Isolate Specificity and Polygenic Inheritance of Resistance in Barley to the Heterologous Rust Pathogen *Puccinia graminis* f. sp. *avenae*

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ABSTRACT

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Barley is a near-nonhost to numerous heterologous (nonadapted) rust pathogens because a small proportion of genotypes are somewhat susceptible. We assessed 66 barley accessions and three mapping populations (Vada × SusPtrit, Cebada Capa × SusPtrit, and SusPtrit × Golden Promise) for response to three Swedish oat stem rust (*Puccinia graminis* f. sp. *avenae*) fungal isolates and determined that barley is a near-nonhost to *P. graminis* f. sp. *avenae* and that resistance was polygenically inherited. The parental

genotypes Vada and Golden Promise were immune to all three isolates, whereas Cebada Capa was immune to two isolates and moderately resistant to the third. Phenotypic data from the Vada × SusPtrit mapping population and the barley accessions tested also demonstrated isolate-specific resistance. In particular, the SusPtrit parent and several other accessions allowed sporulation by isolate Ingeberga but were resistant to isolate Evertsholm. Nine chromosomal regions carried quantitative trait loci (QTL) (*Rpgaql* to *Rpgaq9*) of varying effect, most of which collocated to previously identified QTL for resistance to other heterologous rust pathogens. *Rpgaql* on chromosome 1H (Vada and Golden Promise) was effective toward all isolates tested. Microscopic examination indicated that resistance was prehaustorial in Vada whereas, in SusPtrit, both pre- and posthaustorial mechanisms play a role.

Stem rust, caused by *Puccinia graminis*, attacks many grasses, including the economically important cereals (wheat, barley, and oats) on which severe levels of infection can cause total crop failure. *P. graminis* comprises subspecies and *formae speciales*, based primarily on infection structure morphology and host range (Leonard and Szabo 2005; Niks 1986). Oat stem rust, caused by the *forma specialis* *P. graminis* f. sp. *avenae*, is a major constraint to oat (*Avena sativa* L.) production in Europe and Australia. For instance, Mellqvist and Waern (2010) reported yield losses of up to 30% in untreated Swedish oat fields. Sexual recombination on the alternate barberry (*Berberis vulgaris* L.) host facilitates meiotic recombination, often resulting in new virulence on previously resistant oat cultivars (Berlin et al. 2013; McDonald and Linde 2002). Sexual reproduction in Swedish *P. graminis* f. sp. *avenae* populations has been reported frequently, especially since the repeal of legislation to eradicate barberry (*Berberis* spp.) bushes in the agricultural landscape (Berlin et al. 2013). Nonetheless, in the absence of barberry, the pathogen survives and reproduces on wild oats, volunteer oat plants, and certain grass species (Burdon et al. 1992). Changes in the pathogen populations in areas where alternate hosts are absent are thought to be caused by step-wise mutations, genetic drift, and the immigration of exotic genotypes. The ability of *P. graminis* f. sp. *avenae* to develop virulence for resistance genes in commercial oat cultivars is of particular concern to oat breeders, who now seek more durable sources of resistance.

Disease resistance shown by all genotypes of a particular plant species to a specific pathogen species is known as nonhost resistance.

The concept of nonhost resistance has gained significant research interest from an evolutionary point of view. Due to its presumed durability and lack of specificity toward variants of a pathogen, a better understanding of nonhost resistance may lead to more effective breeding and control options. The differentiation between host and nonhost status is sometimes unclear (Heath 1985; Niks 1987). Within a presumed nonhost plant species, there are occasionally a few genotypes that are moderately susceptible to a normally heterologous (nonadapted) rust pathogen. Such occasional receptivity (Niks 1987) is also referred to as near-nonhost (Atienza et al. 2004) or marginal host status (Jafary et al. 2006). Barley (*Hordeum vulgare* L.) has been reported to be a near-nonhost to several heterologous rust pathogens (Atienza et al. 2004; Mains 1933; Martens et al. 1977; Niks 1987; Niks et al. 1996). Due to the availability of useful genetic resources (Atienza et al. 2004; Jafary et al. 2006, 2008) and recent genomic advancements, the barley-*Puccinia* spp. interaction represents one of the most attractive model systems to study the genetics underlying host and (near-) nonhost status in crop species.

Atienza et al. (2004) developed an experimental barley line, SusPtrit (susceptible to *P. trititica*), by accumulating susceptibility alleles from four unrelated barley accessions that were somewhat susceptible at the seedling stage to the heterologous wheat leaf rust pathogen (*P. trititica*). SusPtrit at the seedling stage was not only as susceptible to *P. trititica* as a regular wheat seedling but, also, more or less susceptible to several other heterologous rust pathogen species (Atienza et al. 2004). Subsequent genetic studies in different mapping populations based on the use of SusPtrit as a parent indicated that resistance to heterologous rust pathogens in immune barley cultivars such as Vada, Cebada Capa, and Golden Promise is polygenically inherited with the rare involvement of a major resistance (*R*) gene (Jafary et al. 2006, 2008; Yeo et al. 2014). The regular nonhost immunity of Vada, Cebada Capa, and Golden Promise to heterologous rust fungi is due mostly to different genes, and there were overlapping specificities when the same quantitative trait loci (QTL)

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in a cultivar might confer resistance to more than one heterologous rust pathogen (Jafary et al. 2006, 2008; Yeo et al. 2014).

Previous studies indicated that *P. graminis* f. sp. *avenae* can infect a few barley accessions at the seedling stage, suggesting that it may be a near-nonhost to *P. graminis* f. sp. *avenae*. A barley selection (73-G1) that was somewhat susceptible to *P. graminis* f. sp. *avenae* demonstrated that resistance was heritable and differed from the T gene (now designated *Rpg1*) conferring resistance to the wheat stem rust pathogen (*P. graminis* f. sp. *tritici*) (Martens et al. 1983). A recent study demonstrated the presence of prehaustorial resistance in a doubled haploid (DH) population based on the Australian barley cultivars Yerong and Franklin in response to three different *P. graminis* f. sp. *avenae* pathotypes from Australia and Canada (Dracatos et al. 2014). The QTL identified in that population (*Rpgal* to *Rpga5*) were located on chromosomes 1HS, 5HL, and 7HS, mainly at locations not previously reported in other barley mapping populations to contain genes for resistance to heterologous rusts (Jafary et al. 2006, 2008; Yeo et al. 2014). Furthermore, no *P. graminis* f. sp. *avenae* isolate specificity was identified in the QTL mapping experiments (Dracatos et al. 2014). Previous studies have determined that, in some cases, resistance to *P. graminis* f. sp. *avenae* in barley is simply inherited (Dracatos et al. 2015; Martens et al. 1983). Dracatos et al. (2015) intercrossed barley cv. Q21861 (*Rpg5*—immune to *P. graminis* f. sp. *avenae*) and the experimental line 73-G1 (*rpg5*) and determined that the *Rpg5* locus on chromosome 5HL conferred resistance to both Australian pathotypes of *P. graminis* f. sp. *avenae* tested. This suggests that *Rpg5* confers resistance to multiple stem rust *formae speciales*, including *P. graminis* f. sp. *avenae* (Dracatos et al. 2015), *P. graminis* f. sp. *tritici* (Jin et al. 1994), and the rye stem rust pathogen (*P. graminis* f. sp. *secalis*) (Sun et al. 1996). The current study was aimed at determining the host status of barley to *P. graminis* f. sp. *avenae*, the extent of isolate specificity of resistance QTL in barley, and the histology of resistance in Vada and SusPtrit barley compared with *P. graminis* f. sp. *avenae* infection on oats.

MATERIALS AND METHODS

Plant materials. A subset (66—due to lack of seed) of the previously described collection of 110 spring barley (*Hordeum vulgare* L.) accessions from diverse geographic origins (Atienza et al. 2004) was used to assess the host status of barley to *P. graminis* f. sp. *avenae*. The collection comprised modern and old cultivars, landraces, and wild accessions for which passport data were available (Atienza et al. 2004). Two F₉ recombinant inbred line (RIL) mapping populations (Vada × SusPtrit and Cebada Capa × SusPtrit and a DH population [SusPtrit × Golden Promise]) (Jafary et al. 2006, 2008; Yeo et al. 2014) were used for genetic studies of resistance to *P. graminis* f. sp. *avenae*. Genetic maps were available for these populations Vada × SusPtrit (152 RILs [Jafary et al. 2006]), Cebada Capa × SusPtrit (113 RILs [Jafary et al. 2008]) and SusPtrit × Golden Promise (122 DH lines [Yeo et al. 2014]).

Pathogen materials. Three field samples of *P. graminis* f. sp. *avenae* (Pattala, Ingeberga, and Evertsholm) were collected from different locations in southern Sweden (Table 1). All field-derived *P. graminis* f. sp. *avenae* isolates could infect SusPtrit barley. Single pustule (SP) isolates were generated for the three field samples. To determine the virulence or avirulence of the three SP-derived *P. graminis*

f. sp. *avenae* isolates, 19 oat differential testers were used for resistance genes including *Pg1*, *Pg2*, *Pg3*, *Pg4*, *Pg8*, *Pg9*, *Pg10*, *Pg13*, *Pg15*, *Pg16*, *Pg17*, *PgSaia*, *PgA*, and the susceptible cultivar Swan. The Evertsholm SP isolate was developed from a random SP on Alfred oat. In initial studies, this SP isolate was unexpectedly not able to infect SusPtrit and only caused numerous pinpoint pale chlorotic flecks but no pustules. For Ingeberga and Pattala field isolates, we selected SP isolates from SPs that appeared on SusPtrit barley, hence assuring their pathogenicity on this barley accession. All SP isolates were subcultured onto adult susceptible Alfred oat plants for spore multiplication. Spore multiplication of SP isolates was performed in different greenhouse compartments, to prevent cross-contamination. Urediniospores of each SP isolate were collected, weighed, placed in a glass vial and were stored in a desiccator at room temperature. Surplus batches of spores were wrapped in aluminum foil, were placed in cryovials, and then, were stored in liquid nitrogen for future use.

Inoculation procedure. Nine days after sowing, the first leaf of each seedling was pinned horizontally to the soil with the adaxial side facing up. Seedlings for each line of the three mapping populations and the 66 diverse barley accessions were then inoculated with freshly collected urediospores in a settling tower. For every box containing 30 to 40 seedlings, 6 mg of urediospores mixed with 48 mg of lycopodium were applied to ensure uniform distribution of about 360 urediospores per square centimeter (Atienza et al. 2004). Following inoculation in a settling tower (Niks et al. 2011), the seedlings were incubated in a humidity chamber overnight at 100% relative humidity at approximately 20°C to permit urediniospore germination. On the next day, the plants were moved to a greenhouse compartment under both artificial and natural light. Rust testing was performed in two consecutive experiments on two to three seedlings per line per isolate. Only one isolate inoculation was performed per day, in order to prevent cross contamination of isolates. Oat differential lines were sown in five-inch pots at four lines (eight to 10 seeds per line) per pot and were inoculated using the settling tower and incubation conditions described above.

Phenotypic assessment of *Puccinia graminis* f. sp. *avenae* on barley. The three mapping populations and diverse barley accessions were assessed phenotypically with three (Ingeberga, Pattala, and Evertsholm) and two (Ingeberga and Evertsholm) Swedish SP *P. graminis* f. sp. *avenae* isolates, respectively. Barley seedlings (three per line) were grown in boxes (37 × 39 cm) along with their parents and susceptible host (oat cv. Alfred). Twelve days after inoculation, the level of infection was quantified by two parameters: visible infection sites per leaf (VIS) (the number of both flecks and pustules per leaf) and infection frequency (IF) (the number of pustules per leaf) (Jafary et al. 2006). For VIS, flecks smaller than 0.5 mm in diameter (like the pinpoint flecks on SusPtrit with Evertsholm) were excluded from the counts. The level of infection of the panel of diverse barley accessions was scored according to a 0 to 5 scale, in which 0 indicates immunity or near-immunity (no visible sign of infection) and 5 (more than 500 pustules per leaf) (Atienza et al. 2004; Table 2). For QTL mapping, we first calculated the average VIS and IF for each line over the two or three seedlings per isolate. Secondly, VIS and IF ratings for each line were scaled by multiplication to obtain equal experiment averages. Correlation coefficients between replicates were also calculated, to establish the reproducibility of the phenotypic data.

TABLE 1. Characteristics of Swedish *Puccinia graminis* f. sp. *avenae* isolates used in this study

Isolate	Virulence on resistance genes ^z	Location of collection		Barley accession			
		Longitude	Latitude	Vada	Golden Promise	Cebada Capa	SusPtrit
Evertsholm	<i>Pg1</i> , <i>Pg2</i> , <i>Pg3</i> , <i>Pg4</i> , <i>Pg9</i> , <i>PgA</i> , <i>Pg17</i>	58.49	16.26	Immune	Immune	Immune	Resistant
Pattala	<i>Pg1</i> , <i>Pg3</i> , <i>Pg8</i> , <i>Pg17</i>	59.05	16.43	Immune	Immune	Resistant	Moderately resistant
Ingeberga	<i>Pg1</i> , <i>Pg3</i> , <i>Pg8</i> , <i>Pg9</i> , <i>Pg13</i> , <i>PgSaia</i> , <i>Pg17</i>	59.65	16.69	Immune	Immune	Resistant	Moderately susceptible

^z All three single-pustule isolates were tested independently on 19 oat differential genotypes for virulence with respect to resistance genes *Pg1*, *Pg2*, *Pg3*, *Pg4*, *Pg8*, *Pg9*, *Pg10*, *Pg13*, *Pg15*, *Pg16*, *Pg17*, *PgSaia*, and *PgA* and the susceptible cultivar Swan.

QTL mapping. QTL analysis was performed on the three mapping populations using MapQTL 6 software (Van Ooijen 2009), using VIS and IF (Jafary et al. 2006), both averaged over the two experiments per mapping population-isolate combination. Mapping consisted of three subsequent steps (Qi et al. 1998). First, interval mapping was used to detect putative QTL (logarithm of odds [LOD] > 3). Markers with the highest LOD values (peak markers) were taken to serve as cofactors in subsequent multiple QTL model (MQM) mapping. When LOD values of some markers on other regions reached the significance level, the MQM was repeated after adding those new peak markers as cofactors, and when LOD values for other markers dropped to lower than the significance level, cofactors were dropped until a stable LOD profile was reached. Finally, restricted MQM mapping using the cofactor markers of the final MQM run was performed. In all methods, a LOD threshold of three was used to declare QTL as significant.

Comparing QTL. To compare QTL positions between linkage maps, the LOD-1 support intervals and peak markers were marked on the high-density integrated genetic map posted on the GrainGenes website (<http://wheat.pw.usda.gov>) (Aghnoum et al. 2010). The Aghnoum et al. (2010) integrated map is based on restriction fragment length polymorphisms (20%), amplification fragment length polymorphisms (AFLP) (20%), simple sequence repeats (SSR) (9%), diversity arrays technology (DArT) (19%), and transcript-derived (TDM) markers. On the integrated map, QTL for partial resistance to leaf rust (*P. hordei*) and nonhost resistance to various heterologous rusts are indicated, enabling comparison of QTL positions to different rust species and isolates (Jafary et al. 2008; Niks et al. 2015). The QTL positions in the Vada × SusPrit and Cebada Capa × SusPrit linkage maps were converted to the positions on the integrated map of Aghnoum et al. (2010). QTL regions associated with resistance to different isolates or in different populations, or both, were considered to be the same if there was overlap between their LOD-1 support intervals on the integrated map.

The SusPrit × Golden Promise genetic map had been constructed on the basis of whole-genome genotyping (TraitGenetics GmbH, Gatersleben, Germany), using an ILLUMINA iSelect 9k barley Infinium chip that carries 7,864 single nucleotide polymorphisms (SNP) (Yeo et al. 2014). The SusPrit × Golden Promise map could not be integrated into the consensus map of Aghnoum et al. (2010), due to the paucity of markers in common. Thus, it was not straightforward to place the SNP peak markers in the SusPrit × Golden Promise population on the integrated map that was used for Vada × SusPrit and Cebada Capa × SusPrit. Each SNP peak marker in SusPrit × Golden Promise was assessed to determine whether it was polymorphic in Vada × SusPrit or in Cebada Capa × SusPrit. For each of these two mapping populations, 87 RILs had been genotyped on the same ILLUMINA chip as was used for SusPrit × Golden Promise. If the population was polymorphic for the relevant SusPrit × Golden Promise marker, we identified the AFLP marker that, for those 87 RILs, cosegregated best with the SNP marker.

Histology of the resistance. Six seedlings, each, of SusPrit, Vada, Cebeco (oat), and Alfred (oat) were inoculated with *P. graminis* f. sp. *avenae* isolate Evertsholm. Six days after inoculation, four leaf samples per accession, each about 3 cm long, were collected. The two remaining seedlings were used to record macroscopic infection types. The collected leaf samples were then stained with Uvitex-B, as described previously (Hoogkamp et al. 1998; Niks 1982), to evaluate development stage and size of 50 infection units per leaf sample, under a Zeiss Axiophot photo microscope with an aniline blue filter. The infection units were classified into three development classes based on the classification by Niks and Kuiper (1983): non-penetrating appressoria, aborted substomatal vesicles, early aborted (EA) colonies (with six or less haustorial mother cells) without or with necrosis, and established (EST) colonies (branched hyphae with more than six haustorial mother cells) with or without necrosis. The latter group included Niks and Kuiper's (1983) class of successful colonies, since it was not possible to judge which infection units, at a

later sampling time, might have succeeded to produce spores. Within the EA and EST colony classes, we recorded whether infection units were associated with necrotic plant cells. Such necrosis was visible as autofluorescence in plant cells.

The rationale of the recording is the following (Niks 1982, 1983; Niks and Kuiper 1983). Of all infection units that penetrate, a proportion is arrested within about 24 h after inoculation (EA), while the remaining establish a feeding relationship to support at least some growth and development of mycelium (EST). These established infection units should have formed at least some haustoria. Some *Rph* genes act very early and cause EA just during the formation of the very first haustoria (Niks and Kuiper 1983). This resistance appears as a high proportion of EA with high association with plant cell necrosis. Other types of resistance, i.e., partial resistance and nonhost resistance, typically reduce the success in haustorium formation, causing high EA essentially without necrosis of plant cells (Niks 1982; Niks and Kuiper 1983). Late acting *Rph* genes arrest mycelial colonies after establishment, also with association with plant cell necrosis. Partial and nonhost resistance also strongly reduce mycelial growth but, typically, as with EA, with very little plant cell necrosis. Therefore, we take (i) a high proportion of EA with hardly any plant cell necrosis and (ii) strongly reduced growth of those infection units that get established with necrosis as markers for prehaustorial resistance mechanisms, and we take EA and EST with strong association with plant cell necrosis as markers for posthaustorial resistance. Low proportions of EA and large size of EST colonies are interpreted as indication for high susceptibility.

The experiment was repeated twice. Per accession, the results are based on approximately 50 infection units × 4 leaf samples × 2 replicates = 400 infection units. The percentage of nonpenetrating infection units was calculated by dividing the number of non-penetrants by the total number of infection units. The proportion of the early abortion class was obtained by dividing the number of EA infection units by the total number of infection units that were EA or established.

RESULTS

Host status of barley with respect to *P. graminis* f. sp. *avenae*. Sixty-six diverse barley accessions were inoculated with two *P. graminis* f. sp. *avenae* SP isolates (Evertsholm and Ingeberga) to quantify the host status of barley to *P. graminis* f. sp. *avenae*, identify possible isolate-specific phenotypic responses in barley, and to compare the responses to other previously tested heterologous rusts (Atienza et al. 2004). The phenotypic data indicated that 89% (Evertsholm) and 59% (Ingeberga) of the barley

TABLE 2. Susceptibility scores (0 to 5) for eight heterologous rust pathogens and adapted *Puccinia hordei* tested on diverse barley accessions

Pathogen	Susceptibility score ^y					
	0	1	2	3	4	5
<i>P. agropyrim</i>	39	7	9	37	8	0
<i>P. triticina</i> Flamingo	56	10	20	13	3	0
<i>P. hordei-secalini</i>	30	37	9	16	7	1
<i>P. graminis</i> f. sp. <i>lolii</i>	72	0	15	11	3	0
<i>P. hordei-murini</i> Rhenen	59	27	3	3	8	0
<i>P. coronata</i> f. sp. <i>avenae</i>	67	29	4	0	0	0
<i>P. graminis</i> f. sp. <i>avenae</i> (Evertsholm) ^z	89	5	1	5	0	0
<i>P. graminis</i> f. sp. <i>avenae</i> (Ingeberga) ^z	59	11	13	17	0	0
<i>P. hordei</i>	3	0	0	5	66	26

^y Susceptibility score (per leaf): 0, immune or near immune (less than three pustules and few flecks); 1, less than three pustules and medium or many flecks; 2, three to 10 pustules; 3, 10 to 100 pustules; 4, more than 100 pustules; 5, more than 500 pustules.

^z The phenotypic data (percentage of lines with the different susceptibility scores) for *P. graminis* f. sp. *avenae* is based on the assessment on a subset of 66 barley accessions as described by Atienza et al. (2004).

accessions did not allow pustule formation at the seedling stage, indicating that barley is a near-nonhost to *P. graminis* f. sp. *avenae* (Table 2). High susceptibility (scores of 4 or 5) was not observed in response to either the Evertsholm or Ingeberga isolates, in contrast to scores obtained with the adapted *P. hordei* and some other heterologous rust pathogens (Table 2). More of the barley accessions were (near) immune to the *P. graminis* f. sp. *avenae* isolate Evertsholm (89% score 0) than to other heterologous rust pathogens, including the Ingeberga isolate (59% score 0). Hence, pathotype specificity was observed for *P. graminis* f. sp. *avenae* on barley. The isolate-specific character of the resistance was observed for barley accessions SusPtrit (Fig. 1; Table 2), SusPmur, and Trigo Biasa (data not shown) that were resistant (pinpoint flecks), scoring 0 with Evertsholm and 3 for Ingeberga. Such isolate specificity may reflect a shared *R* gene in Trigo Biasa and other related accessions. Indeed, Trigo Biasa was one of the ancestors of SusPtrit (Atienza et al. 2004).

Genetic analysis of resistance. All SP isolates used in this study differed with respect to their virulence on 19 Australian oat stem rust differential genotypes (Table 1). The parental lines Vada and Golden Promise were immune to the three SP isolates of *P. graminis* f. sp. *avenae*, whereas Cebada Capa was immune to *P. graminis* f. sp. *avenae* isolate Evertsholm but resistant (few pustules and flecks) to Pattala and Ingeberga (Table 1). SusPtrit was moderately susceptible to isolate Ingeberga, moderately resistant to Pattala, and highly resistant to isolate Evertsholm (Fig. 1A, 1B and 1C). Resistance in SusPtrit in response to isolate Evertsholm was associated with many pinpoint flecks of less than 0.5 mm in diameter (Fig. 1A). Remarkably, isolate Ingeberga had a strong tendency to produce many more telia than uredinia on barley seedlings (Fig. 1B) but not on oat seedlings (Fig. 1D). In all experiments, the susceptible oat control Cebeco was heavily infected by all *P. graminis* f. sp. *avenae* isolates.

Seedlings of all three barley mapping populations, Vada × SusPtrit, Cebada Capa × SusPtrit, and SusPtrit × Golden Promise, were phenotyped for VIS and IF with all three *P. graminis* f. sp. *avenae* isolates. Two experimental replicates were performed for each mapping population-isolate combination, and in all cases, a positive correlation of 0.7 was observed between the two replicates. In experiments with *P. graminis* f. sp. *avenae* isolate Pattala and Ingeberga, SusPtrit was the more or less susceptible parent in all three mapping populations and segregation for VIS and IF was observed, including, in some populations, the presence of some

lines that were more heavily infected than SusPtrit (Fig. 2). VIS (Fig. 2) and IFs (data not shown) observed in the mapping populations inoculated with Pattala and Ingeberga indicated continuous variation, where the level and characteristics of infection ranged from complete immunity to a similar or higher number of flecks and pustules than observed on SusPtrit (Fig. 2). This continuous unimodal variation suggests polygenic inheritance. Because all parental genotypes were either resistant or immune to the Evertsholm isolate, segregation in the mapping populations would only occur if the parents did not share one or more major-effect resistance genes. No segregation was observed for reaction to Evertsholm in the Cebada Capa × SusPtrit and SusPtrit × Golden Promise mapping populations, but 25% of the RILs of the Vada × SusPtrit mapping population allowed at least one sporulating pustule per leaf (Fig. 2G). This segregation suggests that the resistance of Vada to isolate Evertsholm is based on one or more different genes than the resistance of SusPtrit.

QTL analysis of mapping populations. In total, nine chromosomal regions were associated with resistance to at least one isolate of *P. graminis* f. sp. *avenae* (*Rpgaq1* to *Rpgaq9*) (Table 3; Fig. 3). Two resistance QTL were mapped in two populations and were contributed by Vada and Golden Promise (*Rpgaq1*) or by Vada and Cebada Capa (*Rpgaq2*). For one QTL, detected in Vada × SusPtrit (*Rpgaq5*), the resistance allele was contributed by SusPtrit. Four resistance QTL were effective to one isolate and five were effective to two or three isolates. The QTL on 1H (*Rpgaq1*) had a major phenotypic effect (up to 27% of phenotypic variance), was identified in Vada × SusPtrit and SusPtrit × Golden Promise but not in the Cebada Capa × SusPtrit mapping population, and was effective to all isolates tested. On the long arm of chromosome 2H, two resistance loci were identified (*Rpgaq2* and *Rpgaq9*) (Table 3; Fig. 3). *Rpgaq2* was contributed by Vada and Cebada Capa and was effective to both Ingeberga and Pattala isolates. In contrast, *Rpgaq9* was only identified in the Golden Promise parent and was detected only with the Ingeberga isolate. The QTL *Rpgaq5*, at the telomeric region of 7HS for which the resistance allele was contributed by SusPtrit, had a much stronger effect against isolate Evertsholm than to Pattala and had no significant effect against isolate Ingeberga (Table 3). RILs containing the SusPtrit allele of the peak marker had, on average, 5.5 pustules or flecks per seedling leaf, those with the Vada allele had, on average, 43 pustules or flecks with Evertsholm. This contrast was 15 versus 25 for isolate Pattala, respectively. Together, these results indicate

Fig. 1. Infection response of line SusPtrit inoculated with three Swedish single pustule (SP)-derived *Puccinia graminis* f. sp. *avenae* isolates A, Evertsholm, B, Ingeberga, and C, Pattala and D, the Alfred oat control in response to the Evertsholm isolate. All images were taken 12 days after inoculation.

Fig. 2. Frequency distribution of the visible infection site (VIS) phenotype for resistance to three single pustule-derived *Puccinia graminis* f. sp. *avenae* isolates. **A, C, and E**, Pattala, **B, D, and F**, Ingeberga, and **G**, Evertsholm in barley mapping populations Cebada Capa × SusPtrit (A and B), SusPtrit × Golden Promise (C and D), and Vada × SusPtrit (E to G). Only data for the Vada × SusPtrit mapping population is presented for the Evertsholm isolate. Values of the two parental lines are shown by arrows.

TABLE 3. Significant quantitative trait loci (QTL) conferring resistance to one to three *Puccinia graminis* f. sp. *avenae* isolates^w

QTL designation	Population	Isolate	Linkage group	Position (cM) ^x	Peak marker	LOD ^y	% Exp ^z	Donor of resistance allele
<i>Rpgaql</i>	V × S	Evertsholm	1H	53	E33M54-263	5.05	12.5	Vada
<i>Rpgaql</i>	V × S	Ingeberga	1H	53	E33M54-263	5.43	11.5	Vada
<i>Rpgaql</i>	V × S	Pattala	1H	53	E33M54-263	12.5	22.7	Vada
<i>Rpgaql</i>	S × GP	Ingeberga	1H	55.1	SCRI_RS_147042	5.1	12.8	Golden Promise
<i>Rpgaql</i>	S × GP	Pattala	1H	56.8	BOPA2_12_10314	8.27	27.6	Golden Promise
<i>Rpgaql</i>	V × S	Ingeberga	2H	87	E33M61-227	3.64	7.5	Vada
<i>Rpgaql</i>	V × S	Pattala	2H	87	E33M61-227	4.45	7.1	Vada
<i>Rpgaql</i>	CC × S	Ingeberga	2H	135.3	E33M51-121	4.33	15.6	Cebada Capa
<i>Rpgaql</i>	CC × S	Pattala	2H	135.3	E33M51-121	6.31	18.8	Cebada Capa
<i>Rpgaql</i>	V × S	Ingeberga	6H	54	E35M55-217	7.5	16.4	Vada
<i>Rpgaql</i>	V × S	Pattala	6H	54	E35M55-217	4.19	6.6	Vada
<i>Rpgaql</i>	V × S	Ingeberga	7H	126	E35M61-256	3.49	7.2	Vada
<i>Rpgaql</i>	V × S	Pattala	7H	118	E33M61-184	3.4	6.1	Vada
<i>Rpgaql</i>	V × S	Evertsholm	7H	6	E37M50-435	5.37	13.4	SusPtrit
<i>Rpgaql</i>	V × S	Pattala	7H	6	E37M50-435	5.04	8.8	SusPtrit
<i>Rpgaql</i>	CC × S	Pattala	3H	157.4	E35M61-71	3.14	8.7	Cebada Capa
<i>Rpgaql</i>	CC × S	Ingeberga	6H	80.4	E38M54-401	3.32	11.7	Cebada Capa
<i>Rpgaql</i>	CC × S	Pattala	6H	141.4	E32M55-102	4.13	11.7	Cebada Capa
<i>Rpgaql</i>	S × GP	Ingeberga	2H	182.8	SCRI_RS_196748	4.55	11.3	Golden Promise

^w Population refers to the respective mapping populations used in this study abbreviated as follows: Vada × SusPtrit (V × S), Cebada Capa × SusPtrit (CC × S), and SusPtrit × Golden Promise (S × GP).

^x Centimorgan position on the biparental linkage map.

^y LOD = logarithm of odds.

^z Percentage of the explained phenotypic variance.

that barley genotypes share some common resistance genes to *P. graminis* f. sp. *avenae* and that some of the resistance genes are isolate-specific. QTL mapping on the basis of IF instead of VIS gave similar results but lower LOD scores and no other QTL than discovered by mapping on the basis of VIS. Those mapping results are not presented here.

Comparison of *Rpqaq* loci with previously mapped QTL to rust pathogens. The consensus map permitted comparisons of the identified *Rpqaq* loci with previously mapped QTL for resistance to other rust pathogens (Jafary et al. 2006, 2008; Niks et al. 2015; Yeo et al. 2014) in the same mapping populations. We considered two QTL detected for different rusts to colocate when their LOD-1 support intervals overlapped and they were contributed by the same parental line. With the exception of *Rpqaq7* and *Rpqaq9*, all *Rpqaq* loci colocated with previously identified QTL for resistance to heterologous rust pathogens, as summarized in

Table 4. Five *Rpqaq* loci colocated with previously identified resistance QTL to more than one *Puccinia* species, while *Rpqaq5* and *Rpqaq6* colocated with resistance QTL to *P. triticea* and *P. coronata* (derived from *Elymus repens*), respectively. Interestingly, *Rpqaq3* colocated with resistance QTL to two other different *P. graminis* formae *speciales* and *P. triticea*.

Pre- and posthaustorial resistance to *P. graminis* f. sp. *avenae*. Microscopic examination of infected leaf samples of Vada and SusPtrit and two oat accessions, Cebeco and Alfred, indicated that *P. graminis* f. sp. *avenae* urediniospores were able to germinate, locate stomata, and produce appressoria, substomatal vesicles, and infection hyphae on all accessions (Fig. 4). The percentage of stomatal exclusion (appressoria unable to penetrate the stoma on which they formed) varied from 20 to 77%. In both replications, Alfred oat had a significantly higher frequency of appressoria that failed to penetrate stomata than Cebeco and the resistant barley

Fig. 3. Integrated linkage map of barley (Aghnoum et al. 2010) showing the quantitative trait loci (QTL) for resistance to three Swedish *Puccinia graminis* f. sp. *avenae* isolates in the Vada × SusPtrit (black) and Cebada Capa × SusPtrit (gray) mapping populations. The position of the most closely associated markers (bold dashed) with the corresponding QTL identified in the SusPtrit × Golden Promise mapping population were placed on the integrated map. The names on the QTL bars have four components: the provisional name of the gene at the QTL, donor, logarithm of odds (LOD) value, and the *P. graminis* f. sp. *avenae* isolate used to map the specific QTL (I for Ingeberga, P for Pattala, and E for Evertsholm). The ruler on the left side indicates the distances in centiMorgans. The length of the shaded solid bars indicates the LOD-1 confidence intervals (with their corresponding peak markers in the same shade), while the QTL lines are extended to the LOD-2 confidence intervals.

TABLE 4. Summary of resistance quantitative trait loci (QTL) identified in this study and their colocation to previously mapped QTL in response to other rust pathogens

Linkage group	Position (cM)	QTL name	QTL mapped previously to other rusts
1H	48 to 68	<i>Rpqaq1</i>	<i>Puccinia graminis</i> f. sp. <i>avenae</i> (Yerong, Dracatos et al. 2014); <i>P. hordei-secalini</i> (Golden Promise, Yeo et al. 2014; Vada, Jafary et al. 2006)
2H	93 to 118	<i>Rpqaq2</i>	<i>P. triticea</i> (Vada, Jafary et al. 2006)
3H	130 to 145	<i>Rpqaq6</i>	<i>P. coronata</i> of <i>Elymus repens</i> (Vada, Niks et al. 2015)
6H	39 to 67	<i>Rpqaq3</i>	<i>P. coronata</i> of <i>Elymus repens</i> (Cebada Capa, Niks et al. 2015)
	77 to 111	<i>Rpqaq8</i>	<i>P. graminis</i> f. sp. <i>tritici</i> , <i>P. graminis</i> f. sp. <i>lolii</i> , and <i>P. triticea</i> (Jafary et al. 2006)
7H	0 to 43	<i>Rpqaq5</i>	<i>P. hordei</i> (Vada and C. Capa, Jafary et al. 2008; Golden Promise, Yeo et al. 2014); <i>P. coronata</i> of <i>Elymus repens</i> (Cebada Capa, Niks et al. 2015)
	103 to 132	<i>Rpqaq4</i>	<i>P. triticea</i> (SusPtrit, Jafary et al. 2008)
			<i>P. hordei</i> and <i>P. triticea</i> (Vada, Jafary et al. 2006, 2008); <i>P. striiformis</i> f. sp. <i>tritici</i> (Vada, Niks et al. 2015)

genotypes. Percentage of aborted substomatal vesicles was nil (data not presented). Immunity in Vada was mainly due to prehaustorial resistance, as only 15% of the penetrating infection units established branched mycelial colonies (Fig. 4; Table 5). Necrosis did not play an obvious role in the immunity of Vada but was present in at least 64% of the established infection units in SusPtrit. The average diameter of established colonies on barley were about 10× smaller than on the susceptible oat reference cultivars (Fig. 4; Table 5).

DISCUSSION

Host status and histology of barley to *P. graminis* f. sp. *avenae* relative to oat. Different *formae speciales* of *Puccinia*

graminis have coevolved with their respective grass hosts, presumably through the development or acquisition of effectors that permit the establishment of a compatible interaction. *P. graminis* f. sp. *tritici* mainly infects cereals within the family Triticeae, while *P. graminis* f. sp. *avenae* is specialized mainly to *Avena* spp. and other grasses within the Pooideae subtribe (Leonard and Szabo 2005). Barley is a host to *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *secalis* but is not considered a host to *P. graminis* f. sp. *avenae* or *P. graminis* f. sp. *lolii*. Barley is deemed a near-nonhost to numerous heterologous rust pathogen species, based on observed seedling susceptibility in a small proportion of a collection of 110 diverse barley accessions to various rust species, including *P. graminis* f. sp. *lolii* and *P. triticea* (Atienza et al. 2004). The data

Fig. 4. Histological analysis of the development stages of infection using the *Puccinia graminis* f. sp. *avenae*–Evertsholm isolate. **A**, Nonpenetrating infection unit on Alfred oat. **B**, Early abortion without necrosis on Vada. **C**, Early abortion with necrosis on SusPtrit. **D**, Established colony without necrosis on Cebeco oat. **E**, Established colony with necrosis on SusPtrit.

TABLE 5. Average proportion of infection units^x of *Puccinia graminis* f. sp. *avenae* at three development stages on barley and oat leaf segments collected 6 days after inoculation

Rep	Accession	Phenotype	Nonpen	EA (+N) ^y	EST(+N) ^z	Colony diameter (μm)	
						(–N)	(+N)
1	Vada	Immune	0.32a	0.85 (0)a	0.15 (0)	7.12	
1	SusPtrit	Resistant	0.28a	0.69 (0.04)b	0.31 (0.64)	8.31	11.4
1	Alfred (oat)	Very susceptible	0.77b	0.11 (0)c	0.89 (0)	85.64	
1	Cebeco (oat)	Very susceptible	0.35a	0.10 (0)c	0.90 (0)	77.12	
2	Vada	Immune	0.27a	0.84 (0)a	0.16 (0)	6.33	
2	SusPtrit	Resistant	0.2a	0.40 (0)b	0.60 (0.71)	9.13	8.97
2	Alfred (oat)	Very susceptible	0.57b	0.05 (0)c	0.95 (0)	84.64	
2	Cebeco (oat)	Very susceptible	0.42a	0.03 (0)c	0.97 (0)	84.72	

^x The infection units were classified into three development classes based on the classification by Niks and Kuiper (1983): Nonpen = nonpenetrating appressoria, EA = early aborted colonies (with six or less haustorial mother cells) without or with necrosis, EST = established colonies (branched hyphae with more than six haustorial mother cells) with or without necrosis. The latter group included Niks and Kuiper’s (1983) class of successful colonies, since it was not possible to judge which infection units at later sampling times might have succeeded to produce spores. Colony diameter was measured at magnification of 40×. Rep = replicate. Values in each column followed by the same letter are not significantly different ($P \leq 0.05$), according to Duncan’s multiple range test.

^y The values in brackets are average proportions of EA germlings associated with necrosis.

^z First value is the percentage established, being the complement of the percentage of early aborted infection units. The value within the brackets represents the percentage of established colonies associated with plant cell necrosis.

in the present study suggest that barley is also a near-nonhost to *P. graminis* f. sp. *avenae*. The proportion of moderately susceptible barley accessions (11%) to *P. graminis* f. sp. *avenae* from the subset of 66 barleys used by Atienza et al. (2004) was comparable to the proportion previously observed to be somewhat susceptible to *P. tritici* and *P. graminis* f. sp. *lolii*. As reported previously by Dracatos et al. (2014) and as is the case for the other previously tested heterologous rust pathogens, none of the 66 barley accessions were as susceptible as the susceptible oat control Cebeco to either isolate of *P. graminis* f. sp. *avenae*.

Histological analysis using the Evertsholm isolate indicated that *P. graminis* f. sp. *avenae* was able to penetrate stomata on both Vada (immune) and SusPtrit barley (completely resistant) at the same frequency as on the highly susceptible oat cultivar Cebeco. Although largely susceptible, the observed stomatal exclusion in Alfred oat may represent a partial host resistance mechanism to *P. graminis* f. sp. *avenae*. Kochman and Brown (1975) identified different levels of stomatal exclusion between oat accessions to *P. graminis* f. sp. *avenae*.

Our data demonstrated that a substantial proportion of established colonies in SusPtrit were associated with a hypersensitivity reaction, suggesting that the resistance gene at 7H may represent a common *R* gene-mediated resistance mechanism. However, since we did not compare lines that were near-isogenic for the QTL that were discovered, we cannot attribute the hypersensitive reaction in SusPtrit to a particular gene for resistance. The observed high frequency of EA infection units and finding that at least 29% of the established colonies in SusPtrit were not associated with hypersensitivity, indicates that, not only in Vada but also in SusPtrit, prehaustorial resistance plays an important role. This suggests that failed haustorium formation is likely to be a major component of the resistance of barley to *P. graminis* f. sp. *avenae*. High failure rates in haustorium formation is a hallmark of nonhost resistance of plants to rust and powdery mildew fungi (Niks and Marcel 2009; Niks and Rubiales 2002). It is supposed that much of nonhost resistance is explained by pathogen-associated molecular pattern (PAMP)-triggered immunity, which cannot be suppressed by a heterologous pathogen's effectors (Niks and Marcel 2009). Cloning of the resistance genes discovered in this study in SusPtrit and Vada may be of great interest to understand the molecular basis of nonhost resistance.

Polygenic inheritance of resistance to *P. graminis* f. sp. *avenae* in barley. Both simple and complex inheritance of near-nonhost resistance has been reported in various cereal-*Puccinia* pathosystems. Some studies of near-nonhost resistance in wheat to the barley crown rust and barley stripe rust pathogens determined that the resistance was simply inherited and mostly due to single dominant genes (Niu et al. 2014; Rodriguez et al. 2004). The inheritance of resistance to *P. graminis* f. sp. *avenae* in barley was previously found to be simply inherited and was attributed to a single dominant gene (Dracatos et al. 2015; Martens et al. 1983). In contrast, our data demonstrated that near-nonhost resistance to *P. graminis* f. sp. *avenae* in barley, as previously shown by Dracatos et al. (2014), was polygenically inherited. Several studies on the inheritance of near-nonhost resistance in barley to multiple heterologous rust pathogens using the same mapping populations as the present study also determined that resistance was polygenically inherited, with the rare involvement of an *R* gene (Jafary et al. 2006; 2008; Niks et al. 2015; Yeo et al. 2014).

Studies on the inheritance of (near-) nonhost resistance when a mono- or oligogenic inheritance was reported (Golegaonkar et al. 2013; Niu et al. 2014; Rodriguez et al. 2004; Sui et al. 2010) were based on classification of progeny according to an infection type scale of 0 to 4 or 0 to 9. Segregating populations were classified as resistant versus susceptible based on a cutoff in this infection type scale. Frequency distributions were not given, but it is suggested that the distribution was bimodal. We followed a quantitative approach, using a settling tower and counts of flecks and pustules,

and did not find discrete infection types but, rather, a quantitative and not bimodal variation in numbers of uredinia and flecks as reported in previous studies (Jafary et al. 2006; 2008; Niks et al. 2015; Yeo et al. 2014) (Fig. 2). Although we suggest that the method of evaluation may play a role in the ability to detect minor effect QTL, the observed differences between studies may also be due to really different modes of inheritance, depending on the plant accession-pathogen isolate combination.

Isolate specificity. Results from recent QTL mapping studies in barley have shown a high diversity of near-nonhost resistance genes to heterologous rust pathogens with different but, also, overlapping specificities (Dracatos et al. 2014; Jafary et al. 2006, 2008). Some QTL were effective to only one tested heterologous rust fungal species and others to several (Jafary et al. 2006, 2008), as again illustrated in Table 4. In previous studies, usually only one isolate per rust species was tested, so there is not much information on possible isolate specificity of QTL for nonhost resistance. The QTL mapping data reported here were based on SP cultures from three genetically diverse Swedish *P. graminis* f. sp. *avenae* populations that differed in virulence spectrum (Table 1) and occurred in association with barberry (Berlin et al. 2013). The isolates were prone to form telia for sexual reproduction, as evidenced by premature telial formation on barley seedlings, especially by the Ingeberga isolate. Despite the probably great genetic diversity among the isolates, *Rpgaq1* was effective to all isolates and appears to be the same QTL reported by Dracatos et al. (2014), who found it was effective against all *P. graminis* f. sp. *avenae* isolates tested from Australia and Canada. These results indicate that this resistance gene, in case it plays a role in PAMP-triggered immunity, cannot be suppressed by a corresponding effector in any of the tested isolates of *P. graminis* f. sp. *avenae*. *Rpgaq1* is a QTL with a major effect, accounting for up to 27% of the phenotypic variation and, presumably, contributes substantially to the immunity observed in both Vada and Golden Promise, although other, minor-effect QTL were also identified. Furthermore, we identified a QTL on chromosome 6H (*Rpgaq3*) in the Cebada Capa × SusPtrit and the Vada × SusPtrit mapping populations effective to two *P. graminis* f. sp. *avenae* isolates, to *P. graminis* f. sp. *lolii*, and to *P. graminis* f. sp. *tritici*. This is similar to the effectiveness of *Rpg5* to isolates representing at least three *formae speciales* of *P. graminis* (Dracatos et al. 2015). Taken together, this suggests that either the same QTL is effective to multiple *P. graminis formae speciales* or the presence of a cluster of tightly linked barley resistance genes that are specific to one or more *formae speciales*.

In contrast to these apparently nonspecific resistance genes, we also found genes that were clearly *P. graminis* f. sp. *avenae* isolate-specific. Remarkably, seedlings of SusPtrit were moderately susceptible to the Ingeberga and Pattala isolates but completely resistant (only pinpoint flecking) to Evertsholm. This resistance was mainly due to a resistance allele at the *Rpgaq5* locus at the tip of 7HS contributed by SusPtrit, which greatly hampers infection by the Evertsholm isolate, and less so by the Pattala isolate and is ineffective to the Ingeberga isolate. The observed isolate specificity is likely to reflect genetic variation in the allele for the relevant effector within the Swedish *P. graminis* f. sp. *avenae* populations used in this study.

Barley cultivar and accession-specific resistance. The *Rpgaq5* resistance of SusPtrit to isolate Evertsholm was most likely derived from one of the four accessions used for the development of SusPtrit (Menelik, L100, Nigrinudum, and Trigo Biasa). In this study, Menelik, L100, and Nigrinudum were not available. However, Trigo Biasa may be the source of the SusPtrit resistance gene at chromosome 7H, since this line had a similar resistance associated with pinpoint flecks to isolate Evertsholm and was moderately susceptible to the Ingeberga isolate. *Rpgaq1* was identified in the present study in both the Vada × SusPtrit and SusPtrit × Golden Promise mapping populations and collocated with a previously identified QTL for resistance to *P. graminis* f. sp. *avenae* in a DH population based on the Australian barley cultivars Yerong and Franklin (Dracatos et al. 2014). *Rpgaq1* likely occurs in several barley

accessions, including Vada, Yerong, and Golden Promise, but not in Cebada Capa or Franklin. Baker et al. (1970) determined that *Sr18* (present in 95% of common wheats) provided near-nonhost resistance to the heterologous rust pathogen *P. graminis* f. sp. *secalis*. It is likely that similar genes also play a role in near-nonhost resistance in barley to heterologous rust pathogens. In summary, we identified nine different QTL for resistance in the three barley mapping populations studied. *Rpgaq1* and *Rpgaq5* located on chromosomes 1H and 7H, respectively, are well-suited for map-based cloning due to their large size effect and the availability of BAC libraries for both Vada and SusPrit to physically map the responsible regions (Yeo et al. 2016).

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