is coded by (V_{GY}), and residual variance is coded by (V_R). The terms *y* and *r* indicate the number of years and replicates, respectively.

Pearson's correlation coefficients were calculated with *proc corr*, using lsmeans per genotype as input.

Nested association mapping

SNP genotyping was carried out using the barley Infinium iSelect 9K chip consisting of 7,864 SNPs (Comadran et al. 2012). SNPs showing >10% failure rate, >12.5 % heterozygous calls, or being monomorphic over all 1,403 HEB lines were removed from the dataset. SNP filtering resulted in 5,715 informative SNPs used for NAM with an average genetic distance of 0.17 cM and a maximum gap of 11.1 cM between adjacent markers. LD across HEB-25 was calculated as r^2 between all mapped SNPs, excluding heterozygous genotypes, with the software package TASSEL 5.0 (Bradbury et al. 2007). LD decay across intra-chromosomal SNPs was displayed by plotting r^2 between SNP pairs against their genetic distance. A second-degree smoothed loess curve was fitted in SAS with *proc loess*. The population-specific baseline r^2 was defined as the 95th percentile of the distribution of r^2 for unlinked markers (Breseghello and Sorrells 2006). LD decay was defined as the distance at which the loess curve crosses the baseline. An identity-by-state approach was used to differentiate HEB genotypes. Parental genotype information enabled the identification of the exotic donor allele in each segregating HEB family. HEB lines showing a homozygous Barke genotype were assigned a value of 0, HEB lines showing a homozygous exotic genotype were assigned a value of 2, and heterozygous HEB lines were assigned a value of 1. Failed SNP calls were assigned a value using the mean imputation (MNI) approach (Rutkoski et al. 2013). For detailed information see Maurer et al. (2015). Assignment of SNPs to chromosomal positions was based on Maurer et al. (2015).

NAM was performed using 'Model-B' of Liu et al. (2011a) verified to be best suited for GWAS based on family-structured populations (Würschum et al. 2012) and successfully applied in previous HEB-25 studies (Maurer et al. 2015; Maurer et al. 2016a; Saade et al. 2016). 'Model-B' is a multiple regression model including, next to a quantitative SNP effect and a qualitative family effect, quantitative cofactors that correct for population stratification and genetic background noise (Würschum et al. 2012). MTAs were estimated by stepwise forward-backward regression based on minimizing the Bayesian information criterion (BIC, Schwarz 1978) taking all informative SNPs into consideration. Analysis was carried out with SAS 9.4 applying the *proc glmselect* procedure. SNPs were allowed to enter or leave the model at each step until the BIC estimate was not reduced any further. SNPs included in the final model were defined to be significant.

To increase the robustness of identified MTAs, a five-fold cross-validation (CV) was performed. In total, 200 CV runs (40 times five-fold CV) were performed. For this, 200 subsets were extracted out of the full genotype set. Subsets included 80% of genotypes of the full population each, randomly selected per HEB family. The subsets were taken as training sets for the identification of significant MTAs and for estimation of additive effects. The remaining 20% of genotypes were used as the validation set. Subsequently, the count of each significant marker over all training sets was recorded and referred to as detection rate (DR). This value was taken as a measure of robustness of the MTAs. Markers with a DR of >50% were defined as particularly robust and used to assign resistance QTL.

Additive effects for each SNP were extracted as regression coefficient of the respective SNP directly from the NAM model described above. To obtain final estimates, additive effects of significant markers were averaged across all runs. Likewise final R^2 values for significant SNPs were obtained by averaging R^2 values of significant markers across all cross-validation runs. This way, the R^2 value can be interpreted as the percentage of variance explained by the investigated SNP marker. Furthermore, hotspots of MTAs were assigned to chromosome regions by determining the count and the mean additive effect of significant markers within 5 cM.

A standard QTL interval of ±4 cM around the markers with a DR >50% was defined, resembling the LD decay in HEB-25 (S2 File). In case the QTL was composed of more than one marker with a DR >50%, the marker showing the highest DR across all 200 cross-validation runs was defined as peak marker. QTL showing overlapping QTL intervals were combined to a single QTL interval.

To estimate the proportion of phenotypic variance explained by the full model, the unbiased estimator R^2_{adj} (Draper and Smith 1981) was calculated for each subset by simultaneously modeling all of the significant markers in the linear model described above.

To determine the predictive ability R^2_{pred} of the full model for infection severity, the additive effects of markers estimated using the training sets were used to predict the phenotypic value of the remaining 20% of genotypes forming the validation sets (Utz et al. 2000). Following Maurer et al. (2016a) R^2_{pred} was defined to be the squared Pearson product-moment correlation between predicted and observed

phenotypic values. Subsequently, R^2_{adj} and R^2_{pred} values were averaged over all 200 CV runs to obtain final estimates.

Additional to the detection of MTAs across families, parent-specific QTL effects were calculated following the approach of Maurer et al. (2016b). In a first step, the peak marker (SNP with highest DR >50% across all 200 cross-validation runs) of each QTL was selected and placed central in a 26 cM interval (resembling the mean introgression size in HEB-25) to look for significant SNPs in this region. Due to model limitations reported in Maurer et al. (2016b) population-wide QTL located within this interval were pooled into one single parent-specific QTL. Subsequently 'Model-B' SNP effect estimates of all markers within this interval were cumulated for each of the 25 donors, following $\sum_i^n SNP\ (donor)_i * \alpha_i$, where (i) iterates through all significant SNPs (n) in the respective QTL interval. $SNP\ (donor)_i$ represents the quantitative IBS donor genotype (i. e. 0 vs. 2) of the ith significant SNP and α_i denotes the SNP effect estimate of this SNP obtained from 'Model-B'. Since SNPs show different IBS segregation patterns across the donors of HEB families a different cumulated effect was obtained for each donor. This procedure was conducted within each of the 200 cross-validation runs. Subsequently, the mean effect across all cross-validation runs was calculated and taken as the final parent-specific QTL effect estimate.

Comparison with previously identified QTL and analysis of identified QTL intervals

GrainGenes (https://wheat.pw.usda.gov/GG3/) and BARLEX (http://apex.ipk-gatersleben.de/apex/f?p=284:10) databases were searched to obtain marker sequence information on previously reported QTL for net blotch resistance. If available, the marker sequence information was used to check for overlap of net blotch resistance QTL identified in this study with those 23 studies reported before and cited in the introduction. Only those QTL from previous studies were taken into consideration, which were placed in similar chromosomal regions as our QTL. The BARLEYMAP pipeline (Cantalapiedra et al. 2015) was used as a common reference. Using this pipeline, the peak marker as well as flanking markers for known net blotch resistance QTL and markers identified in this study showing a DR >50% were blasted against the POPSEQ map (Mascher et al. 2013) and the barley physical map (IBGS 2012). Markers with a DR >50% identified in this study and located in a genetic distance of less than 4 cM (resembling the LD decay in HEB-25, see S2 File)

to markers of known resistance QTL were defined as potentially corresponding to previously reported resistance QTL. In addition, previously reported QTL for which no marker information could be obtained were compared to QTL detected in this study based on information given in the respective publication.

In addition, the BARLEYMAP pipeline (Cantalapiedra et al. 2015) was used to identify potential candidate genes underlying the particularly robust QTL of this study by aligning the associated markers showing a DR >50% against the barley physical map (IBGS 2012) and the POPSEQ map (Mascher et al. 2013). The gene search was extended to an interval of ±4 cM around markers with a DR >50% to account for the LD decay in HEB-25. Gene ontology (GO) terms defining defense response (0006952, 0050832), apoptotic process (0006915), peroxidase activity (0004601), response to (oxidative) stress (0006979, 0006950), ATP binding (0005524), nucleotide binding (0000166), protein binding (0005515), transporter activity (0005215), and protein kinase activity (004672) were used to validate genes involved in resistance reactions (Serfling et al. 2016). Furthermore, GO terms defining reactions potentially involved, e.g. catalase activity, chitinase activity, cell wall, peroxisome, cell wall modification, and defense response to fungi, were considered (S8 File).

Results

Phenotypic data

In both years the use of the summer hill trial design resulted in an elevated disease pressure across the whole field with spreader strips showing an AO close to 60%. The experimental conditions allowed for an optimal differentiation of the degree of *P*. *teres* f. *teres* resistance between genotypes. A large diversity in *P*. *teres* f. *teres* resistance of genotypes was observed for both traits studied with a highly significant variation ($p < 0.0001$; Tukey-test) between as well as within families of the HEB-25 population (Fig 1A and B; S3 File).

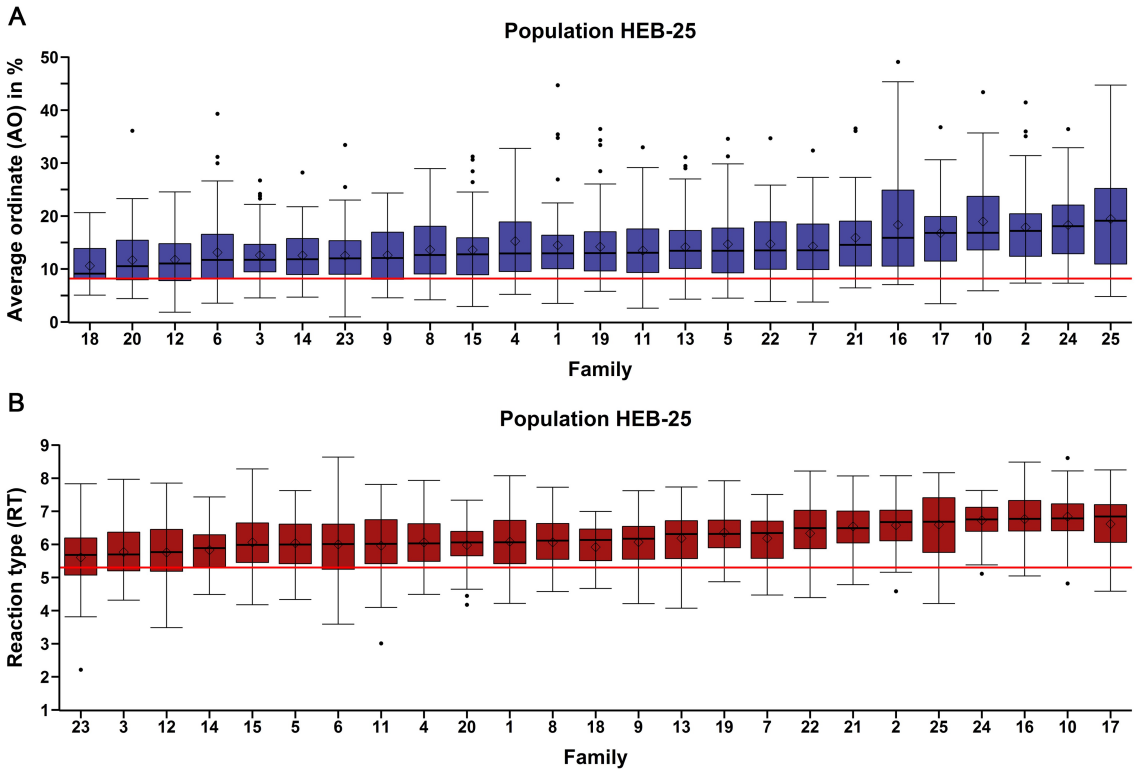

Fig 1. Box-whisker plots per HEB family indicating the variation in genotype responses to net blotch infection. **(A)** average ordinate (AO) and **(B)** reaction type (RT). The y-axis shows the data for each trait; the x-axis depicts the 25 families of HEB-25 (1-25) sorted by ascending median. The red line depicts the value of the resistant check for the respective trait.

A wide range of genotype responses to *P. teres* f. *teres* infection was observed in HEB-25 with a range of 48.2% for AO (0.95-49.1%) and 6.4 for RT (2.21-8.64), respectively (Table 1). Notably, several genotypes of the HEB-25 population showed a higher degree of resistance than the resistant check included in field trials (Fig 1A and B). The top 1% of all genotypes regarding *P. teres* f. *teres* resistance showed a mean AO value of 2.9% and a mean RT value of 3.8 (S3 File). The frequency distributions for both traits were slightly right skewed (S4 File). Because of the HEB-25 population design, the population means are close to the recurrent parent Barke (Table 1). Barke showed a high degree of susceptibility compared to the majority of wild donor parents. Only the wild donor of family 24 (*Hordeum vulgare* ssp. *agriocrithon*), originating from Tibet, China, showed a higher *P. teres* f. *teres* susceptibility than Barke (S5 File).

Table 1. Descriptive statistics for two-year least-squares means (lsmeans) and heritability.

Trait[a]	N[b]	Mean Barke[c]	Mean HEB-25[d]	Min[e]	Max[f]	$SE_{+/-}$[g]	CV[h]	h^2[i]
AO	1403	13.91	14.65	0.95	49.1	0.19	0.48	0.62
RT	1403	6.64	6.20	2.21	8.64	0.02	0.14	0.65

[a]Average ordinate (AO), reaction type (RT).
[b]Number of genotypes analyzed.
[c]Two-year lsmeans of common parent Barke.
[d]Two-year lsmeans of the HEB-25 population.
[e]Minimum.
[f]Maximum.
[g]Standard error.
[h]Coefficient of variation.
[i]Broad-sense heritability.

Two-year broad sense heritability was calculated to be h^2= 0.62 for AO and h^2= 0.65 for RT, respectively (Table 1). High correlations (Pearson's correlation coefficients; p <0.0001) were observed between the two resistance measures AO and RT with r= 0.86 and r= 0.76 for HEB-25 parents and for the HEB-25 population, respectively (S5 File).

Nested association mapping

NAM was performed for the two traits AO and RT, resulting in the identification of a high number of significant MTAs (Fig 2; S6 File). Most MTAs showed a DR below 50% across the 200 cross-validation runs. However, 11 and 13 particularly robust QTL being composed of one or more markers with a DR above 50% were identified for RT and AO, respectively (Table 2). Particularly robust MTAs were identified on all chromosomes except chromosome 1H in case of AO and on all chromosomes for RT. The QTL showing the peak marker with the highest DR (i_SCRI_RS_186193) is located in the centromeric region of chromosome 6H for both traits evaluated (Fig 2; Table 2). This QTL is composed of three SNPs with DR >50% in case of RT and two SNPs in case of AO. In both cases the peak marker showed a negative cross-validated mean effect, i.e. an increase of resistance in the presence of the wild allele compared to the Barke control allele. In general, this chromosome region showed the highest abundance of significant MTAs (Fig 2).

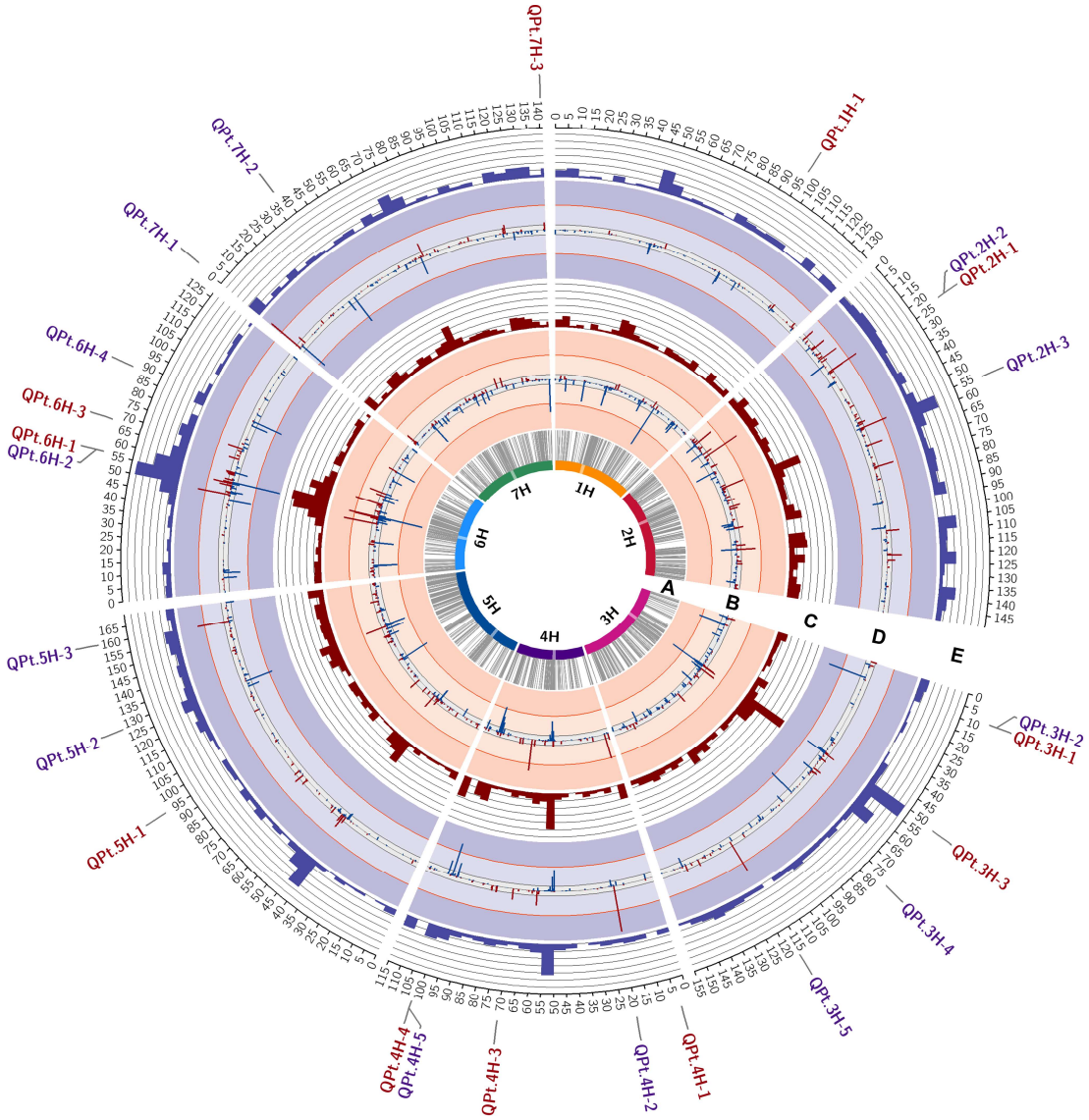

Fig 2. Circos plot indicating QTL involved in net blotch resistance, i.e. average ordinate (AO) and reaction type (RT). The barley chromosomes are arranged as colored bars forming the most inner circle. Centromere regions are highlighted as transparent boxes. **(A)** Grey connector lines represent the genetic position of the 5,715 informative SNPs on the chromosomes with cM positions based on Maurer et al. (2015) given on the scale on the outside of circle E. **(B)** Marker trait associations calculated for reaction type (RT). Bars identify the position and detection rate (DR, height of bars) of significant marker trait associations. Bars in blue, pointing inwards, indicate a population wide trait-decreasing effect exerted by the exotic allele, whereas bars in red, pointing outwards, indicate a population wide trait-increasing effect exerted by the exotic allele. The grey and orange lines depict the DR threshold of 10% and 50% across 200 cross-validation runs. **(C)** Count of significant marker trait associations within 5 cM intervals for the NAM study based on RT data. **(D)** Marker trait associations calculated for average ordinate (AO). Graphical representation as described under (A). **(E)** Count of significant marker trait associations within 5 cM intervals for the NAM study based on AO data. The position of particularly robust QTL with DR >50% are indicated on the scale outside of circle E. QTL detected based on RT are shown in red, whereas QTL detected based on AO are shown in purple.

Table 2. Robust net blotch resistance QTL (DR >50%) detected in the two NAM studies.

QTL	Chr[a]	Markers with DR >50%[b]	Position of peak marker (cM)[c]	DR in 200 CV runs (%)[d]	CV mean R^2 (%)[e]	CV mean allele effect[f]	Corresponding net blotch QTL/genes[g]
Reaction type (RT)							
QPt.1H-1	1H	i_11_10357	95.9	85.5	2.33	-0.28	
QPt.2H-1	2H	i_BK_15	23	78	9.23	+0.50	QRpts2Sa[1] QNFNBAPR.Ar/F-2H[1] QTL_Steffenson[1]
QPt.3H-1	3H	i_11_10112	8.5	54.5	0.87	-0.22	QTL_{UHs}-3H-1[3]
QPt.3H-3	3H	i_11_10966	51.6	73.5	8.64	-0.98	QTL_{UHs}-3H-2[3] QTL_Liu[5]
QPt.4H-1	4H	i_SCRI_RS_206744	3.5	51.5	1.04	+0.21	QRptts-4HS[7]
QPt.4H-3	4H	i_SCRI_RS_175327	70.3	63.5	0.85	+0.51	QRpts4[1] Rpt-4H-5–7[1]
QPt.4H-4	4H	i_SCRI_RS_167808	101.7	53.5	6.64	-0.52	QNFNBAPR.W/AI-4H[1] QNFNBAPR.AI/S-4Hb[1]
QPt.5H-1	5H	i_11_10834	94.7	58	1.63	-0.29	QTL_{UH}-5H-1[2] QRptts-5HL.2[7]
QPt.6H-1	6H	**i_SCRI_RS_186193** i_11_10013 i_SCRI_RS_239642	55.7	90 69 61.5	0.08	-0.68	Rpt5[1]* Rpt-r/-k[1]* Rpt-Nomini/-CIho2291[4]* Spt1[6]* SPN1[5]*
QPt.6H-3	6H	i_SCRI_RS_157316	67.6	56.5	1.37	+0.36	QTL_Liu[5]
QPt.7H-3	7H	i_SCRI_RS_123211	140.7	66	0.10	-0.21	
Average ordinate (AO)							
QPt.2H-2	2H	**i_BK_12** i_BK_13	23	68 51	14.88	+4.74	QRpts2Sa[1] QNFNBAPR.Ar/F-2H[1] QTL_Steffenson[1]
QPt.2H-3	2H	i_SCRI_RS_13639	55.55	58	0.07	+4.05	QTL_Cakir[1] QNFNBAPR.W/AI-2H[1] QRpts2Sb[1]
QPt.3H-2	3H	i_11_10112	8.5	78.5	0.78	-1.59	QTL_{UHs}-3H-1[3]
QPt.3H-4	3H	i_12_10583	77.4	55.5	0.04	-2.86	QRpts3La[1] QNFNBAPR.W/AI-3H[1] QNFNBAPR.AI/S-3H[1]
QPt.3H-5	3H	i_SCRI_RS_146197	117	67	0.12	+5.65	QRpts3L[1] QNFNBAPR.AI/S-3H[1] QNFNBAPR.W/AI-3H[1] QTL_Liu[5] QTL_{PHs}-3H[3]
QPt.4H-2	4H	i_12_30150	19.9	93.5	0.36	+1.92	
QPt.4H-5	4H	i_SCRI_RS_167808	101.7	68	6.50	-3.67	QNFNBAPR.W/AI-4H[1] QNFNBAPR.AI/S-4Hb[1]
QPt.5H-2	5H	i_SCRI_RS_228463	128.2	56	1.50	-2.51	QTL_{PH}-5H-3[2] QRptts5[1]
QPt.5H-3	5H	i_11_21138	159.8	64.5	0.41	+1.72	
QPt.6H-2	6H	**i_SCRI_RS_186193** i_11_10013	55.7	98 65.5	0.39	-5.99	Rpt5[1]* Rpt-r/-k[1]* Rpt-Nomini/-CIho2291[4]* Spt1[6]* SPN1[5]*
QPt.6H-4	6H	i_SCRI_RS_7640	87.9	61.5	0.04	-2.24	
QPt.7H-1	7H	**i_SCRI_RS_200895** i_SCRI_RS_156237	0.6	77 59.5	3.65	+9.64	QNFNBAPR.AI/S-7Ha[1] QTL_{PH}-7H[2]
QPt.7H-2	7H	i_SCRI_RS_179937	37.6	60	1.54	-1.97	

[a]Barley chromosome on which the QTL is located.
[b]SNP name of markers with a detection rate (DR) >50% associated with the QTL. In case the QTL is composed of several markers, the QTL peak marker is shown in bold letters.
[c]Position of the QTL peak marker showing highest DR based on Maurer et al. (2015).
[d]Detection rate of the QTL peak marker in 200 cross-validation runs in percent.
[e]Mean percentage of phenotypic variance explained by the QTL peak marker based on 200 cross-validation runs.
[f]Population-wide mean effect of the QTL peak marker based on 200 cross-validation runs. Positive and negative signs indicate a trait-increasing and trait-decreasing effect of the wild allele compared to the Barke control allele, respectively.
[g]Previously reported net blotch resistance QTL/genes located within the range of LD decay around the QTL marker with DR >50% identified in this study ([1](reviewed in Liu et al. 2011b), [2](König et al. 2013), [3](König et al. 2014), [4](O'Boyle et al. 2014), [5](Liu et al. 2015), [6](Richards et al. 2016), [7](Richards et al. 2017); *for QTL defining the same position see (Gupta et al. 2011; Liu et al. 2011b; Islamovic et al. 2017; Richards et al. 2017)).

A considerable variation in the wild allele effect estimates of adjacent markers was observed with an increase or decrease in trait values compared to the Barke control allele (Fig 2). Notably, the majority of detected QTL are composed of MTAs exhibiting opposed wild allele effect estimates (S6 File). For both traits QTL peak markers exhibited the same effect direction as the mean QTL estimate in all but one case, but differed in effect size (S6 File, 7). Thus, NAM showed that QTL and peak marker effect are not necessarily identical. Across the whole population particularly robust QTL peak markers showed predominantly small to intermediate wild allele effect estimates and low R^2 values. Wild allele effects ranged from -5.99 to 9.64 in case of AO and from -0.98 to 0.51 in case of RT. The peak markers of QPt.7H-1 and QPt.3H-3 showed the highest effect estimate for AO and RT, respectively (Table 2). R^2 values ranged from 0.04 to 14.88% explained variance in case of AO and from 0.08 to 9.23% in case of RT (Table 2). The peak markers of QPt.2H-2 and QPt.2H-1 showed the highest R^2 value for AO and RT, respectively (Table 2).

Parent-specific QTL effects were calculated to obtain an effect estimate resembling the combined effect of all family specific markers the QTL is composed of. Due to previously mentioned model limitations (see Material and methods) QTL QPt.6H-1 and QPt.6H-3 were combined to one single parent-specific QTL (QPt.6H-1/3). Estimation of parent-specific QTL effects revealed a high variation in effect sizes of the wild allele among HEB families (S6 File). In most cases even the effect direction varied. For each trait five QTL (AO: QPt.2H-2, QPt.3H-4, QPt.4H-5, QPt.6H-4, QPt.7H-2; RT: QPt.1H-1, QPt.2H-1, QPt.3H-1, QPt.4H-3, QPt.4H-4) showed the same wild allele effect direction across all families (S6 File). No family showed trait-reducing effects at all parent-specific QTL in case of both traits. The maximum count of parent-specific QTL showing a trait-reducing effect of the wild allele were nine for

AO (family F15) and seven for RT (family F23) (S6 File). For AO the three families F07 (-5.38%), F12 (-4.67%), and F15 (-3.82%), and for RT families F12 (-1.70), F07 (-1.34), and F23 (-1.25) showed the highest trait-reducing effects for wild type alleles summed up over all parent-specific QTL (S6 File).

Between the two NAM studies a considerable overlap was observed. Of the particularly robust 11 and 13 QTL peak markers identified for AO and RT, four peak markers mapped to the same or to a nearby position (Fig 2). In three cases, QTL even shared the same peak marker (Table 2). However, trait-specific QTL existed likewise. Hotspots of MTAs, defined by a high number of significant markers in the respective 5 cM interval, corresponded well with QTL peak marker positions in most cases. Similar to the observations in case of QTL peak markers, considerable overlap between trait hotspot regions of the two traits analyzed existed (Fig 2).

The mean percentage of phenotypic variance explained by the full model (R^2_{adj}) was calculated to be 68.9% for AO and 72.0% for RT (Table 3). Notably, for both traits a considerable fraction of the phenotypic variance was explained by the identified particularly robust QTL peak markers (Table 2). The predictive ability (R^2_{pred}) of the full model for infection severity was calculated to be 42.1% for AO and 43.3% for RT (Table 3).

Table 3. Number of QTL and total phenotypic variance explained.

Trait[a]	QTL[b]	R^2_{adj} (%)[c]	R^2_{pred} (%)[d]
AO	13	68.9	42.1
RT	11	72.0	43.3

[a]Average ordinate (AO), reaction type (RT).
[b]Number of QTL defined for the respective trait.
[c]Mean phenotypic variance explained by the full NAM model.
[d]Mean ability to predict infection severity of independent genotypes.

Comparison with previously identified QTL

Comparison of net blotch resistance QTL identified in this study with those already reported in literature revealed that the majority of identified QTL mapped to chromosome regions known to be linked to net blotch resistance. In case of RT nine out of 11 QTL showed overlap with QTL intervals of previously reported resistance QTL or genes, whereas this was true for nine out of 13 for AO (Table 2). In detail, based on available data no overlap was found for QPt.1H-1, QPt.4H-2, QPt.5H-3, QPt.6H-4, QPt.7H-2, and QPt.7H-3. Out of these QTL, peak markers of QPt.1H-1,

QPt.6H-4, QPt.7H-2, and QPt.7H-3 revealed negative CV mean effects (Table 2) indicating the existence of wild barley alleles conferring net blotch resistance. The alignment of SNPs with DR >50% against the physical barley map by means of the BARLEYMAP pipeline resulted in the identification of a number of genes related to plant defense in the respective QTL intervals. In particular, leucine-rich repeat, NB-ARC, and serine/threonine-protein kinase-like domain genes were found at high frequency. Details are given in S8 File.

In addition, QTL analysis revealed that peak markers of QPt.2H-1 and QPt.2H-2 are SNPs of the barley pseudo-response regulator gene *Ppd-H1*. Based on this finding, other QTL identified in our study were compared to flowering time QTL identified in an earlier HEB-25 study by Maurer et al. (2015). In addition to QPt.2H-1 and QPt.2H-2, overlap of QTL QPt.2H-3, QPt.5H-2, and QPt.7H-2 with flowering QTL QFt.HEB25-2c, QFt.HEB25-5d, and QFt.HEB25-7a of Maurer et al. (2015) was observed. Furthermore, QPt.2H-3, QPt.5H-2, and QPt.7H-2 each showed to include MLOC numbers present in the corresponding flowering time QTL identified in the study by Maurer et al. (2015) and identified to correspond to flowering time related genes *HvCEN*, *Vrn-H1*, and *Vrn-H3,* respectively.

Discussion

The high variation in *P*. *teres* f. *teres* infection severity observed in field trials clearly reflects the high genetic diversity present within the HEB-25 population, which is in line with findings of previous HEB-25 NAM studies focusing on developmental traits (Maurer et al. 2015; Maurer et al. 2016a) and salinity tolerance (Saade et al. 2016). The presence of significant differences not only between families, but also within families demonstrates the high suitability of HEB-25 to identify population-wide as well as parent-specific QTL for *P*. *teres* f. *teres* resistance (Fig 1A and B; S3 File).

Phenotypic results of this study show that the high variation to net blotch resistance can be attributed to combined effects of a diverse set of predominantly highly resistant wild donor parents of HEB-25 and a relatively susceptible recurrent parent Barke. HEB-25 lines identified to possess a higher degree of resistance than the highly resistant check line included in field trials represent suitable candidates for pre-breeding programs (S3 File). Results of earlier studies by Maurer et al. (2015;

2016a) may be considered to select those net blotch resistance conferring HEB lines that combine high *P*. *teres* f. *teres* resistance with favorable yield related parameters. Advantageous is that integration of HEB-25 lines into pre-breeding programs will be faster to achieve than in case of the integration of wild accessions since a backcrossing step with cultivar Barke was already performed during population development.

The summer-hill trial design developed by König et al. (2013) proved to be highly effective, allowing for a clear differentiation of genotype responses, thereby laying the basis for successful QTL identification with NAM. The high correlation between the two infection severity measures applied in this study and the relatively high heritabilities found prove that both measures allow a reliable scoring of genotypic resistance (Table 1; S5 File).

The occurrence of opposed wild allele effect estimates of closely linked markers in this study was also observed in previous HEB-25 studies by Maurer et al. (Maurer et al. 2015; Maurer et al. 2016a; Maurer et al. 2016b) and likely arises from a combination of factors. Firstly, not all SNPs segregate in all genotypes and therefore, markers are likely to reflect only the mean wild allele effect of a fraction of the full population. As a result, closely linked markers segregating in different sets of genotypes of the full population can show opposed effect estimates because of different mean resistance levels of the two sets. Phenotypic results revealed that families differ in their mean resistance level (Fig 1A and B). Therefore, it can be assumed that strongly differing sets are likely to be linked to different families and, thus, opposed effect estimates of closely linked SNPs can be caused by parent-specific alleles. Secondly, the presence of closely linked SNPs showing opposed wild allele effects can be caused by closely linked alleles with contrasting effects on *P*. *teres* f. *teres* resistance. A good example is the centromeric region of chromosome 6H that is known to harbor a number of closely linked *P*. *teres* f. *teres* resistance genes of which some are assumed to be in repulsion (Gupta et al. 2011; Liu et al. 2011b; O'Boyle et al. 2014; Liu et al. 2015; Richards et al. 2016). Therefore, we assume that the high number of closely linked markers with opposed wild allele effect estimates in the centromeric region of chromosome 6H identified in this study is likely to be partially caused by this complex cluster of resistance related genes.

In this study a rather stringent threshold for the acceptance of MTAs was defined. Therefore, minor QTL not passing this threshold but still influencing genotype

response to *P*. *teres* f. *teres* are not considered. Defining a less stringent DR threshold of 10%, as applied in the study of Maurer et al. (2016a), would have resulted in a considerable higher number of QTL (Fig 2; S6 File). Nevertheless, hotspots of MTAs identified in this study may be used to narrow down regions potentially harboring minor QTL involved in the resistance response of genotypes to *P*. *teres* f. *teres.* However, when analyzing the hotspot information it has to be taken into account that in centromeric regions the number of markers is generally high and, therefore, centromeric regions should be interpreted with caution (Fig 2). The detection of QTL despite low estimates across the whole population is a strong proof of the power of the NAM strategy in general, and in particular the suitability and precision of the NAM model applied in this study (Fig 2; S6 File). The mean phenotypic variance explained by the full model and the calculated mean ability to predict the degree of infection of independent genotypes further supports the suitability of the applied model (Table 3).

The high number of QTL linked to net blotch resistance detected in this study, the small CV mean effect estimates as well as the low percentage of phenotypic variance explained by the majority of QTL peak markers indicate a complex inheritance of adult plant *P*. *teres* f. *teres* resistance (Table 2). This supports the conclusion drawn by Liu et al. (2011b) of a highly complex *P*. *teres* f. *teres*–barley interaction. Results of this study are comparable to previous NAM studies focusing on leaf blight in maize (Kump et al. 2011; Poland et al. 2011) that identified variation in resistance to be a result of the accumulation of numerous small effect loci with additive effects. Likewise, NAM studies focusing on rust fungi of wheat (Bajgain et al. 2016; Li et al. 2016) resulted in the identification of a high number of QTL with predominantly small additive effect estimates. In addition, results of this study are comparable to the association study of Tamang et al. (2015) focusing on resistance to the spot form of net blotch (*P. teres* f. *maculata*) and the association study of Richards et al. (2017) focusing on seedling resistance to *P*. *teres* f. *teres*. The authors identified a high number of markers associated with resistance to *P. teres* f. *maculata* and *P*. *teres* f. *teres*, respectively, nearly all explaining only a low percentage of phenotypic variance.

Next to being the result of complex inheritance of *P*. *teres* f. *teres* resistance, small population-wide effects of QTL peak markers may also be attributed to the presence of alleles with differing effects on *P*. *teres* f. *teres* resistance. Namely, in case only a

limited number of HEB-25 lines of the full population show a strong allele effect on resistance or contrasting allele effects among the 25 HEB donor parents exist at a marker position.

The importance of considering the influence of differing allele effects in HEB-25 on estimating a population-wide QTL peak marker effect is supported by results of the parent-specific QTL effect calculation (S6 File). An extreme example is the QTL QPt.7H-1. In this case, the high population-wide effect of the wild allele observed for the peak marker (wild barley allele effect on AO = +9.64) seems to be mainly caused by the strong effect of an allele or allele combination derived from the donor parent of HEB family F16 (wild barley parent-specific allele effect on AO in family F16 = +9.16). Comparable to this study, strongly varying parent-specific allele effects of QTL were observed likewise in the NAM studies of Bajgain et al. (2016) and Li et al. (2016) focusing on the identification of QTL conferring resistance to rust pathogens of wheat. Therefore, especially studies focusing on detailed analysis of specific QTL or the integration of net blotch resistance alleles in modern barley cultivars should use the parent-specific QTL effect information given in this study to select a resistance-carrying HEB line derived from the HEB family in which the estimated favorable QTL effect is maximized. Not including parent-specific QTL effect estimates in the selection decision may result in missing alleles whose strong favorable effect is masked by a high number of parent-specific alleles with an opposed effect on *P*. *teres* f. *teres* resistance (S6 File). However, in this regard it needs to be mentioned that parent-specific QTL effect estimates are likely to be slightly overestimated as each family comprises only a relatively small number of HEB-25 lines (Maurer et al. 2016b). Thus, selection decisions should be based on a combined evaluation of population-wide and parent-specific estimates of the wild allele effect.

Several QTL identified in this study are located at chromosome positions not yet reported to be linked to *P*. *teres* f. *teres* resistance (Table 2). At the same time, QTL were identified that overlap with previously described *P*. *teres* f. *teres* resistance QTL. This fact is a strong proof of the reliability of the identified MTAs. NAM results are further supported by the fact that several QTL regions were independently identified by AO and RT (Fig 2; Table 2).

Out of the QTL that show no overlap some are located in the vicinity of previously reported *P*. *teres* f. *teres* resistance QTL. This is the case for QPt.1H-1 located in the vicinity of a QTL identified by Liu et al. (2015), QPt.7H-2 located close to QTL QTL_{UHs}-

7H identified by König et al. (2014), and QPt.7H-3 located in the vicinity of QTL QNFNBAPR.Al/S-7Hb identified by Lehmensiek et al. (2007). Furthermore, QPt.5H-3 is located in the region of a meta-QTL identified by Schweizer and Stein (2011) effective against several fungal barley pathogens.

It has to be considered that previously reported *P. teres* f. *teres* resistance QTL were identified by the use of different isolates under different environmental conditions and mostly in seedling tests. Only QPt.5H-1, QPt.5H-2, and QPt.7H-1 showing overlap with QTL identified by König et al. (2013) were identified under similar experimental conditions. Therefore, QTL identified in this study showing overlap with previously reported *P. teres* f. *teres* QTL should still be considered as distinct QTL until a test for allelism has been conducted.

Most of the identified particularly robust net blotch resistance QTL showed to be restricted to either AO or RT. These trait-specific QTL showed to be caused partly by the fact that for one trait the markers did not cross the defined DR threshold and thus, were not considered in this study, whereas for the other trait the markers crossed the threshold and were considered (Fig 2). In this case, for both traits DR peaks of markers were observed at the same or very close by positions and a less stringent threshold for the acceptance of MTAs (e. g. >10%, used by Maurer et al. (2016a)) would have resulted in the detection of the QTL based on both traits (Fig 2; S6 File). Furthermore, trait-specific QTL may be caused by the fact that the infection severity measure RT is less influenced by the degree of infection pressure, as a RT score indicative for susceptibility can be observed at a time point at which the fungus covers only a small percentage of the leaf (low AO value). Delaying the last phenotyping date, thus giving the fungus more time to spread across the leaf could have resulted in the detection of QTL regions based on both traits. Next to this study no other studies have been performed comparing AO and RT on the QTL level. Further research is needed to identify the underlying cause of these trait specific QTL.

The information given in this paper regarding genes located in a QTL region may assist in identifying the underlying genetic causes of a QTL effect (S8 File). The presence of leucine-rich repeat, NB-ARC, and serine/threonine-protein kinase-like genes in the QTL intervals at high frequency is in agreement with findings of previous studies indicating an important role of those gene families in the necrotrophic effector triggered reaction to *P. teres* f. *teres* infection (Liu et al. 2015;

Richards et al. 2016). Members of these gene families were also identified in other QTL studies focusing on necrotrophic and hemibiotrophic fungi (Faris et al. 2010; Kump et al. 2011; Poland et al. 2011). The identification of various putative candidate genes by GO-term analysis may be viewed as a valuable source for subsequent studies focusing on the genetic basis of the *P*. *teres* f. *teres*–barley interaction (S8 File).

The overlap of QTL identified in this study with QTL identified to be linked to flowering time related genes (Maurer et al. 2015; Maurer et al. 2016a) points towards the involvement of the flowering time pathway in the resistance reaction to *P*. *teres* f. *teres*. Studies on *Arabidopsis thaliana* (Veronese et al. 2003; Häffner et al. 2010) showed that QTL associated with resistance to the hemibiotrophic fungal pathogen *Verticillium* spp. mapped close to known flowering time genes and that the fungus influenced plant development. Association of flowering time with resistance to a necrotrophic fungus has also been described in a study by Lyons et al. (2015). In this study a positive correlation between late flowering and resistance to *Fusarium oxysporum* in *A. thaliana* accessions was identified and the involvement of the photoperiodic pathway regulator GIGANTEA was shown. Furthermore, a negative correlation between days to anthesis and resistance to the hemibiotrophic maize pathogen *Exserohilum turcicum* has been identified (Balint-Kurti et al. 2010; Poland et al. 2011).

Detailed analysis of the identified overlap of QTL of this study with flowering time related QTL identified by Maurer et al. (2015; 2016a) strongly points towards a negative correlation between flowering time and infection severity. Maurer et al. (2015; 2016a) identified the wild alleles of *Ppd-H1* and *HvCEN* to cause early flowering and, in contrast, the wild alleles of *Vrn-H1* and *Vrn-H3* to induce late flowering. In this study a resistance-decreasing effect of the wild allele was identified for peak markers of QTL overlapping with the *Ppd-H1* and *HvCEN* QTL, and a resistance-increasing effect for peak markers of QTL overlapping with the *Vrn-H1* and *Vrn-H3* QTL. Nevertheless, based on our study only a comparison of QTL localization and QTL effects was possible. Further studies including phenotypic data and trials conducted during the standard growing period are required for final assessment.

Conclusion

The results of this study provide valuable information not only for fundamental studies focusing on elucidating the complex *P*. *teres* f. *teres*–barley interaction, but also for improving net blotch resistance and biodiversity of modern elite barley cultivars. In future, a better understanding of the allelic diversity present at net blotch resistance QTL in HEB-25 will be achieved, after an ongoing exome capture effort will result in detailed information on sequence diversity between 26 parental alleles at each known gene of a QTL region. This way, it is expected to achieve a clearer estimate of haplotype-based allele effects in HEB-25 and to foster the identification and selection of wild barley alleles, which increase net blotch resistance in barley.

Abstract

The biotrophic rust fungi *Puccinia hordei* and *Puccinia striiformis* f. sp. *hordei* are important barley pathogens with the potential to cause high yield losses through an epidemic spread. The identification of QTL conferring resistance to these pathogens is the basis for targeted breeding approaches aiming to improve stripe rust and leaf rust resistance of modern cultivars. Exploiting the allelic richness of wild barley accessions proved to be a valuable tool to broaden the genetic base of resistance of barley cultivars. In this study, SNP-based nested association mapping (NAM) was performed to map stripe rust and leaf rust resistance QTL in the barley NAM population HEB-25, comprising 1,420 lines derived from BC_1S_3 generation. By scoring the percentage of infected leaf area, followed by calculation of the area under the disease progress curve and the average ordinate during a two-year field trial, a large variability of resistance across and within HEB-25 families was observed. NAM based on 5,715 informative SNPs resulted in the identification of twelve and eleven robust QTL for resistance against stripe rust and leaf rust, respectively. Out of these, eight QTL for stripe rust and two QTL for leaf rust are considered novel showing no overlap with previously reported resistance QTL. Overall, resistance to both pathogens in HEB-25 is most likely due to the accumulation of numerous small effect loci. In addition, the NAM results indicate that the 25 wild donor QTL alleles present in HEB-25 strongly differ in regard to their individual effect on rust resistance. In future, the NAM concept will allow to select and combine individual wild barley alleles from different HEB parents to increase rust resistance in barley. The HEB-25 results will support to unravel the genetic basis of rust resistance in barley, and to improve resistance against stripe rust and leaf rust of modern barley cultivars.

Introduction

The biotrophic rust fungi *Puccinia hordei (Ph)*, causing leaf rust, and *Puccinia striiformis* f. sp. *hordei (Psh)*, the causal agent of stripe rust, are important barley pathogens in many barley growing areas worldwide (Chen et al. 1995; Mathre 1997). The ability of the two rust fungi to spread across large distances, rapidly increase in population size, and mutate quickly (Stubbs 1985; Chen 2007; Park et al. 2015) results in a high risk for severe epidemics. Infection can cause yield losses of up to 62% in case of *Ph* and up to 70% in case of *Psh* and reduce grain quality under epidemic conditions (Dubin and Stubbs 1986; Cotterill et al. 1992; Das et al. 2007). Depending on environmental conditions, there is a high variability in disease severity between years. While *Psh* in general has been of minor economic importance over the last several decades the importance of *Ph* has increased (Clifford 1985; Münnich et al. 2000; Chen 2007; Park et al. 2015; Esvelt Klos et al. 2016). Nevertheless, *Psh* remains a major economic threat, especially for barley production in Australia where it is not yet present, as studies in Mexico identified that 70% of Australian barley varieties are susceptible to the aggressive *Psh* race 24 (Wellings et al. 2000; Wellings 2007).

Although both fungi can be controlled by timely fungicide application, emphasis should be laid on resistance breeding as a cost effective, environmental, and consumer-friendly alternative (Chen 2007; Golegaonkar et al. 2009; González González et al. 2013). Most promising in this regard is to breed for cultivars exhibiting both race-specific and non-race specific resistances (Rubiales and Niks 2000).

Up to now 25 major genes (*Rph1-Rph24*) and *Rph*$_{\text{MBR1012}}$ conferring resistance to *Ph* have been reported of which all but one were assigned to chromosome regions (König et al. 2012; Park et al. 2015; Ziems et al. 2017). Out of these, *Rph15*, *Rph16*, and *Rph*$_{\text{MBR1012}}$ (Niks et al. 2000; Perovic et al. 2004; König et al. 2012) are still effective in Europe, whereas in Israel, Morocco, Spain, and the USA *Rph7* has already been overcome by new *Ph* races (Golan et al. 1978; Parlevliet 1981; Steffenson et al. 1993; Shtaya et al. 2006). Given the ability of the fungus to spread across large distances and to mutate quickly, it is only a matter of time until still effective *Rph* genes will be overcome as well. The identification of race non-specific quantitative

resistance and its introgression into modern barley cultivars is therefore of highest importance. Numerous studies focusing on resistance of barley to *Ph* resulted in the identification of a high number of QTL located on all barley chromosomes (Qi et al. 1998; Qi et al. 1999; Kicherer et al. 2000; Qi et al. 2000; Backes et al. 2003; Kopahnke et al. 2004; von Korff et al. 2005; Kraakman et al. 2006; Rossi et al. 2006; Marcel et al. 2007; Hickey et al. 2011; Castro et al. 2012; Schnaithmann et al. 2014; Ziems et al. 2014; Singh et al. 2016).

Over the last several decades less research has been conducted on resistance of barley to *Psh* due to its significantly lower importance compared to *Ph*. However, 26 uniquely different *Rps* (*Resistance to Puccinia striiformis*) major genes (reviewed in Chen and Line 2001) and several QTL (Chen et al. 1994; Thomas et al. 1995; Hayes et al. 1996; Toojinda et al. 1998; Toojinda et al. 2000; Castro et al. 2002a; Castro et al. 2002b; Cakir et al. 2003b; Cakir et al. 2003c; Castro et al. 2003; Vales et al. 2005; Rossi et al. 2006; Yan and Chen 2007; Dracatos et al. 2016; Esvelt Klos et al. 2016) have been reported up to now.

In almost all studies focusing on the identification of QTL and genes conferring resistance to *Ph* or *Psh* bi-parental LM was applied. AM to detect resistance QTL was, to our best knowledge, applied in only one study for resistance to *Psh* (Dracatos et al. 2016) and in two studies for resistance to *Ph* (Hickey et al. 2011; Ziems et al. 2014). Furthermore, Schnaithmann et al. (2014) applied a NAM approach based on an explorative multi-parental NAM population to detect QTL conferring seedling resistance to *Ph*. A large-scale NAM study based on field trials to identify resistance QTL has not been conducted yet for either of the two fungi.

NAM is based on a multi-parental mapping design introduced by Yu et al. (2008) as a genome-wide association strategy to dissect the genetics of complex traits. NAM combines the advantages of conventional LM and AM strategies, namely the high detection power per SNP and the high allelic richness, allowing for an exceptional high mapping resolution (Yu et al. 2008; McMullen et al. 2009; Lu et al. 2010). Next to several studies based on the initial maize NAM population (Yu et al. 2008; Buckler et al. 2009; Lu et al. 2010; Brown et al. 2011; Kump et al. 2011; Poland et al. 2011; Tian et al. 2011; Cook et al. 2012; Peiffer et al. 2013; Peiffer et al. 2014), NAM studies were conducted in a second maize NAM population (Li et al. 2013), sorghum (Jordan et al. 2011), wheat (Bajgain et al. 2016; Li et al. 2016), and barley (Schnaithmann et al. 2014; Maurer et al. 2015; Maurer et al. 2016a; Maurer et al. 2016b; Nice et al.

2016; Saade et al. 2016; Nice et al. 2017; Vatter et al. 2017; Herzig et al. 2018) highlighting the power of this mapping approach.

Until now, the world's first barley NAM population introduced by Maurer et al. (2015) named HEB-25 has not been used to identify QTL linked to resistance to biotrophic fungi. Thus, in this study the genetic diversity present in HEB-25 combined with the exceptional high mapping resolution offered by NAM was used to achieve the following objectives: I) to screen the HEB-25 population for resistance against *Ph* and *Psh*; II) to identify QTL conferring resistance against *Ph* and *Psh*; III) to identify HEB-25 lines with strong resistance, suitable to be introduced in pre-breeding programs; IV) to compare QTL positions detected in this study with those previously reported in literature, and V) to identify putative candidate genes underlying the identified resistance QTL.

Material and methods

Plant material

This study is based on the HEB-25 NAM population (Maurer et al. 2015). HEB-25 comprises 1,420 BC_1S_3 lines in 25 families, originating from crossing 25 highly divergent wild barley accessions (*Hordeum vulgare* ssp. *spontaneum* and *H. agriocrithon*) with the modern spring barley cultivar Barke (*Hordeum vulgare* ssp. *vulgare*). For more detailed information on population development see Maurer et al. (2015). Due to a loss of genotypes during field trials, the analysis is based on 1,401 genotypes of the HEB-25 population.

Field trials

Field trials were conducted at the Julius Kuehn-Institute, Federal Research Centre for Cultivated Plants, in Quedlinburg, Germany, during the seasons 2014 and 2015 using a randomized incomplete block design with two replications. Screening for resistance to *Ph* and *Psh* was performed in separate field trials. Genotypes were sown in double rows of 1 m length with 25 plants per row and spacing of 0.2 m between rows in mid-March in both years. Incomplete blocks were surrounded by spreader strips of susceptible varieties. Spreader strips were spray inoculated with an oil-spore mixture using a hand-held spinning disc sprayer (ULVA+, Micron Sprayers, Bromyard, Herefordshire, U.K.) to ensure homogeneous disease pressure.

A 1:1 mixing ratio of rust spores in mg to oil in ml (Isopar M, ExxonMobil Chemical Company, Spring, TX, USA) and 100 ml of suspension per 30 m^2 was used for inoculation. Starting at shooting, spray inoculation was performed at three dates early in the morning when dew formation was observed. For leaf rust (*Ph*) trials isolate I-80 was used, a very destructive leaf rust isolate overcoming common major resistance genes in the European barley gene pool, except *Rph7*, *Rph15*, *Rph16*, and $Rph_{MBR1012}$ (Niks et al. 2000; Perovic et al. 2004; König et al. 2012). The virulence of I-80 against *Rph17–Rph24* has not been surveyed yet. For stripe rust (*Psh*) trials, the very aggressive race R-24 was used, which is wildly spread in Europe and the Americas (Dubin and Stubbs 1986; Chen et al. 1994; Chen 2007).

Phenotypic data

Percentage of infected leaf area (PILA) was recorded at three subsequent dates according to Moll et al. (2010), starting when disease symptoms were clearly visible in the susceptible spreader strips. A time period of two weeks between phenotyping dates was chosen to allow for sufficient disease development. Based on PILA data the area under the disease progress curve (AUDPC) was calculated for each genotype. AUDPC data were then used to calculate the average ordinate (AO, Moll et al. 1996) for each genotype as a measure of infection severity:

$$AO = \frac{\sum_{i=1}^{N_{i-1}} \frac{(y_i + y_{i+1})}{2} * (t_{i+1} - t_i)}{tp}$$

where (N) is the total number of observations, disease level at the ith observation is coded by (y_i), time at the ith observation is coded by (t_i), and the total trial period in days is coded by (tp).

Statistical analysis

Phenotypic data analysis was performed using the software package SAS 9.4 (SAS Institute Inc., Cary, NC, USA) using *proc mixed*. Genotype, year, and genotype *x* year interaction were set as fixed effects. Design effects were set as random statement. Separate co-variances were set for years to account for the difference in disease pressure between years. To meet the requirements of mixed linear model analysis, phenotypic raw data was $\log_{10}$ transformed before applying the mixed procedure.

Obtained AO $\log_{10}$ least squares means (lsmeans) were used for subsequent nested association mapping (NAM). To estimate variance components to be used for the calculation of broad sense heritability (h^2) all model parameters were set as random. Broad sense heritability across years was calculated as:

$$h^2 = \frac{V_G}{V_G + \frac{V_{GY}}{y} + \frac{V_R}{yr}}$$

where genotypic variance is coded by (V_G), genotype *x* year variance is coded by (V_{GY}), and residual variance is coded by (V_R). The terms *y* and *r* represent the number of years and replicates, respectively.

Pearson's correlation coefficients were calculated with *proc corr*, using lsmeans per genotype as input.

Nested association mapping

SNP genotyping was carried out using the barley Infinium iSelect 9K chip consisting of 7,864 SNPs (Comadran et al. 2012). SNPs showing >10% failure rate, >12.5% heterozygous calls, or being monomorphic over all 1,401 HEB lines were removed from the dataset. SNP filtering resulted in 5,715 informative SNPs used for NAM with an average genetic distance of 0.17 cM and a maximum gap of 11.1 cM between adjacent markers. LD across HEB-25 was calculated as r^2 between all mapped SNPs, excluding heterozygous genotypes, with the software package TASSEL 5.0 (Bradbury et al. 2007). LD decay across intra-chromosomal SNPs was displayed by plotting r^2 between SNP pairs against their genetic distance. A second-degree smoothed loess curve was fitted in SAS with *proc loess*. The population-specific baseline r^2 was defined as the 95th percentile of the distribution of r^2 for unlinked markers (Breseghello and Sorrells 2006). LD decay was defined as the distance at which the loess curve crosses the baseline. An identity-by-state approach was used to differentiate HEB genotypes. Parental genotype information enabled the identification of the exotic donor allele in each segregating HEB family. HEB lines showing a homozygous Barke genotype were assigned a value of 0, HEB lines showing a homozygous exotic genotype were assigned a value of 2, and heterozygous HEB lines were assigned a value of 1. Failed SNP calls were assigned a value using the mean imputation (MNI) approach (Rutkoski et al. 2013). For detailed

information see Maurer et al. (2015). Assignment of SNPs to chromosomal positions was based on Comadran et al. (2012) and Maurer et al. (Maurer et al. 2015).

NAM was performed using 'Model-B' of Liu et al. (2011a) verified to be best suited for GWAS based on family-structured populations (Würschum et al. 2012) and successfully applied in previous HEB-25 studies (Maurer et al. 2015; Maurer et al. 2016a; Saade et al. 2016). 'Model-B' is a multiple regression model including, next to a quantitative SNP effect and a qualitative family effect, quantitative cofactors that correct for population stratification and genetic background noise (Würschum et al. 2012). MTAs were estimated by stepwise forward-backward regression based on minimizing the Bayesian information criterion (BIC, Schwarz 1978) taking into consideration all informative SNPs. Analysis was carried out with SAS 9.4 applying the *proc glmselect* procedure. SNPs were allowed to enter or leave the model at each step until the BIC estimate was not reduced any further. SNPs included in the final model were defined to be significant.

To increase the robustness of identified MTAs, a five-fold cross-validation (CV) was performed. In total, 200 CV runs (40 times five-fold CV) were performed. For this, 200 subsets were extracted out of the full genotype set. Each subset included 80% of genotypes of the full population, randomly selected per HEB family. The subsets were taken as training sets for the identification of significant MTAs and for estimation of additive effects. The remaining 20% of genotypes were used as the validation set. Subsequently, the count of each significant marker over all training sets was recorded and referred to as detection rate (DR). This value was taken as a measure of robustness of the MTAs. Markers with a DR of >50% were defined as robust and used to assign resistance QTL.

Additive effects for each SNP were extracted as regression coefficient of the respective SNP directly from the NAM model described above. To obtain final estimates, additive effects of significant markers were averaged across all runs. Likewise, final R^2 values for significant SNPs were obtained by averaging R^2 values of significant markers across all cross-validation runs. This way, the R^2 value can be interpreted as the percentage of variance explained by the investigated SNP marker. A standard QTL interval of ±4 cM around the markers with a DR >50% was defined, representing the LD decay in HEB-25 (S1 File). In case the QTL was composed of more than one marker with a DR >50%, the marker showing the highest DR across

all 200 cross-validation runs was defined as peak marker. QTL showing overlapping QTL intervals were combined to a single QTL interval.

To estimate the proportion of phenotypic variance explained by the full model, the unbiased estimator R^2_{adj} (Draper and Smith 1981) was calculated for each subset by simultaneously modeling all of the significant markers in the linear model described above.

To determine the predictive ability R^2_{pred} of the full model for infection severity, the additive effects of markers estimated using the training sets were used to predict the phenotypic value of the remaining 20% of genotypes forming the validation sets (Utz et al. 2000). Following Maurer et al. (2016a) R^2_{pred} was defined to be the squared Pearson product-moment correlation between predicted and observed phenotypic values. Subsequently, R^2_{adj} and R^2_{pred} values were averaged over all 200 CV runs to obtain final estimates.

Additional to the detection of MTAs across families, parent-specific QTL effects were calculated following the approach of Maurer et al. (2016b). In a first step, the peak marker (SNP with highest DR >50% across all 200 cross-validation runs) of each QTL was selected and placed central in a 26 cM interval (obtained through simulation studies and representing the mean introgression size in HEB-25) to look for significant SNPs in this region. Due to model limitations reported in Maurer et al. (2016b) population-wide QTL located within this interval were pooled into one single parent-specific QTL. Subsequently, 'Model-B' SNP effect estimates of all markers within this interval were cumulated for each of the 25 donors, following $\sum_i^n SNP\ (donor)_i * \alpha_i$, where ($i$) iterates through all significant SNPs (n) in the respective QTL interval. $SNP\ (donor)_i$ represents the quantitative IBS donor genotype (i. e. 0 vs. 2) of the ith significant SNP and α_i denotes the SNP effect estimate of this SNP obtained from 'Model-B'. Since SNPs show different IBS segregation patterns across the donors of HEB families a different cumulated effect was obtained for each donor. This procedure was conducted within each of the 200 cross-validation runs. Subsequently, the mean effect across all cross-validation runs was calculated and taken as the final parent-specific QTL effect estimate.

Comparison with previously identified QTL and analysis of identified QTL intervals

GrainGenes (https://wheat.pw.usda.gov/GG3/) and IPK Gatersleben (http://www.ipk-gatersleben.de/datenbanken/) databases were used to obtain

marker information of previously reported QTL for *Ph* and *Psh* resistance. If available, this information was used to check for overlap of resistance QTL identified in this study with those already reported. The BARLEYMAP pipeline (Cantalapiedra et al. 2015) was used as a common reference. Using this pipeline, the peak marker as well as flanking markers for known *Ph* and *Psh* resistance QTL and markers identified in this study showing a DR >50% were blasted against the POPSEQ map (Mascher et al. 2013) and the barley physical map (IBGS 2012). Markers with a DR >50% identified in this study and located in a genetic distance of less than 4 cM (representing the LD decay in HEB-25, see S1 File) to markers of known resistance QTL were defined as potentially corresponding to previously reported resistance QTL. In addition, previously reported QTL, for which no marker information could be obtained, were compared to QTL detected in this study based on information given in the respective publication.

In addition, the BARLEYMAP pipeline (Cantalapiedra et al. 2015) was used to identify potential candidate genes underlying the robust QTL of this study by aligning the associated markers showing a DR >50% against the barley physical map (IBGS 2012) and the POPSEQ map (Mascher et al. 2013). The gene search was extended to an interval of ±4 cM around markers with a DR >50% to account for the LD decay in HEB-25. Gene ontology (GO) terms defining defense response (0006952, 0050832), apoptotic process (0006915), peroxidase activity (0004601), response to (oxidative) stress (0006979, 0006950), ATP binding (0005524), nucleotide binding (0000166), protein binding (0005515), transporter activity (0005215), and protein kinase activity (004672) were used to validate genes involved in resistance reactions (Serfling et al. 2016). Furthermore, GO terms defining reactions potentially involved, e.g. catalase activity, chitinase activity, cell wall peroxisome, cell wall modification, and defense response to fungi, were considered too (S2 File).

Results

Phenotypic analysis

Artificial infection resulted in a moderate disease pressure in both years, despite dry weather conditions impeding the initial infection process in the beginning of field trials. Nevertheless, experimental conditions allowed for an unequivocal scoring of

resistance to *Psh* and *Ph*. A large variation concerning resistance was detected in the HEB-25 population for both pathogens. Significant differences ($p < 0.0001$; Tukey-test) were observed between as well as within families in both cases (Fig 1 A, B; S3 File). HEB families 1, 3, and 25 showed the highest resistance to both pathogens based on the AO median.

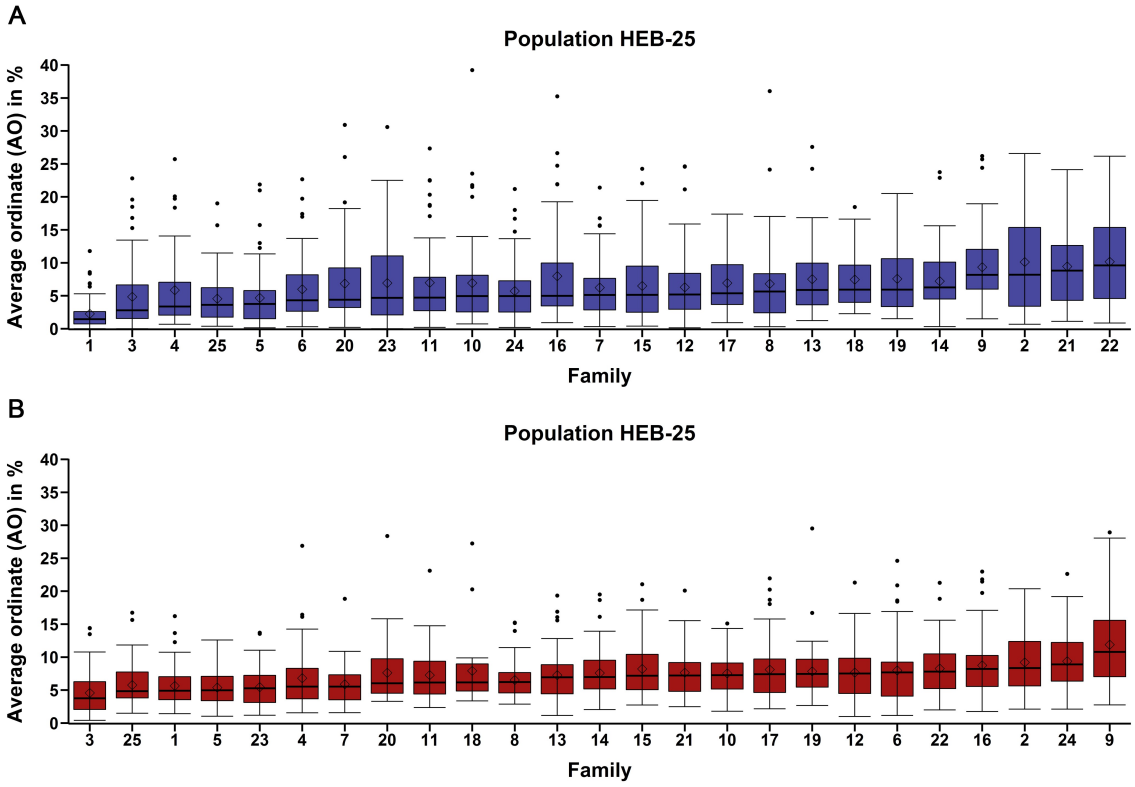

Fig 1. Box-whisker plots per HEB family indicating the variation in genotype response to the two fungi. (A) stripe rust (*Psh*) and **(B)** leaf rust (*Ph*) infection. HEB-25 families (1-25), sorted by ascending median, and rust severity are depicted on x-axis and y-axis, respectively.

AO values ranged from 0% up to 39.2% in case of *Psh* and from 0.4% up to 29.5% in case of *Ph* (Table 1). AO frequency distributions of the HEB-25 population showed to be highly right skewed for both rust fungi, with *Psh* results showing slightly stronger skewness (S4 File). For *Ph*, cultivar Barke showed an intermediate degree of resistance compared to the wild donor parents, whereas in case of *Psh* the common parent Barke showed a very high degree of resistance.

Table 1. Descriptive statistics for two-year field trials in Quedlinburg and heritability.

Trait[a]	N[b]	Mean Barke[c]	Mean HEB-25[d]	Min[e]	Max[f]	$SE_{+/-}$[g]	CV[h]	h^2[i]
AO_{Psh}	1401	3.31	6.72	0	39.23	0.15	0.85	0.70
AO_{Ph}	1401	10.97	7.36	0.40	29.52	0.11	0.58	0.60

[a]Average ordinate for stripe rust (AO_{Psh}) and leaf rust (AO_{Ph}), respectively.
[b]Number of genotypes analyzed.
[c]Mean average ordinate of recurrent parent Barke.
[d]Mean average ordinate of the HEB-25 population.
[e]Minimum.
[f]Maximum.
[g]Standard error.
[h]Coefficient of variation.
[i]Broad-sense heritability.

In most cases, a higher susceptibility of wild accessions to *Psh* than *Ph* was observed. The wild donor of family 25 showed, among all wild donors, the highest resistance to both pathogens (S5 File). In general, only a weak correlation (Pearson's correlation coefficients; $p < 0.0001$; Tukey-test) of $r = 0.28$ between *Psh* and *Ph* infection of genotypes was identified across the whole HEB-25 population. Two-year broad sense heritability was calculated as $h^2 = 0.70$ for *Psh* and $h^2 = 0.60$ for *Ph* (Table 1).

Nested association mapping

NAM was performed separately for each trait and resulted in the identification of numerous MTAs across HEB families (Fig 2; S6 File). However, most of the MTAs showed a DR below 50%. NAM based on *Psh* data resulted in the identification of 12 robust resistance QTL being composed of one or more markers with a DR higher than 50%, whereas NAM based on *Ph* data allowed to identify 11 robust resistance QTL (Table 2). For *Psh* QTL were identified on all chromosomes except chromosome 4H, whereas for *Ph* resistance QTL were identified on all chromosomes except chromosomes 1H and 3H (Table 2). Results of the two NAM studies showed that in most cases resistance QTL for *Ph* and *Psh* map to different chromosome regions. However, three chromosome regions were identified where *Psh* and *Ph* resistance QTL co-locate (Fig 2, Table 2). In detail, co-localization of resistance QTL was observed on the short arm of chromosome 2H (QPs.2H-1; QPh.2H-1), within the centromeric region of chromosome 6H (QPs.6H-1; QPh.6H-2), and on the long arm of chromosome 7H (QPs.7H-1; QPh.7H-3).

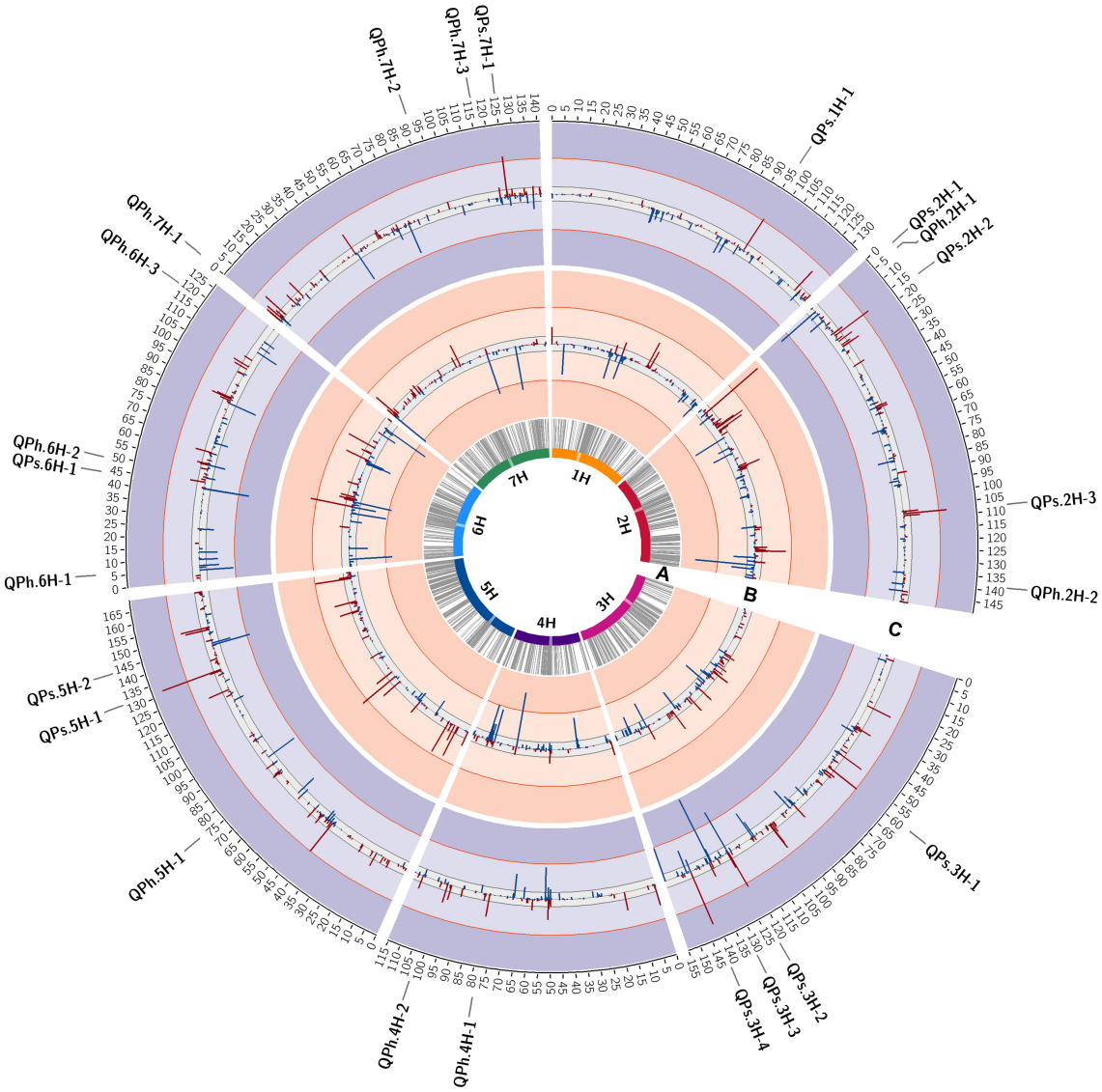

Fig 2. Circos plot indicating QTL controlling stripe rust and leaf rust resistance across HEB families. The barley chromosomes are arranged as colored bars forming the most inner circle. Centromere regions are highlighted as transparent boxes. **(A)** Grey connector lines represent the genetic position of the 5,715 informative SNPs on the chromosomes with cM positions (based on Maurer et al. 2015) given on the scale outside of circle C. **(B)** Marker trait associations calculated for leaf rust data (AO_{Ph}). Bars identify the position and detection rate (DR, height of bars) of significant marker trait associations. Bars in blue, pointing inwards, indicate a population wide trait-decreasing effect exerted by the wild barley allele, whereas bars in red, pointing outwards, indicate a population wide trait-increasing effect exerted by the wild barley allele. The grey and orange lines depict the DR threshold of 10% and 50% across 200 cross-validation runs. **(C)** Marker trait associations calculated for stripe rust data (AO_{Psh}). Graphical representation are the same as described under (A). The position of the 23 robust QTL with DR >50% are indicated on the scale outside of circle C. QTL for stripe rust and leaf rust resistance are coded with QPs and QPh, respectively.

Table 2. Robust stripe rust and leaf rust resistance QTL in HEB-25, detected with DR >50%.

QTL	Chr[a]	Peak marker with DR >50%[b]	Position of peak marker (cM)[c]	DR in 200 CV runs (%)[d]	CV mean R^2 (%)[e]	CV mean effect[f]	Corresponding resistance QTL/genes[g]
Stripe rust (*Psh*)							
QPs.1H-1	1H	i_SCRI_RS_136856	95.6	52.5	0.53	+0.16 (+1.45)	
QPs.2H-1	2H	i_SCRI_RS_165171	2.0	63.0	0.54	-0.34 (-2.19)	
QPs.2H-2	2H	i_SCRI_RS_159228	16.8	58.5	4.39	+0.13 (+1.35)	
QPs.2H-3	2H	i_SCRI_RS_158091	107.9	60.5	0.68	+0.31 (+2.04)	
QPs.3H-1	3H	i_12_30616	59.6	50.5	2.01	+0.13 (+1.35)	
QPs.3H-2	3H	**i_11_20146** i_SCRI_RS_235770	122.3	65.0 55.0	4.25	-0.22 (-1.66)	QTL_Toojinda[1] QTL_Yan/Chen[2]
QPs.3H-3	3H	i_SCRI_RS_209285	131.7	98.0	5.61	-0.28 (-1.91)	
QPs.3H-4	3H	i_12_20198	142.1	80.5	0.27	+0.41 (+2.57)	
QPs.5H-1	5H	i_SCRI_RS_175848	131.7	87.0	12.91	+0.31 (+2.04)	QTL_Cakir[3]
QPs.5H-2	5H	i_SCRI_RS_138608	143.8	53.5	0.09	-0.35 (-2.24)	
QPs.6H-1	6H	**i_SCRI_RS_162771** i_SCRI_RS_196285	43.7	64.0 60.5	2.73	-0.29 (-1.95)	QTL_Hayes[4] QTL6[5] QTL_Toojinda[1]
QPs.7H-1	7H	i_SCRI_RS_220680	125.1	57.0	0.86	+0.13 (+1.35)	*RpsFra*[6] *Rpsx*[7]
Leaf rust (*Ph*)							
QPh.2H-1	2H	i_SCRI_RS_184395	4.5	94.5	6.00	+0.12 (+1.32)	RphQ5[8] *Rph17*[9]
QPh.2H-2	2H	i_SCRI_RS_154135	138.6	81.0	5.48	-0.19 (-1.55)	QRph.sun-2H.2[10]
QPh.4H-1	4H	i_11_20670	78.4	75.0	7.67	-0.15 (-1.41)	Rphq5[11] RphQ8[8] QTL_Hickey[12]
QPh.4H-2	4H	i_SCRI_RS_148773	102.2	50.5	1.00	-0.28 (-1.91)	*Rph21*[13] QLr.S42-4H.a[14]
QPh.5H-1	5H	i_SCRI_RS_212784	75.6	57.5	4.05	+0.08 (+1.20)	
QPh.6H-1	6H	i_11_20882	5.6	59.0	1.26	-0.24 (-1.74)	QTL_Castro[15] QTL_Rossi[16]
QPh.6H-2	6H	**i_SCRI_RS_128460** i_SCRI_RS_128181	49.1	58.5 52.5	3.29	+0.30 (+2.00)	Rphq3[11] QTL_Castro[15] RphQ11[8] QTL_Hickey[12] *Rph24*[17]
QPh.6H-3	6H	i_11_11488	118.6	65.0	0.08	-0.10 (-1.26)	QTL_Backes[18]
QPh.7H-1	7H	i_SCRI_RS_208186	0.2	53.0	0.30	-0.26 (-1.82)	RphQ12[8]
QPh.7H-2	7H	i_12_20611	91.9	56.5	2.14	-0.08 (-1.20)	
QPh.7H-3	7H	i_SCRI_RS_175568	116.1	59.0	2.41	-0.07 (-1.17)	Rphq9[11] QTL_Castro[15]

[a]Chromosomal location of QTL.
[b]ISelect name of peak marker with a detection rate (DR) >50%. In case a QTL is composed of several SNP markers, the peak marker with highest DR is shown in bold letters.
[c]Position of the QTL peak marker based on Maurer et al. (2015).
[d]DR of the QTL peak marker in 200 cross-validation runs in percent.
[e]Mean percentage of phenotypic variance explained by the QTL peak marker, based on 200 cross-validation runs.
[f]Across-family,population-wide mean effect of the QTL peak marker, based on 200 cross-validation runs. Positive and negative signs indicate a trait-increasing and trait-decreasing effect of the wild barley allele compared to the Barke control allele, respectively. Values within the brackets show the effect estimates back-transformed to the original scale.
[g]Previously described stripe rust (*Psh*) and leaf rust (*Ph*) resistance QTL/genes located within the range of LD decay around the QTL marker with DR >50% identified in this study ([1](Toojinda et al. 2000), [2](Yan and Chen 2007), [3](Cakir et al. 2003c), [4](Hayes et al. 1996), [5](Castro et al. 2002b), [6](Dracatos et al. 2016), [7](Castro et al. 2003), [8](Ziems et al. 2014), [9](Pickering et al. 1998), [10](Singh et al. 2016), [11](Marcel et al. 2007), [12](Hickey et al. 2011), [13](Sandhu et al. 2012), [14](von Korff et al. 2005), [15](Castro et al. 2012), [16](Rossi et al. 2006), [17](Ziems et al. 2017), [18](Backes et al. 2003)).

In both NAM studies a broad variation in the wild allele effect estimates of adjacent markers was observed (Fig 2; S6 File). Most of the QTL detected are composed of markers exhibiting opposed wild allele effect estimates, sometimes this holds true even for adjacent markers (S6 File).

The *Psh* resistance QTL showing the peak marker with the highest DR (i_SCRI_RS_209285) is located on the long arm of chromosome 3H. This SNP shows a negative cross-validated mean effect, resembling a decrease of the AO value in the presence of the wild allele compared to the Barke control allele (Fig 2; Table 2). In the *Ph* NAM study, the peak marker with the highest DR (i_SCRI_RS_184395) is located on the short arm of chromosome 2H and shows a positive cross-validated mean effect, representing an increase of the AO value in the presence of the wild allele compared to the Barke control allele (Fig 2; Table 2).

Estimation of wild allele effect estimates of robust *Psh* QTL peak markers across the whole population resulted in only small cross-validated mean effect estimates. Thus, resembling only minor increases or decreases of AO values of genotypes in the presence of the wild allele compared to the Barke control allele. Likewise, analysis of population-wide R^2 values of QTL peak markers resulted in only low to intermediate estimates in the majority of cases (Table 2). Log_{10} based population-wide wild allele effect estimates range from -0.35 to 0.41 When back-transformed to the original scale this represents a maximum change in the AO value of 2.57% in the presence of the wild allele compared to the Barke control allele. The explained variance of a single QTL peak marker (R^2) ranged from 0.09 to 12.91% (Table 2; S6 File).

Similar observations were made in case of *Ph*. Across the whole population robust *Ph* QTL peak markers showed only small wild allele effect estimates and low R^2 values (Table 2). Log_{10} based population-wide wild allele effect estimates ranged from -0.28 to 0.30. When back-transformed to the original scale this represents a maximum change in the AO value of 2% in the presence of the wild allele compared to the Barke control allele. In case of *Ph* the explained variance of a single QTL peak marker (R^2) ranged from 0.08 to 7.67% (Table 2; S6 File).

The peak markers of QPs.3H-4 and QPh.6H-2 showed the highest effect estimate (0.41 and 0.30; log_{10}-value) and the peak markers of QPs.5H-1 and QPh.4H-1 the highest R^2 value (12.91% and 7.67%) for trait AO_{Psh} and AO_{Ph}, respectively (Table 2; S6 File).

After testing for QTL effects across HEB families, parent-specific QTL effects were calculated to obtain an effect estimate representing the combined effect of all family specific markers the QTL is composed of. Due to previously mentioned model limitations (see Material and methods), QTL QPs.3H-2, QPs.3H-3, and QPs.3H-4 were combined to one single parent-specific QTL QPs.3H-2/3/4, as well as QPs.5H-1 and QPs.5H-2 to one single parent-specific QTL QPs.5H-1/2.

For *Psh* as well as *Ph,* data estimation of parent-specific QTL effects revealed considerable variation in the effect size as well as direction of the wild allele between families (S7 File). In case of *Psh* only three parent-specific QTL showed the same effect direction across all families (QPs.2H-2; QPs.3H-1, and QPs.5H-1/2), whereas this was the case for five of the parent-specific QTL identified in the NAM study based on *Ph* data (QPh.2H-1, QPh.4H-2, QPh.5H-1, QPh.6H-1, and QPh.7H-3). No family showed trait-reducing effects at all parent-specific QTL, neither for *Psh* nor for *Ph*. The maximum count of parent-specific QTL showing a trait-reducing effect per family was five out of nine in case of AO_{Psh} and eight out of 11 for AO_{Ph}. For trait AO_{Psh} family F24 (-0.01; log_{10}-based value) and for trait AO_{Ph} family F03 (-0.54; log_{10}-based value) showed the largest reducing effect summed up over all parent-specific QTL (S7 File). Results of the *Psh* and *Ph* NAM study revealed divergence between the QTL peak marker effect and the mean QTL effect based on the parent-specific QTL effects. For both traits QTL peak markers exhibited in most cases the same effect direction as the mean QTL estimate across HEB families, but differed in effect size (S6 File, 7). Thus, NAM showed that QTL mean effect and peak marker effect are not necessarily identical in HEB-25.

The mean percentage of phenotypic variance explained through the full model (R^2_{adj}) was calculated to be 73.5% for *Psh* and 62.6% for *Ph* (Table 3). Notably, in case of both NAM studies a considerable portion of the phenotypic variance is explained by the robust QTL peak markers (Table 2; S6 File). The predictive ability (R^2_{pred}) of the full model for infection severity was calculated to be 42.4% for *Psh* and 32.3% for *Ph* (Table 3).

Table 3. Number of QTL and total phenotypic variance explained.

Trait[a]	QTL[b]	R^2_{adj} (%)[c]	R^2_{pred} (%)[d]
AO_{Psh}	12	73.5	42.4
AO_{Ph}	11	62.6	32.3

[a]Average ordinate for stripe rust (AO_{Psh}) and leaf rust (AO_{Ph}), respectively.
[b]Number of QTL defined for the respective trait.
[c]Mean phenotypic variance explained by the full NAM model.
[d]Mean ability to predict rust infection severity of independent genotypes.

Comparison with previously identified QTL

Comparison of *Ph* resistance QTL identified in this study with those already reported in literature revealed that the majority of identified QTL mapped to chromosome regions known to be linked to *Ph* resistance. Nine out of the 11 QTL identified in this study conferring resistance to *Ph* showed overlap with marker intervals of previously reported *Ph* resistance QTL or genes (Table 2). Based on available data, LD based QTL intervals of QTL QPh.5H-1 and QPh.7H-2 showed no overlap with previously reported *Ph* resistance QTL or genes. In case of *Psh*, less overlap of resistance QTL identified in this study with those already reported was observed. Only four out of the 12 *Psh* resistance QTL identified in this study, namely QPs.3H-2, QPs.5H-1, QPs.6H-1, and QPs.7H-1, overlapped with previously reported *Psh* resistance QTL (Table 2). Four out of ten so far unknown resistance QTL for *Psh* or *Ph*, namely QPh.7H-2, QPs.2H-1, QPs.3H-3, and QPs.5H-2, showed negative CV mean effects (Table 2), indicating the existence of wild barley alleles conferring *Ph* or *Psh* resistance. The alignment of SNPs with DR >50% against the physical barley map by means of the BARLEYMAP pipeline resulted in the identification of a number of genes related to plant defense in the respective QTL intervals. In particular, leucine-rich repeat, NB-ARC, and serine/threonine-protein kinase-like domain genes were found at high frequency. Details are given in S2 File.

Discussion

The strong variation in infection severity of HEB-25 lines infected with *Psh* and *Ph* in field trials demonstrates the high genetic diversity present within the HEB-25 population, and thus, its suitability to identify resistance QTL using NAM. Results of this study are in agreement with results of previous HEB-25 NAM studies that

identified a comparable variation regarding developmental traits (Maurer et al. 2015; Maurer et al. 2016a) and salinity tolerance (Saade et al. 2016). As in case of the previous HEB-25 studies, variation in *Psh* and *Ph* infection severity was detected between as well as within families, clearly indicating the suitability of HEB-25 to not only identify population-wide but also parent-specific QTL effects for resistance to *Psh* and *Ph* (Fig 1A and B; S3 File). The high variation in HEB-25 regarding stripe rust and leaf rust resistance is expected to be a function of the difference in the genetic make-up in the elite parent Barke and the wild donor parents. While wild donors showed in general a higher susceptibility to *Psh* than to *Ph* the opposite was true for the recurrent parent Barke (S5 File).

The evaluation of pathogen resistance in separate field trials for *Psh* and *Ph* allowed the individual phenotypic evaluation of HEB-25 genotypes without a potential bias caused by simultaneous infection of genotypes with both fungi. The integration of susceptible spreader strips and the inoculation of these with aggressive *Psh* and *Ph* isolates proved to be efficient as it allowed a clear and reliable differentiation of genotypes that would have been difficult to achieve under natural infection. This is particularly true for field trials conducted to identify QTL conferring resistance to *Psh*, as this fungus is strongly influenced by environmental conditions. We assume that the relatively high broad sense heritabilities (Table 1), which are a prerequisite for successful QTL identification, would not have been observed if phenotyping had been conducted based on natural infection.

The comparison of phenotypic results of the two field trials facilitated the identification of HEB lines that simultaneously showed a high degree of resistance against *Psh* and *Ph*. Out of these, especially HEB_03_006, HEB_03_015, and HEB_03_142 are valuable candidates to be integrated into barley pre-breeding programs aiming to simultaneously increase *Psh* and *Ph* resistance, as they are among the top one percent of genotypes regarding resistance to both pathogens (S3 File). However, next to a high level of resistance, results of earlier studies by Maurer et al. (2015; 2016a) may be considered during the selection process to select resistant HEB lines, which combine a suitable resistance with elevated yield parameters. It is expected that the integration of favorable wild barley alleles into barley breeding programs will be achieved faster with HEB-25 lines than with wild barley accessions since a backcrossing step with cultivar Barke was already performed during the development of HEB-25.

The occurrence of opposed wild allele effect estimates of closely linked markers identified in this study was also observed in previous HEB-25 studies by Maurer et al. (2015; 2016a; 2016b) and most likely arises from the fact that not all SNPs segregate in all families. Therefore, markers are likely to reflect only the mean wild allele effect of a fraction of the full population. As a result, closely linked markers segregating in different sets of genotypes of the complete population can show opposed effect estimates because of different mean resistance levels of the two sets. Phenotypic results revealed that families differ in their mean resistance level (Fig 1). Therefore, we assumed that strongly differing sets are likely to be linked to different families and, thus, opposed effect estimates of closely linked SNPs can be caused by parent-specific alleles.

Since the focus of this study was to identify robust QTL conferring resistance to *Psh* and *Ph* a rather stringent threshold for the acceptance of MTAs was defined. Minor QTL not passing this threshold but still influencing genotype response to *Psh* and *Ph* are not considered in this study. Defining a less stringent DR threshold of 10%, as applied in the study of Maurer et al. (2016a), would have resulted in a considerable higher number of individual QTL and co-locating QTL for the two rust fungi (Fig 2; S6 File). However, it has to be considered that with a lower DR threshold the risk of false-positive MTAs increases and, therefore, these minor QTL should be interpreted with caution.

The detection of QTL for resistance against *Psh* and *Ph*, despite low estimates across the whole population, is a strong proof of the power of the NAM strategy in general and in particular the suitability and precision of the NAM model applied in this study (Table 2; S6 File). The mean phenotypic variance explained by the full model and the calculated mean ability to predict the degree of infection of independent genotypes further supports the suitability of the applied model (Table 3).

The high number of QTL linked to *Psh* and *Ph* resistance detected in this study, the small CV mean effect estimates, as well as the low percentage of phenotypic variance explained by the majority of QTL peak markers indicate a complex inheritance of adult plant resistance for both pathogens (Table 2; S6 File). QPs.5H-1 with a R^2 value of 12.91% is an exception to the generally low phenotypic variance explained by the majority of QTL peak markers and might indicate the presence of a major resistance gene. Although there may be few lines carrying a major resistance gene, results of this study show that resistance in HEB-25 is predominantly polygenic and is the

result of the accumulation of numerous small effect loci with additive effects. Similar results are reported in studies with other NAM populations focusing on stem rust of wheat (Bajgain et al. 2016) and stem rust, stripe rust, and leaf rust of wheat (Li et al. 2016). In each case, a high number of QTL with small to medium effects were reported and the authors concluded the nature of resistance to be polygenic, with several loci acting additively. However, the maximum allele effect estimates in the study of Li et al. (2016) are higher compared to this study. The same holds true for R^2 values in both studies. It has to be considered that next to being the result of a complex polygenic inheritance of resistance, small population-wide effects of QTL peak markers may also be attributed to the presence of alleles with differing effects on resistance. Namely, in case only a limited number of HEB-25 lines of the full population show a strong allele effect on resistance or contrasting allele effects among the 25 HEB donor parents exist at a marker position.

The importance of considering the influence of differing donor allele effects in HEB-25 on estimated population-wide QTL peak marker effects is supported by the high variation of donor allele effects at parent-specific QTL (S7 File). Results of this study are very similar to the observations made by Bajgain et al. (2016) and Li et al. (2016) focusing on the identification of QTL conferring resistance to rust pathogens of wheat by use of the NAM approach. As in this study the authors identified strongly varying parent-specific allele effects at resistance QTL. Therefore, studies focusing on detailed analysis of specific QTL or on the integration of *Psh* and *Ph* resistance alleles in modern barley cultivars should take into account the parent-specific QTL effect information given in this study to select the most promising resistance-carrying HEB line to be incorporated into a new barley breeding cycle. Not including parent-specific QTL effect estimates in the selection decision may result in missing alleles whose strong favorable effect is masked by a high number of parent-specific alleles with an opposed effect (S7 File). However, it is noteworthy to mention that parent-specific QTL effect estimates may be slightly biased, as each family comprises only a relatively small number of HEB-25 lines (Maurer et al. 2016b). Thus, selection decisions should be based on a combined evaluation of population-wide and parent-specific estimates of wild allele effects.

Most *Ph* resistance QTL and several of the *Psh* resistance QTL identified in this study showed overlap with QTL previously reported to be linked to *Ph* or *Psh* resistance (Table 2). At the same time two QTL for resistance to *Ph* and eight QTL for resistance

to *Psh* identified in this study are located at chromosome positions not yet reported to be involved in resistance against *Ph* or *Psh*, respectively. Several of the *Ph* and *Psh* resistance QTL, although showing no overlap with previously reported *Ph* and *Psh* resistance QTL, were located in chromosome regions known to be linked to resistance to *Ph* or *Psh*. In case of *Ph*, resistance QTL QPh.7H-2 is located in the vicinity of leaf rust resistance QTL Rphq8 identified by Qi et al. (1999) and Marcel et al. (2007) as well as QRph.sun-7H identified by Singh et al. (2016). Likewise, the resistance QTL QPs.1H-1 and QPs.2H-3 identified in the *Psh* NAM study are located in a chromosome region in which Dracatos et al. (2016) identified a QTL linked to *Psh* resistance. Furthermore, QTL QPh.7H-2, QPs.3H-1, and QPs.3H-4 each show overlap with a meta-QTL identified by Schweizer and Stein (2011) effective against several fungal barley pathogens. Based on available data and the QTL intervals defined in this study, all of the 10 QTL identified in this study to show no overlap (Table 2) should be regarded as potentially novel resistance QTL, harboring new and yet undiscovered rust resistance genes. It has to be considered, that the majority of previously reported *Ph* and *Psh* QTL were identified using different rust isolates than those used in this study. Therefore, QTL identified in this study showing overlap with previously reported *Ph* or *Psh* resistance QTL may potentially confer novel resistance alleles at known rust resistance loci.

Only at three chromosomal locations, QTL for stripe rust and leaf rust resistance co-localized. This finding may indicate the existence of rust specific defense mechanisms in HEB-25 rather than a broad-spectrum species-independent pathogen control. This assumption is also supported by a low correlation observed between both traits. The clear preponderance of independent QTL in HEB-25, either specific for *Ph* or *Psh,* is in agreement with Suenaga et al. (2003). The authors detected only one common QTL for leaf rust and stripe rust resistance in wheat. In contrast, studies by McIntosh (1992) and William et al. (2003) showed correlated response of wheat to leaf rust and stripe rust caused by closely linked genes. Likewise, Herrera-Foessel et al. (2011) observed a correlated response to leaf rust and stripe rust of wheat for most of the tested wheat lines caused by either a single gene or very closely linked genes conferring resistance to both pathogens. Furthermore, Krattinger et al. (2009) identified *Lr34*, a broad-spectrum non-race-specific resistance gene that confers resistance to a range of pathogens including leaf rust and stripe rust of wheat. Next to this, William et al. (2006) and Li et al.

(2016) both reported QTL conferring resistance to leaf rust and stripe rust of wheat as well as QTL conferring resistance to only one of the two pathogens. The *Ph* and *Psh* resistance QTL located in close proximity to each other (QPs.2H-1 and QPh.2H-1, QPs.6H-1 and QPh.6H-2, QPs.7H-1 and QPh.7H-3), may represent regions linked to general resistance to rust fungi, and thus, be combined to meta-QTL. However, peak markers of co-locating *Ph* and *Psh* resistance QTL in HEB-25 showed opposed wild allele effects (Table 2). This fact points towards the presence of two independent rust pathogen specific resistance genes located in proximity to each other, rather than the presence of a single resistance gene conferring resistance to both pathogens. The fact that the three co-localized *Ph* and *Psh* resistance QTL were not identified at the same chromosomal position, but were located within a distance of 2.5 (2H) to 9.0 (7H) to each other further supports this assumption.

We found a high frequency of leucine-rich repeat, NB-ARC, and serine/threonine-protein kinase-like genes as putative candidate genes in rust resistance QTL intervals of HEB-25. This finding is in agreement with the important role of those gene families, known as resistance gene analogs (RGAs), in various defense reactions of plants against pathogens (Sekhwal et al. 2015; Andersen et al. 2016). Based on this study the definition of a single candidate gene responsible for the detected QTL effect is not feasible. The final prove, which candidate gene is causing the QTL effect, may be achieved after a high-resolution mapping within the respective QTL interval has been conducted and the identified candidate genes, co-segregating with the resistance phenotype, have been knocked-out or genetically engineered. The various putative candidate genes identified in this study by the use of the BARLEYMAP pipeline and GO-term analysis within the QTL intervals may serve as a starting point for subsequent studies focusing on the genetic basis of resistance of barley to *Ph* and *Psh* (S2 File).

Conclusion

The results of this study provide valuable information not only for basic studies elucidating the molecular basis of *Psh* and *Ph* resistance in barley, but also for improving *Psh* and *Ph* resistance and diversity of modern elite barley cultivars. We expect that in future a better understanding of the allelic diversity present at stripe rust and leaf rust QTL in HEB-25 will be achieved by generating exome capture based SNP and haplotype data for all HEB lines and 26 HEB parents. This way, it is expected to achieve more precise estimates of haplotype-based allele effects in HEB-25 and to increase the power to detect wild barley alleles with favorable effects on barley resistance against stripe rust and leaf rust.

Variation in infection severity

The large variation in infection severity of HEB-25 lines observed in case of all three fungal pathogens, and the variation observed between as well as within families is indicative for the large genetic diversity present within the HEB-25 population. These findings highlight the suitability of HEB-25 for the detection of population-wide and parent-specific QTL conferring resistance against fungal pathogens. This is an added value to the previously identified effective applicability to detect QTL for developmental traits (Maurer et al. 2015; Maurer et al. 2016a; Herzig et al. 2018) and salinity tolerance (Saade et al. 2016). Results presented in this thesis suggest that the high variation does not arise only from the diversity of the wild progenitors serving as donors, but as well from the difference in response to infection between the common recipient Barke and the donors in general. Wild donors showed in general a higher susceptibility to *Psh* than to *Ph*, while the opposite was true for the recurrent parent Barke. Concerning *P. teres* f. *teres*, a higher level of resistance was identified in the wild progenitors. In case of HEB-25 the divergent response of wild donors and the recurrent parent occurred by chance in contrast to the NAM population constructed by Bajgain et al. (2016) that was designed specifically for the detection of QTL conferring resistance against stem rust of wheat. Consequently, the findings of this study further support the high genetic diversity of wild progenitors of barley and their suitability to broaden the genetic basis of modern cultivars.

Given that successful infection and extend of disease pressure strongly depend on the environmental conditions in case of all three fungi (Newton and Johnson 1936; Jordan 1981; Mathre 1997) obtaining reliable results in field trials is challenging. Simultaneous infection of trials with several diseases can further complicate the evaluation of genotype response to a specific pathogen. Xue et al. (1994) identified that *P. teres* f. *teres* and scald (*Rhynchosporium secalis*) often occur together and impede the precise evaluation of genotype susceptibility to *P. teres* f. *teres*. Similar *Psh* can infect barley at low temperatures early in the growth period (Newton and Johnson 1936), while *Ph* requires higher temperatures for optimal infection of barley (Mathre 1997). Therefore, *Psh* can impede the screening of plants for

susceptibility to *Ph* in case both fungi are evaluated in one field trial. Increasing the robustness of results generally requires to perform trials across several years and locations. Given the large size of HEB-25 and the time needed to phenotype the 1420 lines, trials were limited to two replications in two years at one location. The use of artificial infection in field trials, the use of the summer hill trial design in case of *P. teres* f. *teres* (König et al. 2013), and the evaluation of resistance against *Psh* and *Ph* in separate field trials showed to be highly effective, allowing for reliable results despite the limitations mentioned above. Most likely, the relatively high broad sense heritabilities, which are a prerequisite for successful QTL identification, would not have been observed if phenotyping had been conducted based on natural infection. Therefore, the use of artificial infection can be suggested as a standard approach for reliable screening of HEB-25 in field trials when focusing on the detection of QTL conferring resistance to fungal pathogens.

Screening of HEB-25 for resistance against *P. teres* f. *teres*, *Ph*, and *Psh* allowed for the identification of several highly resistant HEB-25 lines. These lines are promising candidates to be integrated into pre-breeding programs aiming to increase disease resistance against fungal pathogens evaluated in the framework of this thesis. Given a backcrossing step with cultivar Barke was already performed during the development of HEB-25, it can be expected that integration of HEB-25 lines into pre-breeding programs will be faster than in case of the integration of wild accessions themselves. Depending on the specific breeding goal, detailed analysis of family F_03 which based on the median showed a relatively high degree of resistance to all three fungi, or selection of lines with strong resistance against a specific fungus might be of interest. Results of *Psh* trials will be of high value for Australian plant breeders, as most Australian barley lines are susceptible to this pathogen (Wellings et al. 2000; Wellings 2007). Straightforward would be the introduction of Barke in breeding programs aiming to increase resistance of Australian barley lines against *Psh*, as results showed it to be highly resistant. However, this might not be the optimal strategy as Barke showed to be relatively susceptible to *P. teres* f. *teres* and *Ph*. Therefore, selecting HEB-25 lines that show a high level of resistance against *Psh* and a limited susceptibility to the other tested fungal pathogens likely represents a better strategy. Suitable candidates might be HEB-25 lines HEB_03_006, HEB_03_015, and HEB_03_142 as they are among the top one percent of genotypes

regarding resistance to *Ph* and *Psh* and are moderately resistant to *P*. *teres* f. *teres*. In this regard it has to be taken into account that results presented in this thesis rely on field trials conducted at one location across two years. Furthermore, selecting lines solely based on the degree of resistance can result in selecting against yield related traits (Brown and Rant 2013; Vyska et al. 2016). GWAS conducted in the framework of this thesis strongly points towards a negative correlation between flowering time, shown to be linked to thousand grain weight (Maurer et al. 2016a), and resistance against *P*. *teres* f. *teres*. However, in this regard it needs to be mentioned that in the framework of this study only a comparison of QTL localization and QTL effects was possible and that further studies based on phenotypic data as well as trials conducted during the standard growing period are required for final assessment.

Combining genotype information presented in this thesis with information on developmental traits and salinity tolerance traits presented in other HEB-25 studies (Maurer et al. 2015; Maurer et al. 2016a; Saade et al. 2016; Herzig et al. 2018) will enable to select resistant HEB-25 lines that fit to the specific growing conditions.

Applicability of HEB-25 for detection of QTL conferring fungal resistance

In the framework of this thesis, HEB-25 was evaluated for the first time for its suitability to detect QTL conferring resistance to fungal pathogens. To achieve QTL detection 'Model-B' was applied, which was identified by Würschum et al. (2012) to be well suited for joint linkage association mapping (JLAM), and verified by Maurer et al. (2015) to be highly effective for conducting GWAS in the NAM population HEB-25. Similar to composite interval mapping (Jansen and Stam 1994; Zeng 1994), 'Model-B' makes use of cofactors to allow for a high QTL detection power. In addition a family effect is included in the model to correct for population stratification (Würschum et al. 2012). The identification of a large number of QTL in case of all three NAM studies conducted in the framework of this thesis circumstantiates the power of this method and is in line with the other HEB-25 studies applying this model (Maurer et al. 2015; Maurer et al. 2016a; Saade et al. 2016; Herzig et al. 2018). In this context, it is noteworthy to mention that in the framework of this thesis only SNP markers being detected in more than 50% of cross-validation runs were used

to assign resistance QTL and analyzed in detail. Defining a less stringent threshold of 10%, as applied in the study of Maurer et al. (2016a), would have resulted in an increase in the number of QTL. Notably, the model allowed for the detection of population-wide QTL conferring resistance despite most of them exhibiting only small mean effect estimates and explaining a low percentage of phenotypic variance. The overlap of several QTL identified in the framework of this thesis with QTL previously identified to be linked to resistance against *P. teres* f. *teres*, *Ph*, and *Psh*, is a strong proof of the reliability of the identified MTAs. Given that overlap between QTL was identified, even though previous resistance QTL were identified by the use of different isolates and under different environmental conditions, it can be assumed that the resistant QTL presented in this thesis are environmentally stable and not isolate specific. Nevertheless, final proof can only be achieved by additional field trials under differing environmental conditions and using varying isolates (Miedaner and Korzun 2012). Furthermore, artificial infection of field trials with mixtures of *Ph* or *Psh* isolates could be considered, instead of only using the currently most aggressive races.

Currently HEB-25 is screened for resistance against *P. teres* f. *teres* and *Ph* in Australia. Furthermore, specific families of HEB-25 are screened for resistance against *P. teres* f. *teres* in Israel. In both cases GWAS will be performed with the same SNP markers and the same reference map constructed by Maurer et al. (2015), as well as the same statistical model used for GWAS studies conducted in the framework of this thesis. It will be interesting to see, if overlap between QTL detected in these two studies with QTL presented in this thesis can be observed. Furthermore, QTL detected in these currently performed studies should be checked for overlap with SNP markers being detected in less than 50% of cross-validation runs in the framework of this thesis. In this regard, the 10% threshold applied in the study of Maurer et al. (2016a) could serve as base for selection of QTL candidates. This will help to identify if these SNP markers are truly associated with fungal resistance and thus should be considered as robust resistance QTL in subsequent studies, or are more likely to represent false-positives. SNP markers that were detected in slightly less than 50% of cross-validation runs in the framework of this thesis, but in more than 50% of cross-validation runs in the studies performed in Australia or Israel are unlikely to represent false-positives.

Allelic diversity and its use for resistance breeding

The presence of QTL with only small effects is in agreement with previous NAM studies focusing on the detection of resistance QTL (Kump et al. 2011; Poland et al. 2011; Bajgain et al. 2016; Li et al. 2016) and is likely to arise partly out of a complex inheritance of adult plant resistance. However, findings of this thesis indicate that small population-wide QTL effects can likewise be caused by the presence of multiple alleles in HEB-25. Estimation of parent-specific QTL effects, performed using the model developed by Maurer et al. (2016b), revealed varying QTL effects between families that likely arise out of the presence of alleles with diverse effects on resistance. This assumption is supported by results of the first HEB-25 study of Maurer et al. (2015) aiming to identify QTL associated with flowering time control. Resequencing of *Ppd-H1* resulted in the identification of twelve haplotypes with differing effects on flowering time of which one was associated with a reduction of flowering time of -11.1 days compared to the elite barley haplotype (Maurer et al. 2015). Parent-specific effects were identified for other QTL associated with developmental traits as well (Maurer et al. 2015; Herzig et al. 2018).

Results of the parent-specific QTL estimates may be used to select families showing a strong effect on the trait under investigation. However, the limited family size might cause a bias in the effect estimates of parent-specific QTL estimates (Maurer et al. 2016b). Furthermore, in the framework of this thesis parent-specific QTL effects were analyzed only for QTL identified in at least 50% of cross-validation runs in the population-wide GWAS. Causative alleles segregating in very few lines might not have been detected in the population-wide GWAS, or MTAs were detected in too few cross-validation runs to be considered in further analysis. Furthermore, the model developed by Maurer et al. (2016b) estimates parent-specific effects based on accumulating SNP effects within a 26 cM window, resembling the mean introgression size in HEB-25. As a result, some of the QTL detected in the population-wide GWAS had to be combined into a single parent-specific QTL. Therefore, in some cases the parent-specific QTL effect estimate reflects a combination of the effects of previously distinct QTL and thus should be interpreted with caution.

The usefulness of QTL conferring resistance for breeders depends on the percentage of phenotypic variance explained by the QTL. In case QTL explain only a small percentage of phenotypic variance, introgression into elite cultivars and MAS is generally not worthwhile. Miedaner and Korzun (2012) define that a QTL should explain at least between 10 to 20% of the phenotypic variance in the original mapping population for MAS. Considering this, only QTL QPs.5H-1 explaining 12.91% of phenotypic variance and QPt.2H-2 explaining 14.88% of phenotypic variance might be of direct interest for breeders. However, the percentage of phenotypic variance explained by the QTL was calculated only on the population-wide level and calculation of parent-specific QTL effects revealed considerable deviations from the population-wide analysis (see Chapter 1, S6; Chapter 2, S7). Therefore, based on information for specific families, MAS and introgression into elite cultivars might be worthwhile for other QTL as well. An example, that focusing on specific families is promising is the QTL QPt.7H-1 identified to be associated with resistance against *P*. *teres* f. *teres*. In case of QTL QPt.7H-1 the high population-wide effect of the wild allele observed for the peak marker (wild barley allele effect on AO = +9.64) seems to be mainly caused by the strong effect of an allele or allele combination derived from the donor parent of HEB family F16 (wild barley parent-specific allele effect on AO in family F16 = +9.16).

The population-wide and parent-specific QTL effects as well as the percentage of phenotypic variance explained by the population-wide QTL presented in the framework of this thesis are based on NAM conducted using the barley Infinium iSelect 9K chip (Comadran et al. 2012). Recently HEB-25 was genotyped using the 50k Illumina Infinium iSelect SNP array for barley, developed by Bayer et al. (2017). The increase in the number of markers will most likely result in a higher number of informative markers segregating in all families. It can be expected, that this will result in more precise effect estimates for population-wide and parent-specific QTL and will influence the estimates of the percentage of phenotypic variance explained by the population-wide QTL. Furthermore, it will be interesting to see if the markers that were detected with highest frequency in the framework of this thesis will remain the same and if additional markers will cross the defined threshold of 50% detection rate. Information obtained by repeating NAM using the phenotypic data obtained in the framework of this thesis and the new 50k SNP array (Bayer et al. 2017) data will further increase the value of results for studies focusing on the

detailed analysis of specific QTL or on the integration of resistance alleles in modern barley cultivars.

Identification of putative candidate genes

The BARLEYMAP pipeline (Cantalapiedra et al. 2015) was used to identify potential candidate genes underlying the particularly robust QTL of this study. Identification of potential candidate genes was achieved by aligning the associated markers detected in more than 50% of cross-validation runs against the barley physical map (IBGS 2012) and the POPSEQ map (Mascher et al. 2013). This approach resulted in the detection of leucine-rich repeat, NB-ARC, and serine/threonine-protein kinase-like genes in the QTL intervals in case of all three fungi. The presence of these genes in the QTL intervals at high frequency is in agreement with the important role of these gene families, referred to as resistance gene analogs (RGAs), in plant defense response (Sekhwal et al. 2015; Andersen et al. 2016). Members of these gene families were identified in QTL studies focusing on different fungal pathogens (Brueggeman et al. 2008; Faris et al. 2010; Kump et al. 2011; Poland et al. 2011). Furthermore, previous studies indicate an important role in the necrotrophic effector triggered reaction to *P. teres* f. *teres* infection (Liu et al. 2015; Richards et al. 2016).

In the framework of this thesis, the definition of a single candidate gene responsible for the detected QTL was not feasible. This is due to the fact that the LD across HEB-25 was calculated to be 7.85 cM (Maurer et al. 2015). Therefore, candidate gene search was limited to this resolution and thus, a selection of a specific candidate gene out of all putative candidate genes within the defined QTL interval of ±4 cM around the peak marker could not be achieved. Identifying which specific candidate gene is causing the QTL effect will require high-resolution mapping within the respective QTL intervals. Subsequently, final proof could be achieved by knock-out of the identified candidate genes, co-segregating with the resistant phenotype. Powerful methods that may be applied to achieve the targeted gene knock-out are the use of zinc finger nucleases, transcription activator-like effector nucleases, or the CRISPR/Cas9 system (Bortesi and Fischer 2015).

Fine mapping generally requires the development of specific mapping population to obtain additional recombinants, which is time consuming and labor intensive (Tian et al. 2015). In case of HEB-25 the utilization of heterogeneous inbred families (HIFs) is presumably the most straightforward approach for performing fine mapping. The use of HIFs for fine mapping was first described by Tuinstra et al. (1997). HIFs are developed by selfing a RIL that is heterozygous at the SNP locus of interest. In case of HEB-25, this generally would be the peak marker of a specific resistance QTL, but could also be another marker in the QTL interval that was detected in a similar high percentage of cross-validation runs. Selfing of the specific RIL results in a segregation of 1:2:1 at the SNP locus of interest. Since a RIL is selfed, the remaining genome remains essentially unaltered. The population derived by this method represents a HIF which can be used for fine mapping the QTL of interest. In case of HEB-25, HIFs are directly available, as HEB-lines were derived from BC_1S_3 plants and are homozygous at the majority of loci, while still segregating at certain loci. Furthermore, seed multiplication for each HEB-line is performed in field plots. Therefore, all plants within the plot used for multiplication of a specific HEB-line constitute a HIF. The high power of utilizing HIFs for fine mapping was shown in the multi-parental mapping population developed by Liller et al. (2017). Use of HIFs allowed for fine mapping of a major QTL for awn length in barley to a <0.9 cM interval and defining a prime candidate gene for the awn length phenotype.

For successful fine mapping a high marker density is required (Yu et al. 2011). Currently exome-capture is performed on HEB-25 parents and is anticipated to result in the generation of millions of additional markers (K. Pillen, pers. com.). Due to the specific design of NAM populations, the obtained marker data can subsequently be assigned to all 1420 HEB-lines. The availability of exome-capture data in combination with the available reference genome sequence (Mascher et al. 2017) will be a major step towards the detailed characterization of the resistance QTL identified in the framework of this thesis and the identification of specific genes associated with genotype resistance to *P*. *teres* f. *teres*, *Ph*, or *Psh*.

Summary

Net blotch, caused by the fungus *Pyrenophora teres* f. *teres*, *Puccinia hordei*, causing leaf rust, and *Puccinia striiformis* f. sp. *hordei*, the causal agent of stripe rust, are important fungal diseases of barley with the potential to cause severe yield losses and a reduction in feed and malting quality. The identification of QTL conferring resistance is the basis for targeted breeding approaches aiming to improve resistance of modern barley cultivars against these fungi. Initial domestication from its wild progenitor *Hordeum vulgare* subsp. *spontaneum* and extensive breeding resulted in a loss of genetic diversity in modern elite barley varieties. Many modern barley varieties are similar in their genotype resulting in an increased risk of occurrence of severe epidemics. There is an urgent need to broaden the genetic basis of resistance of modern barley cultivars to ensure a stable production. Wild barley accessions possessing high allelic richness have shown to be a valuable source of resistance alleles no longer present in the current breeding pool.

In the framework of this thesis, the NAM population HEB-25 was utilized to detect QTL conferring resistance against net blotch, leaf rust, and stripe rust. Screening of HEB-25 in two-year field trials revealed the presence of a high genetic diversity within HEB-25 and allowed for the identification of HEB-lines with a high degree of resistance to one or more of the evaluated fungi.

NAM applying the 9k iSelect barley chip performed independently for all three fungi resulted in the identification of a high number of QTL conferring resistance on all chromosomes with predominantly small effect. In case of net blotch six QTL, eight QTL for stripe rust and two QTL for leaf rust, are considered novel showing no overlap with previously reported resistance QTL.

Estimation of parent-specific QTL effects indicates the presence of alleles with increasing or decreasing effect on genotype resistance, respectively. Leucine-rich repeat, NB-ARC, and serine/threonine-protein kinase-like genes were found at high frequency in the QTL intervals and due to their important role in plant defense response represent putative candidate genes causing the QTL effect.

HEB-25 showed to be well suited for the detection of QTL conferring resistance to net blotch, leaf rust, or stripe rust and represents a valuable source for improving genetic diversity and resistance of modern barley cultivars against these fungi.

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Chapter 1) A nested association mapping population identifies multiple small effect QTL conferring resistance against net blotch (*Pyrenophora teres* f. *teres*) in wild barley

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S1 File Summer hill trial design at an early and a later developmental stage.
S2 File LD decay of intra-chromosomal markers across HEB-25.
S3 File Net blotch least squares means (lsmeans) averaged across two years for average ordinate (AO) and reaction type (RT).
S4 File Frequency distribution of two-year lsmeans for trait average ordinate (AO) and reaction type (RT).
S5 File Correlation between trait average ordinate (AO) and reaction type (RT) based on two-year lsmeans of HEB-25 parents and all HEB-25 lines.
S6 File GWAS and cross validation results on net blotch measures of average ordinate (AO) and reaction type (RT).
S7 File Estimates of mean wild allele QTL effects and donor-specific QTL effects for net blotch measures of average ordinate (AO) and reaction type (RT).
S8 File Alignment of SNPs (DR >50%) with known genes based on BARLEYMAP.

Chapter 2) Identification of QTL conferring resistance to stripe rust (*Puccinia striiformis* f. sp. *hordei*) and leaf rust (*Puccinia hordei*) in barley using nested association mapping (NAM)

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S1 File LD decay of intra-chromosomal markers across HEB-25.
S2 File Alignment of markers with DR >50% using BARLEYMAP.
S3 File Two-year least squares means (lsmeans) of HEB lines for traits AO_{Psh} and AO_{Ph} (i.e. stripe and leaf rust symptoms, respectively).
S4 File Frequency distribution of two-year lsmeans for trait for traits AO_{Psh} and AO_{Ph} (i.e. stripe and leaf rust symptoms, respectively).
S5 File Average ordinate (AO) values of HEB-25 parents.
S6 File GWAS results for resistance against strip rust (*Psh*) and leaf rust (*Ph*) in HEB-25.
S7 File Estimates of parent-specific QTL effects and QTL mean effects across HEB families for or traits AO_{Psh} and AO_{Ph} (i.e. stripe and leaf rust symptoms, respectively).

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Eidesstattliche Erklärung / *Declaration under Oath*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

_____ _____

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Publications

Vatter T, Maurer A, Perovic D, Kopahnke D, Pillen K, Ordon F (2018) Identification of QTL conferring resistance to stripe rust (*Puccinia striiformis* f. sp. *hordei*) and leaf rust (*Puccinia hordei*) in barley using nested association mapping (NAM). PLOS ONE 13:e0191666

Vatter T, Maurer A, Kopahnke D, Perovic D, Ordon F, Pillen K (2017) A nested association mapping population identifies multiple small effect QTL conferring resistance against net blotch (*Pyrenophora teres* f. *teres*) in wild barley. PLOS ONE 12:e0186803

Vatter T, Neuhäuser B, Stetter M, Ludewig U. (2015). Regulation of length and density of Arabidopsis root hairs by ammonium and nitrate. Journal of Plant Research 128:5, pp 839–848. DOI 10.1007/s10265-015-0733-8

Conference contributions

2017 German Society for Plant Breeding (GPZ) Meeting of AG Genomanalyse - Status of translating genomics into application, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. Poster: Mapping QTL for resistance to net blotch (*Pyrenophora teres* f. *teres*) in a wild barley nested association mapping (NAM) population.

2016 9th Young Scientists Meeting, Federal Research Centre for Cultivated Plants (Julius Kühn-Institut), Quedlinburg, Germany. Oral: Mapping QTL for resistance to net blotch (*Pyrenophora teres* f. *teres*) in a wild barley nested association mapping (NAM) population.

2015 XVIII. International Plant Protection Congress - Mission possible: food for all through appropriate plant protection, Berlin, Germany. Poster: Identification of QTLs conferring resistance to net blotch (*Pyrenophora teres* f. *teres*) in barley using a nested association mapping population

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