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ORIGINAL PAPER

Golden SusPtrit: a genetically well transformable barley line for studies on the resistance to rust fungi

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Abstract
Key message **We developed 'Golden SusPtrit', i.e., a barley line combining SusPtrit's high susceptibility to non-adapted rust fungi with the high amenability of Golden Promise for transformation.**
Abstract Nonhost and partial resistance to *Puccinia* rust fungi in barley are polygenically inherited. These types of resistance are principally prehaustorial, show high diversity between accessions of the plant species and are genetically associated. To study nonhost and partial resistance, as well as their association, candidate gene(s) for resistance must be cloned and tested in susceptible material where SusPtrit would be the line of choice. Unfortunately, SusPtrit is not amenable to *Agrobacterium*-mediated transformation. Therefore, a doubled haploid (DH) mapping population ($n = 122$) was created by crossing SusPtrit with Golden Promise to develop a 'Golden SusPtrit', i.e., a barley line combining SusPtrit's high susceptibility to non-adapted rust fungi with the high amenability of Golden Promise for transformation. We identified nine genomic regions occupied by resistance quantitative trait loci (QTLs) against four non-adapted rust fungi and *P. hordei* isolate 1.2.1 (*Ph*.1.2.1). Four DHs were selected for an *Agrobacterium*-mediated transformation efficiency test. They were among the 12 DH lines most susceptible to the tested non-adapted rust fungi. The most efficiently transformed DH line was SG062N (11–17 transformants per 100 immature embryos). The level of non-adapted rust infection on SG062N is either similar to or higher than the level of infection on SusPtrit. Against *Ph*.1.2.1, the latency period conferred by SG062N is as short as that conferred by SusPtrit. SG062N, designated 'Golden SusPtrit', will be a valuable experimental line that could replace SusPtrit in nonhost and partial resistance studies, especially for stable transformation using candidate genes that may be involved in rust-resistance mechanisms.

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Introduction

Nonhost resistance implies immunity of all members of a plant species against a potential pathogen species (Niks et al. 2011). The resistant plant species is referred to as nonhost, and the would-be pathogen species is referred to as heterologous or non-adapted pathogen. Classification of a plant species as nonhost or host against certain potential pathogen species is not easy (Niks 1987; Niks et al. 2011). Some plant species have few accessions with an intermediate level of susceptibility to a particular pathogen. Such

plant species are referred to as having a near-nonhost status (Niks 1987; Niks et al. 2011). The rare susceptibility of those few accessions may occur only at the seedling stage or under a very severe infection pressure (Niks 1987). Barley appears to be a near-nonhost to several non-adapted rust fungal species, such as *Puccinia triticina* and *P. hordei-murini*. Through the accumulation of genes for susceptibility to *P. triticina* from rare barley accessions with moderate susceptibility at the seedling stage, an experimental barley line called SusPtrit was developed. This line is extraordinarily susceptible to several grass rust fungi that are unadapted to barley (Atienza et al. 2004). SusPtrit facilitated the development of the barley–*Puccinia* rust fungus model system to study the inheritance of nonhost resistance in plants. Two mapping populations—Vada/SusPtrit (V/S) and Cebada Capa/SusPtrit (C/S)—were developed using SusPtrit as one of the parents (Jafary et al. 2006, 2008).

Partial resistance is a type of host resistance that slows down epidemic development despite a compatible infection type (Niks et al. 2011). On partially resistant barley, the pathogen realizes a lower infection frequency, has a lower sporulation rate and has a longer latency period (Parlevliet 1979). The partial resistance of barley against *P. hordei* (the homologous or adapted rust) is one of the most extensively studied cases of this type of resistance [reviewed by St. Clair (2010)].

Nonhost and partial resistance to *Puccinia* rust fungi in barley are both polygenically inherited. Nonhost and partial resistance quantitative trait loci (QTLs) against different rust fungi have been mapped in different bi-parental mapping populations (Qi et al. 1998, 1999; Jafary et al. 2006, 2008; Marcel et al. 2007, 2008). Nearly all of the reported QTLs were effective against only one to three rust species, and hence, were rust-species specific, and some QTLs were even rust-isolate specific (Atienza et al. 2004; González et al. 2012; Jafary et al. 2006; Marcel et al. 2008). The QTLs for nonhost resistance to rust fungi tended to map in the same genomic regions as the QTLs for partial resistance to *P. hordei* (González et al. 2010; Jafary et al. 2008). There is evidence that nonhost and partial resistance of barley to rust fungi share important features: both are principally prehaustorial (Hoogkamp et al. 1998; Niks 1983; Niks and Marcel 2009), show a high diversity between accessions of the plant species (Qi et al. 2000; Jafary et al. 2006, 2008) and are genetically associated (Hoogkamp et al. 1998; Zhang et al. 1994). The association was also shown in a transcriptional study wherein barley (cv. Ingrid) was exposed to a pair of adapted and non-adapted rust fungi and a pair of adapted and non-adapted powdery mildews. The induced transcriptional changes overlapped not only for the responses of cv. Ingrid to the adapted and non-adapted fungal pathogen, but also for the responses to the two different pathosystems (Zellerhoff et al. 2010).

SusPtrit is useful for studying the association between nonhost and partial resistance of barley. This valuable experimental line is not only susceptible to *P. triticina* and several other non-adapted rust fungi, but also extremely susceptible to the adapted *P. hordei* (Atienza et al. 2004; Jafary et al. 2006). QTLs conferring nonhost and partial resistance in other barley accessions have been introgressed into SusPtrit to develop near-isogenic lines (NILs) (Yeo et al., unpublished). These QTL-NILs allow the testing of individual QTLs against non-adapted rust fungi and *P. hordei* without the interference of other QTLs. Subsequently, sub-NILs are developed to fine-map the responsible gene(s) to a small genetic window, which may be spanned by bacterial artificial chromosome (BAC) clones. The candidate gene(s) explaining the resistance QTLs are identified from the sequenced BAC clones, isolated and verified through complementary functional studies.

Functional studies of candidate genes may be conducted via either transient overexpression or transient silencing of genes by particle bombardment, as applied in the studies of candidate resistance genes against powdery mildew (Douchkov et al. 2005; Miklis et al. 2007). For candidate genes of barley against rust fungi, this approach is not feasible because the carriers of the gene constructs cannot reach the mesophyll cells, which are the main cell types that rust fungi target. Transient overexpression and silencing in barley are also feasible through virus-mediated overexpression (VOX) and virus-induced gene silencing (VIGS) (Lee et al. 2012). These approaches, however, are difficult for quantitative resistance because phenotyping with replication is not possible as each individually treated plant is unique. Stable transformation is another alternative for the functional study of candidate genes, although also this approach has its caveats and limitations. Primary transformants transmit the transgene to their offspring through the grains. This transmission to offspring is important because it allows the transgenic line to have multiple identical plants with the transgene, which are necessary to test the line for its level of partial resistance. Stable transformation is possible in barley, but the efficiency of barley transformation is genotype-dependent (Hensel et al. 2008). To date, barley cv. Golden Promise (GP) is the first choice for the standard method of *Agrobacterium*-mediated transformation using immature embryos (IEs). The transformation efficiency of GP can be as high as 86.7 transformants per 100 IEs when the co-cultivation medium is supplemented with L-cysteine and acetosyringone and the *Agrobacterium* strain AGL-1 is used (Hensel et al. 2008).

In a functional study, the resistance allele can be silenced in a resistant plant. In polygenic nonhost resistance, however, an immune plant may have several redundant genes for resistance. A barley accession, such as cv. Vada, may be immune to most, if not all, non-adapted rust fungi, and

the resistance is encoded by several genes (QTLs) (Jafary et al. 2006). Silencing one of the resistance QTLs in Vada might be insufficient to compromise the nonhost resistance enough to alter the immunity to some level of susceptibility. Therefore, testing of the candidate genes for resistance in a susceptible line may be a better option when SusPtrit is the line of choice. SusPtrit is, unfortunately, not amenable to *Agrobacterium*-mediated transformation using the established procedure (Hensel et al. 2008) with minor modification (FKS Yeo and G Hensel, unpublished data). Although the non-inoculated IEs of SusPtrit appeared to be responsive to callus induction media, the callus growth of *Agrobacterium*-inoculated IEs ceased after approximately 2 weeks. A similar situation was observed by W.A. Harwood on cultivars Optic, Oxbridge and Tipple [unpublished data, reviewed by Harwood (2012)].

In this study, a new bi-parental population of doubled haploids was created by crossing SusPtrit with GP. The objective was to find a line, 'Golden SusPtrit', that combines the susceptibility of SusPtrit to non-adapted rust fungi with the amenability of GP for *Agrobacterium*-mediated transformation. In addition, the population was used to map nonhost and partial resistance QTLs, which were compared with QTLs identified earlier in other mapping populations.

Materials and methods

Development of recombinant doubled haploids and general outline

SusPtrit was crossed with cv. GP. F_1 grains were sent to PLANTA Angewandte Pflanzengenetik und Biotechnologie, Germany, to develop a DH population. The haploid/DH plantlets were regenerated from embryogenic pollen cultures, with the DH plants obtained through spontaneous genome duplication. Fertility of spikes was used to indicate the restoration of the diploid condition. The population was inoculated with selected non-adapted and adapted leaf rust fungi, and QTLs were mapped (see below). The most susceptible DH lines were selected and tested for amenability to *Agrobacterium*-mediated transformation.

Whole genome genotyping and linkage map construction

Fresh young leaves of seedlings of the SusPtrit, GP and the DH populations were used to extract genomic DNA according to the CTAB-based protocol of Stewart and Via (1993). The DNA samples were sent for whole genome genotyping (TraitGenetics GmbH, Gatersleben, Germany). Genotyping was performed using an ILLUMINA iSelect 9k barley infinium chip which carries 7,864 SNPs. JoinMap 4.1 (van Ooijen 2006) was used for linkage analysis and map construction. Map distance calculations were made based on Kosambi's mapping function. The linkage groups were assigned to their respective chromosomes based on the linkage map for the Morex/Barke recombinant inbred line population, which was previously genotyped using the same chip (Comadran et al. 2012). The linkage map was used for QTL mapping. The biggest gap in the linkage map was 16 cM on chromosome 6H.

Mapping QTLs for nonhost and partial resistance at the seedling plant stage

Four non-adapted leaf rust fungi, *P. hordei-murini* isolate Rhenen (*Phm*.R), *P. hordei-secalini* isolate France (*Phs*.F), *P. persistens* isolate Wageningen (*Pp*.W), and *P. triticina* isolate Flamingo (*Pt*.F), as well as one adapted leaf rust fungus, *P. hordei* isolate 1.2.1 (*Ph*.1.2.1), were used for disease tests. Inoculum of these pathogens was produced on their respective host plants (Atienza et al. 2004).

The disease tests were carried out in a greenhouse. The seedlings of the SusPtrit/GP (S/G) population were inoculated with the above-mentioned leaf rust fungi in consecutive experiments. For each leaf rust fungus, three consecutive disease tests (series) were performed. In each series, every DH line of the S/G population was represented by one seedling.

Grains of the DH lines, SusPtrit and GP were sown in boxes (37 × 39 cm). Twelve-day-old seedlings with unfolded primary leaves were fixed horizontally with the adaxial side facing up. For non-adapted leaf rust fungi, seven milligrams of spores per box per series were used, amounting to approximately 400 spores deposited per cm^2. For *Ph*.1.2.1, 1 mg of spores (approximately 60 spores per cm^2) per box was applied. The spores were diluted with 10 times their volume of lycopodium spores before inoculating the box in a settling tower (Niks et al. 2011). The inoculated boxes were incubated overnight for 8 h in the dark in a dew chamber set at 18 °C with 100 % relative humidity. Following incubation, the boxes were moved to a greenhouse compartment set at 20 ± 3 °C with 70 % relative humidity.

For non-adapted leaf rust fungi, the infection frequency (IF; number of pustules per cm^2 leaf area) was scored at 12 days post-inoculation. For *P. hordei*, the latency period (LP50S) of the leaf rust fungus was scored and calculated as described by Parlevliet (1975). From the day the first pustules became visible, a mid-section of each seedling leaf was delimited by marker pen, and mature pustules in this section were counted daily using a pocket lens (10×), until the number did not increase anymore (5 or 6 days). The LP of the pathogen on each seedling was evaluated by estimating the number of hours from inoculation to the moment

Table 1 Primer sequences used for the PCR analysis of putative transgenic regenerants

Primer	Sequence 5′–3′	Primer binding site
35S-F2-Catrin	CATGGTGGAGCACGACACTCTC	Bp 331–352 of enhanced *CaMV 35S* promoter
Bie475	TTTAGCCCTGCCTTCATACG	Bp 1421–1440 of *ZmUBI1* promoter
GH-GFP-F1	GGTCACGAACTCCAGCAGGA	Bp 680–661 of *gfp* gene
GH-HYG-F1	GATCGGACGATTGCGTCGCA	Bp 896–877 of *HPT* gene

at which 50 % of the ultimate number of uredinia was visible. Relative infection frequency (RIF) and relative latency period (RLP50S) were calculated relative to the average IF and LP50S, respectively, of all SusPtrit seedlings in each series.

The RIF and RLP50S data were used to map QTLs using MapQTL®6 (van Ooijen 2009). The logarithm of the odds (LOD) threshold to declare a QTL was determined by a permutation test. Only QTLs mapped in at least two of the three series and in the data averaged over the three series were reported. The confidence interval of a QTL is the estimated LOD-2 support interval. When the LOD-2 support interval of two QTLs overlapped, either between QTLs mapped in the different series of one rust fungus or of different rust fungi, they were considered one QTL. Declared nonhost resistance QTLs were designated as *Rphmq*/*Rphsq*/*Rppq*/*Rptq* followed by a number. Partial resistance QTLs to *P. hordei* were designated as *Rphq* (host QTL) followed by a number.

Selection of S/G DH lines for *Agrobacterium*-mediated transformation

The S/G DH lines were ranked based on their RIFs in the first series of disease tests against *Phs*.F and *Pt*.F. Preliminary data on the infection levels of *Phm*.R were available and were used to provisionally rank the DH lines for selection of the most susceptible DH lines. The ten most susceptible DH lines according to the ranking, with an infection severity similar to SusPtrit against the three rust fungi, were selected for *Agrobacterium*-mediated transformation efficiency tests.

Of the ten selected DH lines, four were used to test the amenability to genetic transformation. Growth of donor plants and the transformation protocol were essentially the same as described elsewhere (Hensel et al. 2009). Briefly, developing caryopses were harvested at 12–16 days post-pollination and surface sterilized. The IEs were excised and either pre-cultured on liquid barley co-culture medium for 1 day or directly inoculated with the *Agrobacterium tumefaciens* strain AGL-1 harboring the plasmid pGH215. The plasmid contains the selectable marker gene *HYGROMYCIN PHOSPHOTRANSFERASE* (*HPT*) driven by the doubly enhanced *CaMV 35S* promoter and the synthetic *green fluorescent protein* (*gfp*) gene under the control of the maize *UBIQUITIN 1* promoter with first intron. The agrobacteria were grown, inoculated and co-cultured with the IEs (Hensel et al. 2009). After co-culture, the IEs were transferred to barley callus induction medium supplemented with either 20 or 50 mg/L Hygromycin B (Roche, Mannheim, Germany) to induce calli under selective conditions. After two rounds of 2-week incubations in the dark at 24 °C, the calli were transferred to barley regeneration medium supplemented with 25 mg/L Hygromycin B, then transferred to light.

All regenerants (T_0) were transferred to soil, and genomic DNA was isolated and used for PCR with primers specific for the selectable marker and reporter genes, as described below (Table 1). The transformation efficiency was evaluated based on the number of independent transgenic regenerants per 100 IEs (transgenics/100 IEs).

DNA gel blot analysis and analysis of reporter gene expression

Twenty-one transgenic plants (T_0) from the most efficiently transformed line, SG062N, were randomly selected and subjected to DNA gel blot analysis to determine the transgene copy number. At least 25 µg of genomic DNA was digested with *Hin*dIII, separated by agarose gel electrophoresis and blotted onto a Hybond N membrane. A gene-specific probe (*GFP* or *HPT*) was labeled with digoxigenin-dUTP (DIG-11-dUTP), as recommended by the supplier (Roche, Mannheim, Germany). The 21 T_0 plants of SG062N produced 21 T_1 populations by selfing. Between 21 and 59 T_1 grains harvested from the 21 transgenic T_0 plants of SG062N, as well as from the SG062N wild-type control, were surface sterilized, germinated on solid B5 medium (Gamborg et al. 1968) and incubated under a 16/8 h light/dark regime at 24 °C. After 10–14 days, root tips were screened for GFP fluorescence using a Leica MZFLIII fluorescence microscope equipped with the GFP Plant filter set (Leica Microsystems, Wetzlar, Germany). Genomic DNA of four plants from each T_1 population, pre-selected by the presence/absence of GFP fluorescence in the root tip, was extracted from ~100 mg of snap-frozen leaf tissue, as described in Pallotta et al. (2000). Multiplex-PCR was designed based on the amplification of 100 ng of template primed by the sequences listed in Table 1. Amplicons were separated by agarose gel electrophoresis and

visualized by staining with ethidium bromide. From each of the 21 T_1 populations, three plants that tested positive in the PCR assays, as well as one plant that had lost the transgene via segregation, were subjected to DNA gel blot analysis, as described above, to estimate the transgene copy number and to characterize the integration site(s) regarding linked/unlinked copies.

Results

DH population and linkage map construction

Of the 308 in vitro cultured plantlets, 137 survived and were fertile, thus producing grains. Through whole genome genotyping, duplicate genotypes were identified and eliminated, resulting in a population of 122 unique DH lines.

From the 7,864 SNPs on the chip, 2,943 SNP markers were polymorphic between SusPtrit and GP. Before linkage analysis was performed, 2,257 markers with identical segregation patterns were removed from the data set. We used 686 markers to construct the linkage map. At LOD threshold 10, seven linkage groups corresponding to seven barley chromosomes were detected (Supplemental Fig. 1). The total map length was 1,175 cM. The map length of individual linkage groups ranged from 130 cM (4H, 84SNP markers) to 202 cM (5H, 122 SNP markers).

At our lab we also genotyped two other barley mapping populations (Vada/SusPtrit and Cebada Capa/SusPtrit) with this SNP array, and in addition obtained calculated map positions for 6,534 of the SNP markers from TraitGenetics. The map positions of 4,880 SNP markers that were polymorphic and mapped in at least one of our three mapping populations and also in the TraitGenetics data had excellent agreement in relative genetic distance and linear order on the linkage groups. Only 38 markers had a conflicting assignment to linkage group.

Of the 686 SNP markers, 351 (51 %) exhibited segregation patterns that significantly deviated from the expected 1:1 ratio. Segregation was skewed towards the SusPtrit allele for 213 markers and towards the GP allele for 138 markers. The markers showing distorted segregation occurred in clusters (14 clusters). On linkage groups corresponding to chromosomes 2H and 3H, all of the distorted segregation was skewed towards the SusPtrit allele. In contrast, all of the distorted segregation for markers on chromosome 4H was skewed towards the GP allele.

Disease resistance of S/G recombinants against non-adapted and adapted leaf rust fungi

SusPtrit is susceptible to all four non-adapted leaf rust fungi and to *Ph*.1.2.1. GP is immune to all four non-adapted leaf rust fungi and causes 5 % higher RLP50S (approximately 8 h longer LP) of *Ph*.1.2.1 than SusPtrit. Segregation in the level of resistance among the S/G population was quantitative, suggesting a polygenic inheritance pattern (Fig. 1).

The infection levels observed in the S/G population with the four non-adapted leaf rust fungi ranged from immune to either as susceptible as or more susceptible than SusPtrit. Correlations between the average RIF values for the four non-adapted rust species ranged from 0.5 to 0.7. These values indicate a moderate association in the genetic basis of resistance to these four rust species. This result is consistent with the conclusion made by Jafary et al. (2006, 2008) that genes underlying nonhost resistance have overlapping specificities. Due to the moderate association of susceptibilities to different rust fungi, there were several lines with high susceptibility to more than one rust fungus. Table 2 lists 21 S/G DH lines that were for at least two non-adapted rust fungi among the 20 most susceptible lines of the mapping population.

Generally, the pustules formed by the non-adapted rust fungi on the SG lines were of the compatible type, i.e., they were not associated with chlorosis or necrosis. This was true even for the lines on which few pustules appeared, i.e., lines with fair levels of resistance. For all non-adapted rust types, fewer than 15 lines tested with *Pt*.F, *Phs*.F, or *Phm*.R and fewer than 30 lines tested with *Pp*.W displayed some chlorosis or necrosis; however, in most cases, these reactions were inconsistent over experimental runs.

Nonhost and partial resistance QTL mapping in the S/G population

For each non-adapted rust fungus, the results obtained in the three disease test series correlated well ($r = 0.6$–0.9); however, the correlation between the three disease test series was low for *Ph*.1.2.1 ($r = 0.2$–0.4). Based on permutation tests, a LOD threshold between 2.9 and 3.2 was used for QTL declaration in each mapping attempt.

We found two nonhost resistance QTLs for *Phm*.R, four for *Pt*.F, five for *Phs*.F and six for *Pp*.W (Table 3). As previously described by Jafary et al. (2008), declaring QTLs from LOD profiles may become arbitrary when multiple peaks are observed in the same genomic region. Such was the case for *Pp-nhq5* and *Pp-nhq6* located on chromosome 7H (Table 3; Fig. 2), which we chose to report as two QTLs. These QTLs co-localized with two QTLs against *Phs*.F, *viz. Phs-nhq4* and *Phs-nhq5*, which were indicated by two clearly separated peaks in the LOD profile.

The QTLs mapped for the different non-adapted rust fungi occupied nine genomic regions, among which only one region on 7H affected resistance to all four non-adapted rust species. Four regions had a QTL that significantly contributed to resistance to only one particular

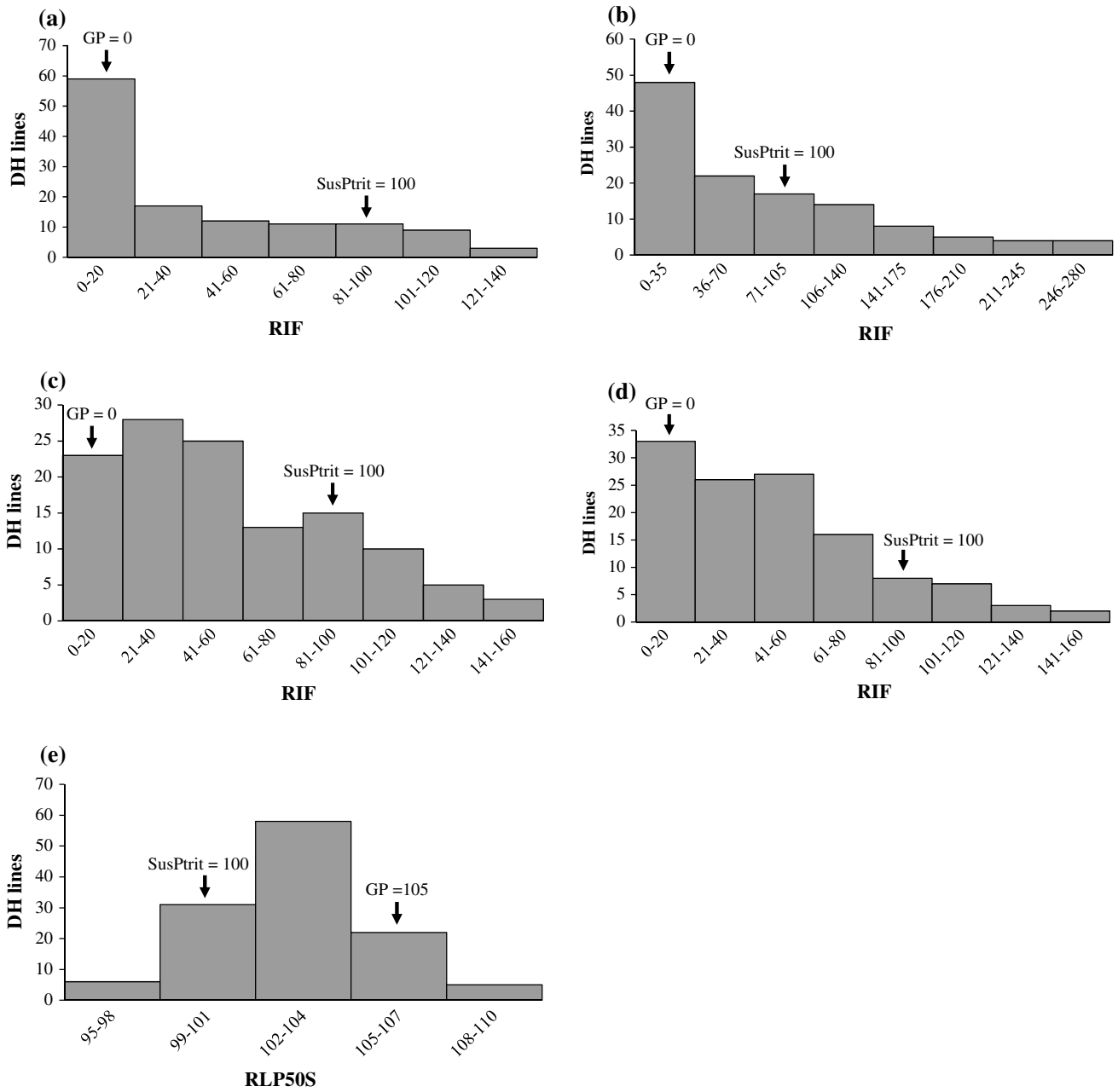


Fig. 1 Frequency distribution of average relative infection frequency (RIF) for **a** *Phm*.R **b** *Phs*.F **c** *Pp*.W **d** *Pt*.F, and **e** frequency distribution of average RLP50S for *Ph*.1.2.1. Values of the parental lines are indicated by *arrows*

non-adapted rust fungus. The four other regions had a QTL that was found to confer resistance to two or three rust fungi (Table 3; Fig. 2). This tendency of QTL regions to affect susceptibility to more than one non-adapted rust fungus may explain why many DHs with high IF to one non-adapted rust fungus also exhibit relatively high IFs to the other non-adapted rust fungi.

Among the QTLs, *Phm-nhq2* at 7H had such a high LOD score (LOD = 21) and such a large effect (explaining 51 % of the phenotypic variation) that it could be considered a major resistance gene. Of the remaining QTLs, seven explained 10–18 % and nine explained <10 % of the phenotypic variation. The immune parent, GP, is the sole resistance allele donor for all of the QTLs mapped for *Phm*.R, *Pp*.W and *Pt*.F. For the QTLs affecting resistance to *Phs*.F, GP donated a resistance allele for four of the QTLs and SusPtrit donated the resistance allele for one QTL. This observation is consistent with the intermediate resistance against *Phs*.F seen in SusPtrit (Fig. 1b).

Table 2 Twenty-one S/G DH lines with level of susceptibility similar to or higher than SusPtrit for more than one rust species

DH line	*Phm*.R	*Phs*.F	*Pt*.F	*Pp*.W	*Ph*.1.2.1	No. of species
SG019N	4	18	8	20	–[a]	4
SG020N	7	1	11	1	–[a]	
SG037N	14	2	4	7	–[a]	
SG048N	19	16	12	9	–[a]	
SG062N	10	10	9	19	–[a]	
SG117N	2	15	17	3	–[a]	
SG038N	14	7	–[a]	16	8	
SG093N	8	5	–[a]	6	15	
SG088N	20	19	6	–[a]	–[a]	3
SG130N	–[a]	6	16	15	–[a]	
SG109N	18	–[a]	2	2	–[a]	
SG047N	12	3	–[a]	4	–[a]	
SG010N	6	4	–[a]	–[a]	–[a]	2
SG078N	17	8	–[a]	–[a]	–[a]	
SG097N	–[a]	14	20	–[a]	–[a]	
SG119N	–[a]	9	18	–[a]	–[a]	
SG068N	–[a]	–[a]	5	8	–[a]	
SG113N	–[a]	–[a]	1	10	–[a]	
SG133N	–[a]	–[a]	7	11	–[a]	
SG075N	11	–[a]	–[a]	17	–[a]	
SG051N	–[a]	12	–[a]	12	–[a]	
SusPtrit	13	38	13	21	17	

Ranking was based on the average of RIF over three series of disease tests

[a] The DH line ranked over 20 based on the average of RIF over three series of disease tests

Only one QTL for partial resistance to *P. hordei* was detected; this QTL mapped to chromosome 6H and explained approximately 14 % of the total phenotypic variation. The resistance allele is donated by GP, as expected. The QTL is designated as *Rphq3* (as explained below). This QTL co-localizes with *Pp-nhq4* and *Pt-nhq3* (Table 3).

Among the non-adapted rust fungi, Jafary et al. (2008) mapped nonhost resistance QTLs with the same isolates used in this study for *Phs* and *Pt* but with different isolates for *Phm* (*Phm* isolate Aragón) and *Pp* (*Pp* isolate RN-8) in the mapping populations V/S and C/S. Jafary et al. (2006) mapped QTLs for partial resistance to *Ph*.1.2.1 in the V/S mapping population. Recently, V/S and C/S were re-genotyped using the same ILLUMINA iSelect 9 k barley infinium chip used for the present S/G map, and new SNPs linkage maps were generated for these two populations (unpublished data). The V/S, C/S and S/G maps were integrated [(Martín Sanz et al., in prep.; Rients Niks (PI), Wageningen University], and we compared the positions of nonhost and partial resistance QTLs mapped in S/G with those mapped in V/S and C/S. Seven out of the nine QTL regions detected in S/G coincided with QTL intervals detected in V/S and C/S. The coinciding QTLs are effective against either the same or to different rust species (Table 3; Fig. 2). On chromosome 6H, the only QTL conferring partial resistance to *P. hordei* of S/G coincided with the nonhost resistance QTLs of Jafary et al. (2008). On the barley integrated map [Barley, Integrated, Marcel 2009 available at http://wheat.pw.usda.gov/GG2/index.shtml; (Aghnoum et al. 2010)], the nonhost resistance QTLs of Jafary et al. (2008) on chromosome 6H coincided with *Rphq3*, a QTL for partial resistance to *P. hordei* mapped in L94/Vada (Marcel et al. 2008; Niks et al. 2000; Qi et al. 1998). It is possible that the partial resistance QTL of S/G also coincided with *Rphq3*; hence, the same name was given.

Amenability of pre-selected DH lines for *Agrobacterium*-mediated transformation

The four DH lines chosen for the *Agrobacterium*-mediated transformation efficiency test were among the 12 most susceptible lines (average ranking) to *Phm.R*, *Phs.F* and *Pt*.F (Supplemental Table 1). Three of the four tested DH lines were amenable to transformation. The efficiency of transformation ranged from 1 to 17 T_0 plants/100 IEs (Table 4). The most efficient DH line was SG062N (11–17 T_0/100 IEs), and its T_0 plants were further analyzed to determine the number of T-DNA copies that were integrated (see below). The transformation efficiency for these lines was approximately 6 % less than for the GP line. Compared to other barley genotypes tested for transformation efficiency using IEs, SG062N had a transformation efficiency better than the 9 barley accessions tested by Hensel et al. (2008)

Table 3 Summary of nonhost and partial resistance QTLs detected in this study at seedling stage in the S/G population and the overlapping QTLs reported in Jafary et al. (2006, 2008)

Chr	Position (cM)	*Phm*.R	*Phs*.F	*Pp*.W	*Pt*.F	*Ph*.1.2.1	Previously mapped QTLs for rust resistance[b]
1H	43–68		*Rphsq1* (4, 11 %, G)[c]				*Phs-nhq* (6, 12 %, V)
2H	40–68			*Rppq1* (4, 10 %, G)	*Rptq1* (4, 11 %, G)		Pp^{RN}-*nhq* (4, 6 %, C)
	98–141		*Rphsq2* (4, 10 %, G)	*Rppq2* (3, 8 %, G)	*Rptq2* (5, 12 %, G)		Pp^{RN}-*nhq* (5, 8 %, V)
3H	112–176	*Rphmq1* (3, 5 %, G)					Phm^{A}-*nhq* (6, 10 %, C) *Phs-nhq* (7, 12 %, C) Pp^{RN}-*nhq* (7, 14 %, C) *Pt-nhq* (8, 22 %, C)
4H	52–75		*Rphsq3* (3, 8 %, S)				*Pt-nhq* (3, 7 %, S) *Phs-nhq* (5, 11 %, S) Pp^{RN}-*nhq* (6, 12 %, S) Phm^{A}-*nhq* (8, 14 %, S)
5H	73–110			*Rppq3* (4, 10 %, G)			
6H	56–88			*Rppq4* (4, 9 %, G)	*Rptq3* (4, 9 %, G)	*Rphq3* (4, 14 %, G)	Pp^{RN}-*nhq* (4, 5 %, C) *Pt-nhq* (11, 19 %, V) Phm^{A}-*nhq* (6, 12 %, C) *Rphq3* (16, 21 %, V)
7H	92–121		*Rphsq4* (4, 9 %, G)	*Rppq5*[a] (6, 14 % G)			*Phs-nhq* (3, 6 %, V) Pp^{RN}-*nhq* (5, 10 %, V) *Pt-nhq* (11, 21 %, V) *Rphq8* (4, 6 %, V)
	141-168	*Rphmq2* (21, 51 %, G)	*Rphsq5* (5, 12 %, G)	*Rppq6*[a] (4, 9 %, G)	*Rptq4* (7, 18 %, G)		

Phm^{A} is a *P. hordei-murini* isolate from Aragón, Spain. Pp^{RN} is a *P. persistens* isolate from Netherlands collection number RN-8. These isolates were used in Jafary et al. (2008) and different from the isolates used in this study

[a] The LOD-2 interval of these QTLs overlapped but they are still considered as two QTLs

[b] Results extracted from Jafary et al. (2006, 2008) except for *Rphq3* extracted from Qi et al. (1998)

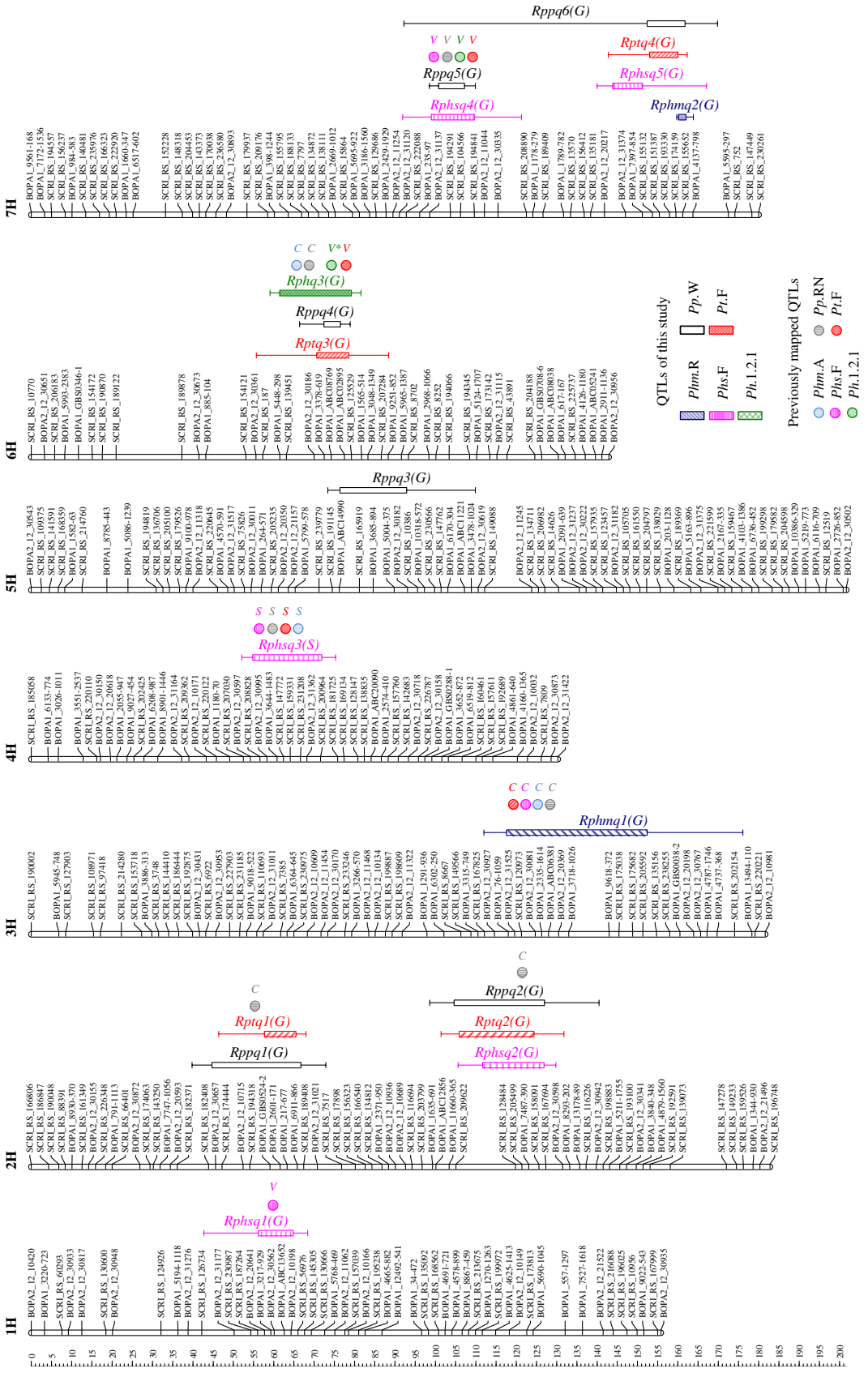
[c] In parenthesis, it gives the QTL LOD score, percentage of explained phenotypic variation and the donor. The LOD score and the percentage of explained phenotypic variation for each QTL from this study were extracted from the mapping result on the average data. The QTL donor for resistance: *C* cebada capa, *G* GP, *S* SusPtrit, *V* Vada

and other barley accessions reviewed in Goedeke et al. (2007).

We compared the genotypic composition of the lines that were tested for transformation efficiency, in particular the lines SG133N that allowed no transformation and SG062N, that appeared as most efficient (Table 4). These lines may indicate the chromosome regions on which genes are located of which the GP alleles enhance transformation efficiency. Presuming that SusPtrit-derived segments in SG062N and GP-derived segments in SG113N are unlikely candidates for carrying important genes for transformation efficiency, we identified six regions that may harbor the genes that provide GP its amenability to transformation. These regions are positioned in chromosome 1H, 2H, 3H, 4H and 6H (Supplemental Fig. 2).

Twenty-one SG062N T_0 plants from three independent transformation attempts were randomly selected to determine the number of T-DNA copies integrated in their genomes (Supplemental Fig. 3). We detected 1–5 integrated T-DNA copies in the genomes of the 21 T_0 plants using *HPT* and *gfp* probes (Table 5). The *gfp* probe suggested that 8/17 of the T_0 plants had single-copy integrations, while the *HPT* probe suggested 10/21 T_0 plants had single-copy integrations. At T_1, GFP fluorescence (Supplemental Fig. 4) indicated that 6 out of the 17 T_1 populations, instead of the expected 8 T_1 populations, were segregating for a

Fig. 2 Skeleton linkage map with position of nonhost resistance QTLs and one partial resistance QTL mapped in this study and the co-localizing partial and nonhost resistance QTLs of Jafary et al. (2006, 2008). Only on chromosome 6H, *V** is a QTL for partial resistance to *P. hordei* mapped in Qi et al. (1998). The QTL bars represent the QTLs of this study. The *bars* correspond to the rMQM LOD-1 and the extended lines correspond to the rMQM LOD-2 confidence interval. The letter inside *parenthesis* indicates the QTL donor (*G* = GP; *S* = SusPtrit). The QTL dots represent the approximate position of peak markers of previously mapped QTLs. The *letter above the dots* represent the QTL donor (*C* Cebada Capa, *S* SusPtrit, *V* Vada). The *ruler on the left side* shows the distance in cM calculated according to Kosambi


Rppq6(G)
Rptq4(G)
Rppq5(G)
Rphsq5(G)
Rphsq4(G)
Rphmq2(G)
Rphq3(G)
Rppq4(G)
Rptq3(G)
Rppq3(G)
Rphsq3(S)
Rphmq1(G)
Rppq2(G)
Rptq2(G)
Rphsq2(G)
Rptq1(G)
Rppq1(G)
Rphsq1(G)
QTLs of this study
Phm.R
Phs.F
Ph.1.2.1
Pp.W
Pt.F
Previously mapped QTLs
Phm.A
Phs.F
Ph.1.2.1
Pp.RN
Pt.F
7H
6H
5H
4H
3H
2H
1H

single-copy T-DNA. This result is because there were two T_1 populations (BG398E21 and BG398E22) that gave segregation ratios of 15:1 (with reporter gene expression:no reporter gene expression), indicating that two copies of T-DNA were segregating. It is possible that the two independently integrated T-DNAs in BG398E21 and BG398E22 cannot be distinguished based on *Hin*dIII DNA digestion. Nine other T_1 populations (excluding BG398E21 and BG398E22) segregated for two or more T-DNA copies, and among the nine, six showed segregation of linked T-DNA copies (Table 5; Supplemental Fig. 5).

Susceptibility of SG062N to non-adapted rust fungi and Ph.1.2.1

The selection of DH lines for *Agrobacterium*-mediated transformation tests was based on preliminary infection data. Additional series of experiments to quantify the susceptibility level were performed following the initiation of the transformation experiments.

This study identified nine genomic regions occupied by resistant QTLs against the four non-adapted rust fungi and *Ph*.1.2.1. At the nine genomic regions, SG062N carried six susceptibility alleles and three resistance alleles. The resistance alleles conferred resistance to *Pp*.W, *Pt*.F and *Phs*.F, and each explained approximately 10 % of the phenotypic variation.

Based on the three series of disease tests for QTL mapping, the level of infection in SG062N for the four non-adapted rust fungi is either similar to or higher than the level of infection in SusPtrit (Table 6). The LP50S against *Ph*.1.2.1 conferred by SG062N is as short as that conferred by SusPtrit in additional disease tests (data not shown). This result is expected because SG062N has the susceptibility allele for *Rphq3*, the only partial resistance QTL detected in this study.

Table 4 Summary of transformation experiments for the four selected DH lines, expressed as number of transformed plants per 100 plated IEs

DH line	Attempt	Total IEs	Number of T_0 plants[d]	T_0 plants/100 IEs
SG062N	BG398-1	210	35	17
	BG398-2	430	49	11
	BG398-3	122	15	12
			Average T_0 plants/100 IEs	**13**
SG047N	BG396-1	420[a]	36	9
	BG396-2	300[b]	7	2
			Average T_0 plants/100 IEs	**6**
	BG399-1	310	30	10
SG093N	BG399-2	180	16	9
	BG399-3	110[b]	1	1
			Average T_0 plants/100 IEs	**8**
	BG400-1	220	0	
SG133N	BG400-2	420[b]	0	
	BG400-3	210[b]	0	
GP	BG405-1	200	36[c]	18
	BG405-2	200	40[c]	20
			Average T_0 plants/100 IEs	**19**

Co-cultivation 2–3 days and selection on 50 mg/L Hygromycin B

[a] 210 IEs were pre-cultured 1 day before co-culture

[b] The IEs were pre-cultured 1 day before co-culture

[c] The number of regenerants positive for root GFP fluorescence detection. Ten randomly selected regenerants were positive for PCR detection of *gfp* and *HPT* genes

[d] The number of regenerants positive for root GFP fluorescence, and for PCR detection of *gfp* and *HPT* genes

Discussion

Genetics of nonhost and partial resistance

Nine chromosomal regions were found to segregate for nonhost resistance in S/G, and of these regions, one co-locates with the only partial resistance QTL, *Rphq3*, mapped in this study. Among the nonhost resistance QTLs mapped in this study, *Phm-nhq2* on chromosome 7H had the largest effect and may be considered a major gene for resistance. The resistance conferred by *Phm-nhq2* is not associated with a hypersensitivity response. The confidence interval of *Phm-nhq2* overlapped with the estimated position of the major gene for resistance to *P. hordei*, *Rph19.ah* (Marcel 2007). This result suggests that either *Phm-nhq2* is an allelic version of *Rph19.ah* or that they are simply at two closely linked loci. GP is not known to carry *Rph19.ah*.

Rphq3 was the only partial resistance QTL found in this study. GP gave an average of 5 % (8 h) longer LP50S than SusPtrit, a relatively low level of partial resistance to *P. hordei* compared to Vada, which has a high level of partial resistance. The LP50S of *P. hordei* on Vada is approximately 25 % longer than on the susceptible accessions SusPtrit (Jafary et al. 2006) and L94 (Qi et al. 1998). Additionally, Vada has three QTLs effective at the seedling stage (Jafary et al. 2006; Qi et al. 1998). It is not surprising, then, to find only *Rphq3* in S/G; however, there may be other QTLs with effects too small to be detected.

GP is immune to non-adapted rust fungi and contains many genes (nine chromosomal regions) for resistance to the four non-adapted rust species tested. This finding is

Table 5 Copy number of integrated T-DNA for the 21 SG062N T_0 plants and segregation of their T_1 populations

T_0			T_1 populations			
Transformants (T_0)	Copy number according to *gfp* probe	Copy number according to *HPT* probe	Reporter gene expression vs. no expression	Segregation observed (assumed)	X^2 value	Likelihood (P) according to X^2 test
BG398E06	1	1	39:18	2.2:1 (3:1)	1.92	>0.10
BG398E07	1	1	42:17	2.5:1 (3:1)	0.64	>0.40
BG398E09	1	1	27:12	2.2:1 (3:1)	0.70	>0.40
BG398E10	1	1	37:12	3.1:1 (3:1)	0.03	>0.90
BG398E11	1	1	36:15	2.4:1 (3:1)	0.54	>0.40
BG398E14	1	1	25:14	1.8:1 (3:1)	2.43	>0.10
BG398E21	1	1	35:3	12:1 (15:1)	1.33	>0.20
BG398E22	1	1	48:1	48:1 (15:1)	1.53	>0.20
BG398E01	2	2	21:0	21:1 (15:1)	3.40	>0.05
BG398E12	2	2	44:4	11:1 (15:1)	0.36	>0.60
BG398E03	2	3	26:12	2.2:1 (3:1)	2.17	>0.10
BG398E18	3	1	44:0	44:0 (15:1)	3.02	>0.05
BG398E17	3	2	46:9	5:1 (3:1)	2.17	>0.10
BG398E16	3	3	53:0	53:0 (63:1)	2.59	>0.10
BG398E20	3	4	52:6	9:1 (15:1)	4.09	>0.05
BG398E19	4	4	45:0	45:0 (15:1)	3.00	>0.05
BG398E05	5	5	36:12	3:1 (3:1)	0.00	>0.99
BG398E04	nd	1	nd	nd	nd	nd
BG398E02	nd	2	nd	nd	nd	nd
BG398E08	nd	2	nd	nd	nd	nd
BG398E15	nd	2	nd	nd	nd	nd

Table 6 The susceptibility of SG062N relative to SusPtrit against the four non-adapted rusts tested over three series per rust species

Non-adapted rusts	Average relative infection frequency	
	SusPtrit	SG062N
Phm.R	100	104
Phs.F[a]	100	189
Pp.W	100	100
Pt.F	100	105

[a] SusPtrit has one resistance QTL

very similar to the results obtained by Jafary et al. (2006, 2008) for Vada and Cebada Capa. It is, however, possible to find DH lines with susceptibility as high as, or higher than, that of SusPtrit.

Among the nine chromosomal regions with resistance QTLs identified in S/G, five regions conferred resistance to different rust fungi, suggesting that the responsible genes have effects on multiple rust species. Jafary et al. (2006) observed that QTLs affecting multiple rust species do not tend to be effective against taxonomically related rust species. Based on the phylogenetic tree of the rust species constructed by Jafary et al. (2006), we observed three genomic regions where the co-localization only involved QTLs affecting resistance to closely related rust species (*Phs*.F, *Pp*.W and *Pt*.F) (Table 3). In the other three regions, the QTLs were effective against less closely related rust species (QTLs for *Phm*.R and *Ph*.1.2.1 overlapped with QTLs for *Phs*.F, *Pp*.W and *Pt*.F). Co-localization of the QTLs for nonhost and partial resistance suggests an overlap of gene sets for these types of resistance in barley. Jafary et al. (2008) also observed similar QTL co-localization. Furthermore, several other studies (Hoogkamp et al. 1998; Zellerhoff et al. 2010; Zhang et al. 1994) have suggested that nonhost and partial host resistance may partly involve the same genes.

The tendency for co-localization of QTLs for different rust fungi may be due to either several closely linked genes, each involved in resistance to only one or two rust species, or to a single gene that contributes to resistance to multiple rust species. Fine-mapping is required to distinguish between these two possibilities. Ultimately, cloning the gene(s) can demonstrate their effectiveness to one or more diseases as demonstrated by the cloning of ATP-binding cassette (ABC) transporter genes (Krattinger et al. 2009).

SG062N, a new experimental line for nonhost and partial resistance studies

As in most monocotyledonous plant species, barley transformation efficiency is limited by genotype, explant, and media components, among other factors [reviewed in (Cheng et al. 2004; Goedeke et al. 2007; Harwood 2012)]. To improve transformation efficiency, adjusting treatment and tissue culture variables can be tried [e.g., the use of different *Agrobacterium* strains or the application of acetosyringone and L-cysteine (Hensel et al. 2008)]. Improving transformation efficiency for one genotype (e.g., GP) is helpful, but the transformable line may not be ideal for studying specific traits—in this case, GP is not suitable for the functional study of nonhost resistance. The line of choice to study nonhost resistance, SusPtrit, was unsuccessfully tested for amenability to *Agrobacterium*-mediated transformation (data not shown). Therefore, we applied a breeding approach to combine the amenability of GP for *Agrobacterium*-mediated transformation with the susceptibility of SusPtrit to non-adapted rust fungi.

Theoretically, the S/G mapping population can be used to locate genetic factors affecting transformation efficiency, as described in Cogan et al. (2002, 2004). It is not practical, however, to apply the *Agrobacterium*-mediated transformation procedure used in our study to a mapping population because of the labor and greenhouse space required. The high non-genetic variation in the transformation efficiency of a single line between experimental runs is another factor that complicates the mapping of such genes in barley. This variation can be attributed to variables such as the actual environmental conditions for transformation and tissue culture, the quality of explant donor plants and the individual handling of the experiment (Hensel et al. 2008). The high transformation efficiency of GP is likely a result of several genes, as in *Brassica oleracea* (Cogan et al. 2002, 2004). Hence, quantitative variation was observed in the transformability of the four pre-selected DH lines. We compared the genotypes of SG062N (highest transformation efficiency) and SG133N (not transformable) and found six chromosomal regions potentially involved in the transformation efficiency of barley (Supplemental Fig. 2).

To date, GP is the line of choice for standard barley transformation. Notably, GP is a gamma-ray-induced mutant derived from cultivar Maythorpe (Forster 2001). The efficiency for transformation of GP is most likely not a result of the mutation, as Maythorpe can be transformed approximately as efficiently as GP. The transformation efficiency of Maythorpe ranges from 6 to 19 % (G. Hensel and J. Kumlehn, unpublished data) and has been reported to reach 25 % in one experiment [W.A. Harwood, unpublished data (John Innes Centre, Norwich, UK)]. In the Germplasm Resources Information Network (GRIN, http://www.ars-grin.gov/npgs/holdings.html), the ancestors of GP/Maythorpe are traced back to Chevalier, Hana and Gull (Supplemental Fig. 5). Tracing the ancestor that has donated the genetic factors for efficient transformation can provide valuable information.

By crossing SusPtrit with GP, the susceptibility of SusPtrit to non-adapted and adapted (*P. hordei*) rust fungi and the amenability of GP to *Agrobacterium*-mediated transformation were easily combined. Simple screening of the progeny for individual lines that had inherited traits of both SusPtrit and GP was sufficient to verify that we had achieved our objective of obtaining the valuable new experimental line—SG062N (Golden SusPtrit). The optimized transformation procedure for GP can be applied directly to Golden SusPtrit to obtain approximately 47 % of transformants with single-copy T-DNA integration (based on the *gfp* probe), which is fairly comparable to the proportion (50 %) reported by Hensel et al. (2008).

Golden SusPtrit is as susceptible as SusPtrit to *P. hordei* and to the four tested non-adapted rust fungi. As such, Golden SusPtrit will replace SusPtrit as a valuable experimental line for future nonhost and partial resistance studies, especially for stable transformation with candidate genes that might be responsible for resistance.

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Conflict of interest The authors declare that they have no conflict of interest.

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