Infection of *Brachypodium distachyon* with Selected Grass Rust Pathogens

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The model temperate grass *Brachypodium distachyon* is considered a nonhost for wheat rust diseases caused by *Puccinia graminis* f. sp. *tritici*, *P. triticina*, and *P. striiformis*. Up to 140 *Brachypodium* accessions were infected with these three rust species, in addition to *P. graminis* ff. spp. *avena* and *phalaridis*. Related *B. distachyon* lines showed similar cytological nonhost resistance (NHR) phenotypes, and an inverse relationship between *P. graminis* f. sp. *tritici* and *P. striiformis* growth was observed in many lines, with accessions that allowed the most growth of *P. graminis* f. sp. *tritici* showing the least *P. striiformis* development and vice versa. Callose deposition patterns during infection by all three rust species showed similarity to the wheat basal defense response while cell death that resulted in autofluorescence did not appear to be a major component of the defense response. Infection of *B. distachyon* with *P. graminis* f. sp. *avena* and *P. graminis* f. sp. *phalaridis* produced much greater colonization, indicating that *P. graminis* rusts with Poace hosts show greater ability to infect *B. distachyon* than those with Triticeae hosts. *P. striiformis* infection of progeny from two *B. distachyon* families demonstrated that these NHR phenotypes are highly heritable and appear to be under relatively simple genetic control, making this species a powerful tool for elucidating the molecular basis of NHR to cereal rust pathogens.

The mechanisms of plant resistance to adapted microbial species are becoming increasingly understood, with the roles of pathogen-associated molecular patterns (PAMPs) and effector molecules and their recognition by plant PAMP receptors and plant resistance genes, respectively, established in many species (Jones and Dangl 2006). In contrast, the interaction between plants and nonadapted pathogens (i.e., nonhost resistance [NHR]) is more poorly defined, although significant mechanistic overlap appears to exist between host and NHR mechanisms (Fan and Doerner 2012; Schulze-Lefert and Panstruga 2011). Current models suggest that layers of defense exist to prevent nonadapted pathogens from colonizing nonhost plants, which range from passively preformed physical and chemical defenses to active pathogen recognition followed by defense activation (Fan and Doerner 2012; Heath 2000; Jones and

Dangl 2006). This model is best illustrated in the infection process of powdery mildew, where several plant genes essential for epidermal penetration resistance to nonadapted mildews have been identified (*PEN1* to *PEN3*) (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). Underlying this penetration barrier is a second defense layer involving genes such as *EDS1*, *PAD4*, and *SAG101*, which are components of salicylic acid (SA)-mediated defenses (Fan and Doerner 2012). The *PEN2* gene has also been implicated in NHR of *Arabidopsis* to *Magnaporthe oryzae* (Nakao et al. 2011).

The growth of rust pathogens on nonhost plant species has been examined for several plant–pathogen interactions. In two cases, these have involved a nonhost plant that is not closely related to the hosts of the rust pathogens, i.e., *Uromyces vignae* (the bean rust pathogen) and *Puccinia triticina* (the wheat leaf rust pathogen) growth on *Arabidopsis* (Mellersch and Heath 2003; Shafiei et al. 2007). In both cases, modest pathogen growth was apparent that was dependent upon the genotype of the nonhost plant, and the resistance response involved both SA and reactive-oxygen-mediated defenses. In contrast to this relatively extreme divergence between pathogen host and nonhost, in an analysis of “near NHR”, in which the pathogen can grow on a few accessions of a species, the growth of *P. triticina* on barley was examined. Resistance was polygenic, with quantitative trait loci (QTL) showing map locations similar to genes conferring partial resistance to the adapted barley rust pathogen *P. hordei* (Jafary et al. 2008).

Previously, we have examined the interaction between cereal rust pathogens and rice (Ayliffe et al. 2011). Rice is atypical in that it is an agricultural species that is a nonhost of all known rust species. The resistance of rice to rust is not predominantly due to a basic incompatibility or prehaustorial defense mechanisms, because five different cereal rust pathogen species were shown to infect and enter the rice leaf through stomata and colonize underlying plant mesophyll cells with haustoria (Ayliffe et al. 2011). The NHR response of rice to cereal rust infection was shown to be an active response involving the production of hydrogen peroxide and the deposition of callose. Genetic variation in the NHR response was observed, with some rice cultivars supporting more pathogen growth than others (Ayliffe et al. 2011). However, this variation was relatively subtle and prone to environmental effects, making identification of the underlying genes a difficult proposition.

In contrast to rice, the model grass *Brachypodium distachyon* is a host to a rust pathogen species, *P. brachypodii*, which infects a number of *Brachypodium* spp. (Barbieri et al. 2011). Phylogenetic studies based upon ribosomal RNA intergenic spacer sequences indicate that *P. brachypodii* is more closely related to *P. striiformis* than other rust species, including *P.*

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graminis and *P. triticina* (Zambino and Szabo 1993). Resistance to an isolate of *P. brachypodii* was shown to be quantitatively inherited in a cross between *B. distachyon* accessions Bd3-1 and Bd1-1, with three QTL identified that collectively explained 39 to 54% of the variance in seedlings and 22% of the variance in older plants (Barbieri et al. 2012).

The NHR interaction of *Brachypodium* spp. with *P. hordei*, *P. triticina*, and *P. striiformis* ff. spp. *tritici* and *hordei* has previously been reported to involve macroscopic symptoms (Draper et al. 2001). Several *Brachypodium* accessions showed tissue browning upon *P. striiformis* infection, and sporulating pustules were observed on two hexaploid accessions, ABR100 and ABR105 (Catalan et al. 2012; Draper et al. 2001). These hexaploid accessions have subsequently been classified as a separate species, *B. hybridum* (Catalan et al. 2012). Upon infection with *P. triticina*, symptoms were less obvious, with only a single accession showing brown flecking, whereas several accessions showed browning to *P. hordei* (Draper et al. 2001). The ability of *P. striiformis* to sporulate on *Brachypodium* accessions was subsequently confirmed in a later study of 119 *Brachypodium* accessions challenged with *P. striiformis* ff. spp. *tritici*, *hordei*, and *bromi* (Barbieri et al. 2012). In all, 79% of the plant accession-rust isolate interactions produced no macroscopic symptoms, while 29% produced small or large dark flecks, and a single accession (PI227011) produced sporulating uredinia upon challenge with either *P. striiformis* f. sp. *hordei* or *P. striiformis* f. sp. *bromi* but not *P. striiformis* f. sp. *tritici* (Barbieri et al. 2012). This accession is also hexaploid (Vogel et al. 2006) and, hence, now also considered as *B. hybridum* (Catalan et al. 2012).

More recently, infection of eight accessions of *B. distachyon* (Bd1-1, Bd2-3, Bd3-1, Bd18-1, Bd21, Bd21-3, Bd29-1, and Bd30-1) with *P. graminis* ff. spp. *tritici*, *lolii*, and *phlei-pratensis* was reported (Figueroa et al. 2013). Sporulating lesions were observed on most accession upon infection with *P. graminis* ff. spp. *lolii* and *phlei-pratensis* but rarely with *P. graminis* f. sp. *tritici*. Prehaustorial resistance mechanisms were suggested to play a major role against this latter pathogen species (Figueroa et al. 2013).

The small genome size of *B. distachyon* and increasing genomic resources of this species potentially makes it a model system for the molecular analysis of NHR mechanisms to cereal rust pathogens. A number of collections of *Brachypodium* accessions are publically available, with many accessions derived from the Mediterranean region. Some collections have used a unique prefix (e.g., ABR, Bd, and BdTr) followed by an accession number to designate each accession (Filiz et al. 2009; Garvin et al. 2008; Vogel et al. 2006, 2009). Other collections have assigned different prefixes within a collection to reflect the geographical location where the plants were collected (e.g., Gaz, Tek, Adi, and so on) (Vogel et al. 2009). The phylogenetic relationship among many of these plants is known from simple-sequence repeat marker analyses (Vogel et al. 2009). These accessions were essentially divided into two main groups by Vogel and colleagues (2009), with one group consisting of TEK1-10 (excluding TEK3), BdTr1-1, BdTr7a and BdTr7b, and six BdTr8 accessions (hereafter referred to as the TEK group), and the remainder of the accessions forming a second group (hereafter referred to as group 2). These two groups were further divided into smaller clades, with the number of accessions within a single clade ranging from 1 to 17 (Vogel et al. 2009).

To further understand the NHR interaction of cereal rust pathogens with *B. distachyon*, we have undertaken an extensive cytological analysis of over 140 *B. distachyon* accessions infected with *P. graminis* ff. spp. *tritici*, *avenae*, and *phalaridis*; *P. striiformis* f. sp. *tritici*; and *P. triticina*. These microscopic analyses provide further biological insight into the range of

macroscopic lesions and immunity previously reported to occur upon challenge of *Brachypodium* spp. with wheat rust pathogen species (Barbieri et al. 2012; Draper et al. 2001; Figueroa et al. 2013). In addition, in two *B. distachyon* families, we show that the NHR phenotypes to *P. striiformis* infection are heritable and appear to be controlled by relatively few genes.

RESULTS

Infection of *Brachypodium* spp. with rusts of the Triticeae *P. graminis* f. sp. *tritici* infection.

In all, 140 different *B. distachyon* accessions were infected with *P. graminis* f. sp. *tritici*, and the development of rust symptoms was analyzed cytologically 4 weeks postinoculation. Macroscopic symptoms varied from apparent immunity in some lines to the formation of lesions ranging from sparse to common (Fig. 1A). In some instances, small pustules were also produced (discussed below). In all, 25% of accessions showed no symptoms, 19% showed relatively obvious lesions, and 7% of accessions produced small sporulating uredinia. The remaining lines had an intermediate phenotype (Supplementary Table S1).

All accessions were analyzed microscopically and each had leaves infected with urediniospores that had germinated and successfully entered the *Brachypodium* leaf via the formation of an appressorium over a stomate and subsequent production of substomatal vesicles, infection hyphae, and haustoria, regardless of whether macroscopic symptoms were obviously apparent or not. No accession had the ability to completely prevent growth of this *P. graminis* f. sp. *tritici* isolate via prehaustorial defense mechanisms. In contrast, Figueroa and colleagues (2013) suggested that prehaustorial resistance mechanisms played a major role in the response of eight *B. distachyon* accessions to *P. graminis* f. sp. *tritici*. Six of these accessions were included in our study, three of which showed relatively extensive *P. graminis* f. sp. *tritici* growth, including sporulation in one instance (Bd2-3, Bd3-1, and Bd21-3). These differences may reflect *P. graminis* f. sp. *tritici* race specificity in the *B. distachyon* response or, alternatively, may be due to differences between infection conditions used in the two studies.

On accessions showing no obvious macroscopic symptoms, such as Bd21, a range of infection site sizes was observed that varied from little more than a substomatal vesicle to larger infections that encompassed multiple mesophyll cells, with small sites and larger sites in close juxtaposition (Fig. 1C1 to C5). The frequency of these larger infection sites was significantly lower when compared with small infection sites (Fig. 2, Bd21/*P. graminis* f. sp. *tritici*). Very occasionally, much larger infection sites were observed that encompassed several hundred mesophyll cells and which resulted in macroscopic lesion formation; however, the rarity of these infections sites made them difficult to discriminate visually from unrelated wounding events that occur occasionally on glasshouse grown plants.

Microscopic analysis of *B. distachyon* accessions that showed obvious macroscopic symptoms, such as BdTr13g, also identified a range of infection site sizes from very small to large, with small infection sites generally far more abundant (Fig. 1D1). However, among these smaller infection sites were much larger infections that encompassed hundreds of mesophyll cells and which produced small sporulating pustules in some instances (Fig. 1D1 and D2). Almost invariably, the occurrence of macroscopic lesions on the leaf surface of *B. distachyon* accessions corresponded precisely to the production of underlying large rust infection sites (Fig. 1D3 and D4). The full development of macroscopic symptoms took between 3 and 4 weeks postinfection, which is substantially longer than

the development of *P. graminis* f. sp. *tritici* on susceptible wheat. After this period of time, many infected *B. distachyon* leaves had begun to undergo age-dependent senescence, suggesting that more growth of this obligate biotrophic pathogen was unlikely to occur.

Visual symptoms therefore represent only a small fraction of *P. graminis* f. sp. *tritici* infections on *B. distachyon* and a con-

tinuum of infection site sizes was observed on all accessions. Difference in the relative frequency of large infection sites enables macroscopic differentiation between apparently immune accessions and those allowing more rust growth. The presence of macroscopic lesions on *B. distachyon* accessions upon *P. graminis* f. sp. *tritici* infection is not indicative of an extreme resistance response but, rather, increased fungal growth com-

pared with other infection sites. Two exceptions to this generalization were observed. Some members of the BdTr10 group produced small brown lesions that were not associated with large infection sites (described below), while some members of the BdTr8 group, even when uninfected, produced spreading gray lesions.

A strong correlation between the genetic relationship of these accessions and the phenotypes observed upon *P. graminis* f. sp. *tritici* infection was apparent. All members of the TEK group (Vogel et al. 2009) showed very restricted pathogen development when infected with *P. graminis* f. sp. *tritici* (Table 1). In contrast many, although not all (e.g., Bd21 and BdTr10), accessions in group 2 allowed extensive pathogen growth and sporulation in some instances, often with all members within a single clade showing very similar phenotypes (Table 1). In some accessions, a clear developmental gradient of NHR to *P. graminis* f. sp. *tritici* infection was observed, particularly among the BdTr13 group. Here, a basipetal gradient of *P. graminis* f. sp. *tritici* growth was often observed on single leaves, with a greater frequency of large infection sites being present at the leaf base and a progressively diminishing frequency of large infection sites occurring toward the leaf tip (Fig. 1D1, F1, and F2). This fungal growth pattern reflects the age gradient of leaf cells, suggesting that the youngest leaf cells are less capable of suppressing the growth of the non-adapted pathogen.

P. striiformis f. sp. *tritici* infection.

A subgroup of 32 *B. distachyon* accessions representative of the genetic diversity identified by Vogel and associates (2009) were infected with an Australian *P. striiformis* f. sp. *tritici* isolate, 104 E137 A– (Table 1). A largely inverse relationship was observed in infection phenotypes when compared with *P. graminis* f. sp. *tritici* infection, with those accessions allowing the most *P. striiformis* f. sp. *tritici* growth generally being the most restrictive of *P. graminis* f. sp. *tritici* growth and vice versa. This observation is exemplified by Bd21, where minimal growth of *P. graminis* f. sp. *tritici* occurred (Fig. 1C1) but extensive growth of *P. striiformis* f. sp. *tritici* was observed (Fig. 1B, E1, and E2). The distribution of infection site sizes of each rust species on this accession is shown in Figure 2.

All members of the TEK group that were tested supported extensive *P. striiformis* f. sp. *tritici* development (Fig. 2, Tek4/*P. striiformis* f. sp. *tritici*) but these same accessions were among the most restrictive of *P. graminis* f. sp. *tritici* growth (Table 1). In contrast, many group 2 accessions (e.g., the BdTr13

group) that allowed extensive *P. graminis* f. sp. *tritici* development (Fig. 1D1, F1, and F2) were highly restrictive of *P. striiformis* f. sp. *tritici* growth (Table 1). These data indicate a strong relationship between plant genotype and NHR response to *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici*. This correlation, although compelling, is not absolute, because some members of group 2 (e.g., Bd21) showed extensive *P. striiformis* f. sp. *tritici* growth and restricted *P. graminis* f. sp. *tritici* development, similar to that of the TEK group (Table 1).

For accessions on which extensive *P. striiformis* f. sp. *tritici* growth was apparent, macroscopic lesions were observed (Fig. 1E1 and E2), consistent with previous reports of macroscopic stripe rust lesions (Barbieri et al 2012; Draper et al. 2001). Conversely, accessions that severely restricted *P. striiformis* f. sp. *tritici* growth showed no obvious macroscopic symptoms (not shown). Hence, as observed for *P. graminis* f. sp. *tritici*, the presence of large lesions on *B. distachyon* upon *P. striiformis* f. sp. *tritici* infection is indicative of extensive fungal colonization (Fig. 1E1 and E2) rather than an extreme resistance response. Unlike *P. graminis* f. sp. *tritici*, however, no sporulating pustules were ever produced by the Australian *P. striiformis* f. sp. *tritici* isolate used in this study on any accession over multiple experiments when grown under glasshouse conditions at 21°C. However, subsequently, it became apparent that *P. striiformis* f. sp. *tritici* sporulation did occur on accession Bd21 and TEK4 (other accessions were untested) when the maximum daily temperature was reduced to 18°C (Fig. 1E3), which is the first report of *P. striiformis* f. sp. *tritici* sporulation on this diploid (2x = 10) species, and consistent with *P. striiformis* sporulation reported on *B. hybridum* (Barbieri et al. 2012; Draper et al. 2001). The development of *P. striiformis* f. sp. *tritici* symptoms on *Brachypodium* spp. was also substantially delayed when compared with wheat, with symptoms being maximal 3 to 4 weeks postinfection, after which time many leaves began to initiate developmental senescence.

A second subgroup of *Brachypodium* accessions was infected with a European *P. striiformis* f. sp. *tritici* isolate, 08/501. Five of these accessions had also been screened with the Australian *P. striiformis* f. sp. *tritici* isolate and very similar infection outcomes were observed for both stripe rust pathogen isolates on these common accessions, indicating little evidence of race specificity. An inverse relationship was also observed for *P. graminis* f. sp. *tritici* growth and growth of the European *P. striiformis* f. sp. *tritici* isolate on Bd1-1, Bd2-3, Bd3-1, Bd21, Bd21-3, and Gaz6 and Gaz4 members of the Koz group. However, this relationship was not apparent among Adi and Kah

Fig. 1. Growth of rust pathogens on *B. distachyon*. **A**, Macroscopic symptoms observed on leaves from *B. distachyon* accession BdTr3b 4 weeks postinoculation (wpi) with *Puccinia graminis* f. sp. *tritici*. **B**, Symptoms present on leaves of Bd21 plants 4wpi with *P. striiformis* f. sp. *tritici*. **C**, Microscopic analysis of Bd21 tissue 4wpi with *P. graminis* f. sp. *tritici*. C1 shows small *P. graminis* f. sp. *tritici* infection sites on the leaf. Most sites consisted of appressoria (C2) and substomatal vesicles with several small infection hyphae (C3). Larger infection sites were also observed (C5) that produced haustoria (C4 and C5 arrowhead). C2/C3 and C4/C5 are the same infection sites, respectively. A 20-μm scale bar is shown in C2 to C5. **D**, Growth of *P. graminis* f. sp. *tritici* on *B. distachyon* accession BdTr13g 4wpi. A mixture of large and small infection sites are apparent (D1), with the largest sites producing urediniospores (D2). Macroscopic symptoms on the leaf (D3, cleared leaf) correlate with large *P. graminis* f. sp. *tritici* infection sites (D4). D3 and D4 are the same region of leaf tissue. **E**, Extensive growth of *P. striiformis* f. sp. *tritici* on *B. distachyon* accession Bd21. Obvious macroscopic symptoms are seen on the leaf surface (E1) that correspond to infection hyphae ramification throughout the leaf (E2). When grown under an 18°C maximum temperature, *P. striiformis* f. sp. *tritici* uredinia were observed (E3). **F**, Basipetal gradient of *P. graminis* f. sp. *tritici* growth on leaves from *B. distachyon* BdTr13 accessions. F1 and F2 show a leaf from accession BdTr13f and BdTr13k, respectively, infected with *P. graminis* f. sp. *tritici*. A higher frequency of large infection sites is seen at the leaf base (left-hand side of each figure) in each case. A similar gradient of growth is observed in D1 on a BdTr13g leaf infected with *P. graminis* f. sp. *tritici*. **G**, Growth of *P. triticina* on Bd21 4wpi. A distribution of infection site sizes is apparent, ranging from small to medium. **H**, Growth of *P. graminis* f. sp. *phalaridis* on Bd21 4wpi. Sporulating infection sites shown in H1 are surrounded by macroscopic browning. Extensive ramification of infection hyphae occurs throughout the leaf beneath *P. graminis* f. sp. *phalaridis* pustules (H2). **I**, Extensive growth of *P. graminis* f. sp. *avenae* on *B. distachyon* Bd21 4 wpi. Several heavily infected leaves are shown with extensive growth of infection hyphae and frequent production of sporulating uredinia. **J**, Macroscopic lesions present on leaves of BdTr10h 4 wpi with *P. striiformis* f. sp. *tritici*. **K**, Small *P. graminis* f. sp. *tritici* infection sites present on a leaf from BdTr10h. Spores and spore germ tubes can also be seen on the leaf surface. **L**, Pigmentation in cells surrounding *P. striiformis* f. sp. *tritici* infections shown in K. Note that browning is not associated with rust infection structures on the leaf surface. **M**, *P. striiformis* f. sp. *tritici* infection site on leaf tissue of BdTr10h. **N**, Autofluorescence associated with the *P. striiformis* f. sp. *tritici* infection shown in M. All images showing microscopic analysis of rust infection were stained with wheat germ agglutinin conjugated to fluorescein isothiocyanate.

accessions and most members of the Gaz group, with each accession showing only small amounts of growth of both rust pathogens. These latter groups were not tested with the Australian *P. striiformis* f. sp. *tritici* isolate.

P. triticina infection.

B. distachyon accessions Bd21, Bd21-3, Bd2-3, BdTr3h, BdTr10h, BdTr10m, BdTr13g, BdTr13h, TEK4, TEK7, and

Fig. 2. Distribution of infection site areas of rust pathogens on *Brachypodium distachyon* accessions. Each graph shows the percent of rust infection sites (y axis) with a given infection site area in 1,000 μm^2 (x axis). Infection site areas were determined by measuring microscopically the length and width of each infection site and then calculating the area based upon the assumption that each site is oval in shape. The *B. distachyon* accession and cereal rust used for infection are indicated on each graph as “accession/abbreviated rust pathogen”, as is the number of infection sites measured (N). Infection sites depicted as black bars did not produce rust spores while those depicted as gray bars did sporulate. Note that the y axis has a different scale on the first two graphs compared with the remainder.

TEK12 were infected with *P. triticina* isolate 104-1236(7)11. Unlike *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici*, this rust pathogen didn't produce as much variation in *B. distachyon* infection phenotypes among this relatively small group of accessions, with obvious macroscopic symptoms absent on all accessions analyzed. Microscopic analysis identified a distribution of *P. triticina* infection sites on each accession which ranged from little more than a substomatal vesicle up to infection sites that encompassed over 100 mesophyll cells, with different-sized infection sites in close juxtaposition (Fig. 1G). The extent of growth of this *P. triticina* isolate was not as pronounced on any accession as was the growth of *P. graminis* f. sp. *tritici* or *P. striiformis* f. sp. *tritici* on some of these *B. distachyon* lines.

Infection of *Brachypodium* spp. with *P. graminis* rusts that have Poae hosts.

All 140 *B. distachyon* accessions were inoculated with *P. graminis* f. sp. *phalaridis*, a stem rust forma specialis that parasitizes *Phalaris aquatica* (phalaris grass). In contrast to *Puccinia graminis* f. sp. *tritici* infection, where the formation of small uredinia was relatively rare on most accessions, *P. graminis* f. sp. *phalaridis* rust frequently produced sporulating uredinia on most *B. distachyon* accessions, with only a single accession (BdTr7a) not producing pustules. Often associated with these small uredinia was a ring of brown plant tissue surrounding the uredinium but which did not encompass the extensive underlying infection hyphae (Fig. 1, H1 and H2).

Similar infection was seen with *P. graminis* f. sp. *avena*, the special form of stem rust pathogen that infects oat, with sporulating uredinia observed on all of the *B. distachyon* accessions examined (Fig. 1I). However, on two accessions, BdTr7a and Bd1-1, uredinia were rare and very small when formed. In contrast, on some accessions, uredinia were up to 1.5 mm in size, with large numbers of urediniospores produced (Fig. 1I). On cultivars showing significant *P. graminis* f. sp. *phalaridis* and *P. graminis* f. sp. *avena* pustule production (e.g., Bd21), a distribution of infection site sizes was observed microscopically with many small infection sites also present (Fig. 2).

These data indicate that *B. distachyon* support more extensive colonization by *P. graminis* rusts that parasitize the family Poae compared with *P. graminis* f. sp. *tritici*. *B. distachyon* could even be considered partial hosts of Poae stem rusts, given that sporulating uredinia occurred on most accessions. For comparative purposes, the median infection site area observed for different rust pathogen species on several different *B. distachyon* accessions is shown (Fig. 3A); these being Bd21, which allows extensive growth of *P. striiformis* f. sp. *tritici*, *P. graminis* f. sp. *avena*, and *P. graminis* f. sp. *phalaridis* but is restrictive of *P. graminis* f. sp. *tritici* growth; BdTr10h, which is restrictive of *P. striiformis* f. sp. *tritici*; and TEK4, which allows extensive *P. striiformis* f. sp. *tritici* growth. In addition, the median infection site area of wheat stem rust pustules that have just erupted on a fully susceptible wheat host is included. Although these latter infection sites on the host plant have not reached full size, they are at least eightfold larger than nonadapted rust growth on *B. distachyon*. Therefore, those *B. distachyon* accessions that allowed the most growth of the rust pathogens examined were still significantly restrictive when compared with the fully susceptible host.

The nonhost response to rust pathogen infection.

For all five rust pathogens described above, cell death around infection sites that resulted in autofluorescence was not common on *B. distachyon* accessions regardless of the extent of fungal growth, indicating that hypersensitive cell death was usually not involved in the NHR response. Even on those

accessions where autofluorescent cell death was more common, the extent of autofluorescence was often limited to just one or two cells (Fig. 3B). An exception to this generalization occurred with BdTr10h (Fig. 3B), which is further described below.

In contrast, when callose deposition was examined after *Brachypodium* infection with *P. graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici*, or *P. triticina*, it was present at most infections sites, regardless of infection site size (Fig. 4A to C). However, an obvious difference appeared to exist in the number of callose-producing cells at different-sized infection sites on the same leaf, with small infection sites appearing to have a proportionally greater number of callose-producing cells compared with large infection sites (Fig. 4A to C).

To further characterize the callose deposition patterns observed, the size of rust infection sites was measured microscopically and the number of callose-producing cells counted at each site. This process was undertaken for *P. graminis* f. sp. *tritici* infection sites on BdTr13h, *P. striiformis* f. sp. *tritici* sites on Bd21, and *P. triticina* sites on Bd21-3 and Bd21. These accessions were chosen due to their propensity to allow the growth of both large and small infection sites of each rust pathogen species. A linear relationship was observed between log2 of the infection site area versus log2 of the infection site area divided by the number of callose-producing cells (essentially equivalent to the proportion of noncallose-producing cells) (Fig. 5A).

These data suggest that large rust infection sites on *B. distachyon* increase in size due to an ability to either suppress callose deposition or avoid detection and subsequent callose deposition in some mesophyll cells. Whether this avoidance or suppression is transient or fixed is unknown; however, the absence of callose production in infected cells in the center of large infection sites (i.e., the earliest infected cells) suggest a long-term ability to suppress the callose deposition response (Fig. 4A1 and A2). The absence of callose in some cells at large infection sites does not appear to be due to differential callose turnover because most small infection sites remained surrounded by callose-producing cells for the entire infection period.

Pathogen arrest by callose deposition in the absence of cell death is also a feature of host basal defense mechanisms against adapted pathogens. To compare the relationship between this response for cereal rusts on host and nonhost species, fully susceptible wheat cultivars were infected with the same isolates of *P. graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici*, and

P. triticina rust used to infect *B. distachyon*. Wheat leaves were harvested from these susceptible cultivars after pathogen sporulation had occurred. Microscopic examination indicated that the vast majority of rust infection sites had successfully sporulated. However, occasional infection sites were present that did not manage to sporulate and were significantly smaller

Fig. 3. A, Median infection site areas of rust pathogen infections and salicylic acid analysis. Median infection site areas shown for Bd21 (21) infected with *Puccinia graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici*, *P. graminis* f. sp. *avena*, and *P. graminis* f. sp. *phalaridis*; BdTr10h (10h) and TEK4 (T4) infected with *P. striiformis* f. sp. *tritici*; and Sonora wheat infected with *P. graminis* f. sp. *tritici*. This latter measurement on the host plant was taken immediately after pustule eruption and these infection sites would have become significantly larger with increased infection time. **B**, Analysis of *P. striiformis* f. sp. *tritici* growth on *Brachypodium distachyon* accessions. The average number of autofluorescent cells per *P. striiformis* f. sp. *tritici* infection site is shown. Numbers above each data set indicate the number of infection sites measured while + to ++++ indicates an increasing level of *P. striiformis* f. sp. *tritici* growth observed. BdTr10h can be seen to produce significantly more autofluorescent cells per infection site ($P < 0.05$) than all other accessions tested. **C**, Salicylic acid (SA) concentration in uninfected (gray bars) and *P. graminis* f. sp. *tritici*-infected (white bars) leaf tissue of *B. distachyon* isolates TEK4, Bd21, and BdTr3b. Tissue was harvested 3 days postinoculation. No significant difference in SA concentration was observed between infected and uninfected tissue of the same *B. distachyon* accession. A highly significant difference (t test, $P < 0.05$) in SA concentration was observed between pairwise comparisons of Bd21/TEK4 and Bd21/BdTr3b but not TEK4/BdTr3b. Standard deviations are indicated.

in size than most sporulating infection sites (Fig. 4F1). These smaller sites varied in size, which presumably reflects the point in development when further growth was prevented by basal resistance. When infection site areas and number of callose-producing cells were quantified at these unsuccessful infection sites (Fig. 4F1 and F2), a very similar relationship was

observed on wheat compared with the *B. distachyon* nonhost (Fig. 5B). These data suggest that similar mechanisms prevent rust pathogen growth during wheat basal defense and the *B. distachyon* NHR response.

No correlation between the presence of rust haustoria and callose deposition was observed, with haustorium-containing

Fig. 4. Callose deposition upon rust pathogen infection. **A** to **C**, Large (**A1**) and small (**B1** and **C1**) *Puccinia graminis* f. sp. *tritici* infection sites present on leaves of *Brachypodium distachyon* accession BdTr13h. Callose-producing cells at each infection site are shown in **A2**, **B2**, and **C2**, respectively. **D**, *P. graminis* f. sp. *tritici* haustorium (**D1**, marked with an arrow) present in a callose-producing BdTr13h cell (**D2**). **E**, Two *P. graminis* f. sp. *tritici* haustoria (**E1**, marked with arrows) present in non-callose producing BdTr13h cells. Other mesophyll cells surrounding this infection site do not contain haustoria but produce callose. **F**, *P. tritici*-infected wheat leaf tissue from 'McKellar' is shown in **F1**. Two small infection sites on this susceptible plant (marked with arrows) show proportionally greater callose deposition compared with surrounding large infection sites (**F2**).

cells showing both callose deposition and callose absence at infection sites on both wheat (not shown) and *B. distachyon* (Fig. 4D and E) for all three wheat pathogen species. Small infection sites that did not develop haustoria on *B. distachyon* (i.e., little more than a substomatal vesicle) were often surrounded by mesophyll cells containing callose (Fig. 4B and C). The activation of a defense response in the absence of haustoria at these infection sites suggests that recognition of the nonadapted pathogen is mediated either by PAMP molecules or, alternatively, from yet-to-be-identified effectors secreted from infection hyphae or substomatal vesicles.

To determine the role of SA accumulation in the *B. distachyon* NHR response to *P. graminis* f. sp. *tritici*, two *B. distachyon* lines were selected that were restrictive of *P. graminis* f. sp. *tritici* growth (Bd21 and TEK4) and one line that allowed extensive *P. graminis* f. sp. *tritici* growth (BdTr3b). At minimum, three pots were infected with *P. graminis* f. sp. *tritici* and a similar number of pots left uninfected. SA concentration was determined per gram of tissue harvested from each pot and values pooled for identical treatments. From these experiments, no increase in SA accumulation was observed in any *B. distachyon* line in response to *P. graminis* f. sp. *tritici* infection (Fig. 3C). However, a fourfold difference in SA content was observed between Bd21 and the remaining two lines that did not correlate with the extent of rust growth observed on these plants (Fig. 3C). Similar variation in SA content has been observed among different rice lines (Raskin et al. 1990; Silverman et al. 1995).

The BdTr10 group is atypical.

The BdTr10 group and accession Gaz5 appeared atypical upon rust infection for several reasons. For most other accessions, the presence of macroscopic lesions upon infection with either *P. graminis* f. sp. *tritici* or *P. striiformis* f. sp. *tritici* invariably correlated with large underlying rust infection sites. In contrast, upon infection with either of these two rust pathogen species, the BdTr10 group and Gaz5 produced numerous small lesions on the leaf surface (Fig. 1J) which, upon microscopic examination, corresponded to small infection sites (Fig. 1K and L). These macroscopic lesions were due to the accumula-

tion of brown pigment in mesophyll cells surrounding the infection site (Fig. 1L). Among the BdTr10 group, this observation was most pronounced on BdTr10h, BdTr10m, and BdTr10d, with the remaining accessions tending to show less obvious phenotypes.

In addition, unlike other *B. distachyon* accessions, infection sites on BdTr10h were almost always accompanied by autofluorescent cell death (Fig. 1M and N), with a significantly greater average number of autofluorescent cells per infection site (Fig. 3B) (Other accessions in the BdTr10 group were not examined in detail for autofluorescence.) The presence of brown pigment in autofluorescent cells tended to suppress the fluorescence, indicating that this pigment and the fluorescent phenolic compounds localized in the plant cell wall after death were different (Fig. 1M and N). Large rust infection sites were rare on these accessions but were observed occasionally. Only a proportion of small infection sites resulted in lesion formation (approximately half), with many similar-sized infection sites producing no macroscopic symptoms. Therefore, a significant phenotypic difference was apparent in these lines relative to all other accessions in response to *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* infection.

However, BdTr10 lines and Gaz5, like most *B. distachyon* accessions, allowed more extensive growth of both *P. graminis* f. sp. *avena* and *P. graminis* f. sp. *phalaridis*, with frequent, small sporulating uredinia produced. In contrast, growth of *P. tritici* was modest on BdTr10h and BdTr10m but infection sites were not associated with pigmentation of adjacent plant cells. Therefore, the atypical response of the BdTr10 group to *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* is unique to these two rust pathogens and again demonstrates that the genetically defined NHR outcome is dependent upon both the *B. distachyon* genotype and rust genotype or species.

Inheritance of the NHR response to *P. striiformis* f. sp. *tritici*.

To determine the heritability of NHR phenotypes among *B. distachyon* accessions, two families were produced by crossing *B. distachyon* lines that showed differential infection phenotypes. The first cross was produced between BdTr13k and

Table 1. Inverse relationship between *Puccinia striiformis* f. sp. *tritici* and *P. graminis* f. sp. *tritici* infection site sizes (ifs) on *Brachypodium distachyon* accessions

Accession	Group	<i>P. striiformis</i> f. sp. <i>tritici</i> 104E137A-growth	<i>P. graminis</i> f. sp. <i>tritici</i> growth
BdTr1a	2	Very small ifs	Large ifs
BdTr1c	2	Very small ifs	Large ifs
Bd2-3	2	Very small ifs	Pustules
BdTr3b	2	Small ifs	Pustules
BdTr3c	2	Very small ifs	Pustules
BdTr3n	2	Very small ifs	Large ifs
BdTr5b	2	Very small ifs	Pustules
BdTr5d	2	Small ifs	medium ifs
BdTr9h	2	Very small ifs	Pustules
BdTr9i	2	Very small ifs	Pustules
BdTr9k	2	Very small ifs	Large ifs
BdTr13c	2	Very small ifs	medium ifs
BdTr13g	2	Small ifs	Pustules
BdTr13h	2	Very small ifs	Pustules
Bd21	2	Large ifs	Very small ifs
BdTr12a	2	medium ifs	Very small ifs
BdTr10d	2	Very small ifs	Very small ifs
BdTr10h	2	Very small ifs	Small ifs
BdTr10m	2	Very small ifs	Very small ifs
TEK4	TEK	Large ifs	Very small ifs
TEK7	TEK	Large ifs	Very small ifs
TEK12	TEK	Large ifs	Very small ifs
Bd1-1	TEK	Large ifs	Small ifs
BdTr8a	TEK	Large ifs	Small ifs
BdTr8m	TEK	Large ifs	Small ifs

Bd21 while the second cross was produced between BdTr10h and TEK4. Both families were expected to segregate for NHR phenotypes to *P. striiformis* f. sp. *tritici*, given the phenotypes of each parent described above. *B. distachyon* is inherently difficult plant species to cross; hence, only a single F1 plant was obtained for each cross from many attempts. The F1 status of each plant was confirmed by the inheritance of restriction fragment length polymorphism markers from each parent and the subsequent segregation of these markers among F2 progeny (Fig. 6).

Seedlings from the F4 generation of the BdTr13k × Bd21 family were infected with *P. striiformis* f. sp. *tritici* isolate 104 E137 A– (with an 18°C maximum day temperature) and obvi-

ous differences in *P. striiformis* f. sp. *tritici* infection phenotypes were observed among the 316 F4 plants tested. In this experiment, two F4 seedlings were infected from each of 158 F3 lines. Plants were readily classified into either of two groups.

Table 2. Segregation of *Puccinia striiformis* f. sp. *tritici* infection phenotypes among F4 progeny of selected BdTr13k × Bd21 F3 lines

F3 line	Number of F4 progeny tested	Immune plants	Extensive <i>P. striiformis</i> f. sp. <i>tritici</i> growth
7	19	15	4
43	15	13	2
56	20	15	5
83	9	5	4
236	20	19	1
256	12	9	3

Fig. 5. Relationship between the frequency of callose-producing cells and rust pathogen infection site area. **A**, Graph ($\log^2$) showing cereal rust infection site area (x axis) relative to the infection site area divided by the number of callose-producing cells at each infection site (y axis). A small Y value indicates that proportionally more cells at a given infection site produced callose while a large Y value indicates that much fewer cells produced callose at that infection site relative to its area. For each *Brachypodium distachyon* accession–rust pathogen interaction, it can be seen that there are proportionally fewer callose-producing cells as infection sites become progressively larger. Measurements for Bd21/*Puccinia tritricina* are indicated by squares, for Bd21-3/*P. tritricina* by diamonds, for Bd21/*P. striiformis* f. sp. *tritici* by Xs, and for BdTr13h/*P. graminis* f. sp. *tritici* by triangles. The line of best fit for each dataset is indicated. **B**, The same graph shown in A, with measurements superimposed for ‘McKellar’ wheat infected with *P. tritricina* (black circles), ‘Sonora’ wheat infected with *P. graminis* f. sp. *tritici* (black diamonds), and ‘Morocco’ wheat infected with *P. striiformis* f. sp. *tritici* (black crosses). The line of best fit for these datasets are indicated and labeled 1 to 3, respectively. All *B. distachyon* data are shown in gray and are identical to those shown in A, while all wheat data are shown in black.

Fig. 6. **A**, Restriction fragment length polymorphism (RFLP) analysis of *Brachypodium distachyon* families. DNA blot analysis of *B. distachyon* DNAs restricted with *Bam*HI. Total DNAs were extracted from lane 1, BdTr13k; lane 2, an F1 plant produced from crossing BdTr13k and Bd21; lane 3, Bd21; and lanes 4 to 16, 13 F2 plants derived from the F1 plant. RFLP bands unique to each parent DNA and which segregate in the F2 progeny are highlighted with arrows. **B**, RFLP analysis of lanes 4 to 16, DNAs from 13 F2 progeny derived from lane 2, an F1 plant produced by crossing lane 1, BdTr10h, with lane 3, TEK4. DNAs were restricted with *Xho*I and transferred to nylon membranes. RFLP unique to each parent and which segregate among F2 progeny are indicated with arrows on the left-hand side of the image. Membranes in both panels were hybridized with a probe encoding nucleotides 3,605 to 4,161 of an *Oryza sativa* phenylalanine ammonia lyase gene (GenBank accession X87946). Arrowheads on the right-hand side of each panel indicate molecular weight mobilities of 12, 5, 3, and 2 kb, respectively.

In total, 88 F4 seedlings showed extensive *P. striiformis* f. sp. *tritici* symptoms on the leaf, including sporulation in some instances (Supplementary Fig. S1) similar to the Bd21 parent, while 228 plants showed immunity, as did the BdTr13k parent. Little evidence of intermediate phenotypes was observed. Among the 158 F3 lines tested, in 107 cases, both F4 seedlings were immune; in 37 cases, both F4 seedlings showed obvious *P. striiformis* f. sp. *tritici* symptoms; whereas, for 14 F3 lines, one F4 seedling showed immunity while the other showed obvious *P. striiformis* f. sp. *tritici* symptoms. From 6 of the 14 F3 lines in which segregation of *P. striiformis* f. sp. *tritici* immunity was observed, additional F4 seedlings were tested. In each small F4 family analyzed, segregation was again observed for either immunity or extensive *P. striiformis* f. sp. *tritici* symptom development, with no intermediate phenotypes again observed (Table 2).

Microscopic analysis of all 316 F4 plants described above confirmed that each plant was heavily challenged with *P. striiformis* f. sp. *tritici* and that immune plants contained many small *P. striiformis* f. sp. *tritici* infection sites consisting of substomatal vesicles, infection hyphae, and haustoria. In contrast, F4 plants with obvious macroscopic symptoms showed extensive *P. striiformis* f. sp. *tritici* growth, with frequent production of uredinia upon microscopic examination. These latter infection sites again corresponded precisely with macroscopic lesions.

This phenotypic data is not consistent with immunity to *P. striiformis* f. sp. *tritici* infection being conferred by many genes with small additive effects because intermediate infection phenotypes were not observed among these plants. Rather, immunity appears to be a trait with a relatively simple mode of inheritance. The segregation ratios observed in this F4 family are most consistent with a two gene model, with one dominant and recessive gene each independently conferring immunity; however, additional genetic analyses are required to confirm this gene model.

Infection with *P. striiformis* f. sp. *tritici* isolate 104 E137 A– was also undertaken on 213 F2 seedlings derived from the BdTr10h × TEK4 cross. In this case, three phenotypes were observed macroscopically. In all, 115 seedlings were immune; 44 seedlings showed very small brown lesions, as did the BdTr10h parent; while 54 seedlings showed extensive *P. striiformis* f. sp. *tritici* infection symptoms, with sporulation at some infection sites, as did the TEK4 parent. Microscopic analysis of plants showing either immunity or having small brown lesions on the leaf surface indicated that both groups contained small *P. striiformis* f. sp. *tritici* infection sites similar in size to the BdTr10h parent, whereas extensive *P. striiformis* f. sp. *tritici* growth was observed on plants that showed large macroscopic symptoms. In this family, little evidence of intermediate amounts of *P. striiformis* f. sp. *tritici* growth relative to the parental lines was also observed. These data are consistent with a single, dominant gene present in the BdTr10h parent severely restricting *P. striiformis* f. sp. *tritici* growth relative to the TEK4 parent (i.e., 159 plants with restricted growth versus 54 plants with extensive growth, $\chi^2 P = 0.905$).

As described above, the BdTr10 group is atypical in that small brown lesions are often associated with both *P. striiformis* f. sp. *tritici* and *P. graminis* f. sp. *tritici* infection sites even though BdTr10 accession are highly restrictive of growth by both of these nonadapted rust pathogens (Fig. 1J to N). In most other *B. distachyon* accessions, the presence of macroscopic symptoms invariably corresponds to extensive *P. striiformis* f. sp. *tritici* or *P. graminis* f. sp. *tritici* growth. Interestingly, the 159 BdTr10h × TEK4 F2 plants that showed extreme inhibition of *P. striiformis* f. sp. *tritici* growth segregated for this brown lesion trait, with this segregation consistent with a single, recessive gene conferring the browning response (115

immune versus 44 small brown lesions, $\chi^2 P = 0.606$). These data suggest that, segregating independently of extreme *P. striiformis* f. sp. *tritici* resistance, a second gene is conferring browning, but this latter gene is not required for immunity. It is difficult to determine whether this brown-lesion phenotype is also apparent in the remaining F2 plant group that showed extensive *P. striiformis* f. sp. *tritici* growth, given the severity of the symptoms associated with these infection sites.

DISCUSSION

These data demonstrate phenotypic variation among different *B. distachyon* accessions in the NHR response to infection by several rust pathogens. These phenotypes are often conserved between genetically related accessions and also dependent upon the rust pathogen used. These observations are exemplified by the inverse relationship between *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* growth on the TEK group and many group 2 accessions, with *P. striiformis* f. sp. *tritici* growth most pronounced on the former group and *P. graminis* f. sp. *tritici* sporulation occurring only on the latter. Similarly, the atypical response of the BdTr10 group, typified by increased autofluorescent cell death and pigmentation occurring at small infection sites, is specific to *P. graminis* f. sp. *tritici* and *P. striiformis* f. sp. *tritici* infection but is not apparent in response to challenge by *P. triticea*, *P. graminis* f. sp. *avena*, or *P. graminis* f. sp. *phalaridis*. Therefore, the NHR response to cereal rust growth is dependent upon both the nonhost genotype and the rust pathogen species.

Microscopic analyses indicated that callose deposition during the NHR response could be induced in adjacent mesophyll cells of *B. distachyon* by as little as a rust substomatal vesicle (Fig. 4D and E). Given the absence of haustoria and haustorial effectors (Rafiqi et al. 2012) at these sites, the defense response, presumably, is PAMP triggered. In addition, a similar relationship was observed between callose production and infection site area for both the *B. distachyon* NHR response and wheat basal defense to all three wheat rust pathogen species, with the latter host response also likely based upon PAMP-triggered immunity (Jones and Dangl 2006).

The observation that larger rust infection sites contain proportionally fewer *B. distachyon* cells producing callose is consistent with these infection sites having a transient ability to suppress or evade the NHR response. The absence of callose in some cells located in the middle of large infection sites which have been infected for 4 weeks suggests that suppression of callose deposition in these cells may be irreversible. Why cells in juxtaposition at the same infection site show such variation in callose deposition patterns is unknown and highlights the remarkably cell-autonomous nature of the NHR response at these larger infection sites.

Variation was also observed for rust pathogen infection site size on the same leaf even though clonal spores are used for the infection. Similar variation in infection outcomes is also apparent for these same rust species on susceptible hosts, with a small proportion of infection sites failing to circumvent basal resistance. A consequence of these differential outcomes is that, in both nonhost and host interactions, a continuum of infection site sizes is observed. It is the range and median of this distribution that distinguishes host and nonhost interactions to rust pathogen infections and also explains the macroscopic variation in the nonhost response observed among different *B. distachyon* lines.

Plant cell death that resulted in autofluorescence was neither common nor extensive at rust infection sites on *B. distachyon*, whereas callose deposition was frequently observed. These observations are similar to cereal rust infection of rice (Ayliffe et

al. 2011). In spite of the possibly very different evolutionary history of these two plant species with rust pathogens, they respond with apparently similar NHR responses. Rice may have evolved in the absence of rust pathogens (Leppik 1961; Wahl et al. 1984), whereas *B. distachyon*, being indigenous to the Mediterranean region (Filiz et al. 2009; Garvin et al. 2008), has co-evolved with the rust pathogens of cereal progenitors and is parasitized by rust species.

Although apparently similar NHR responses occur on rice (Ayliffe et al. 2011) and *B. distachyon* upon infection with nonadapted rust pathogens, the extent of rust growth is far greater on *B. distachyon*. This observation is unsurprising given that *B. distachyon* is believed to have diverged from the Triticeae family 35 to 40 million years ago (Bossolini et al. 2007) whereas rice diverged approximately 50 million years ago (Paterson et al. 2004). The greater colonization of *B. distachyon* by stem rust pathogens that parasitize the Poaceae family (*P. graminis* f. sp. *phalaridis* and *P. graminis* f. sp. *avena* [this study] and *P. graminis* ff. spp. *lolii* and *phlei-pratensis* [Figueroa et al. 2013]) suggests that these plant species may be more closely related to the Poaceae than the Triticeae family, in spite of apparently equidistant phylogenetic relationships (Song et al. 2007). Arguably, the *B. distachyon*–*P. graminis* f. sp. *tritici* interaction is a true nonhost interaction while *B. distachyon* is a partial host for stem rust pathogens of the Poaceae family, given the formation of sporulating uredinia on most accessions examined.

Ultimately, an understanding of the NHR response of *B. distachyon* to rust pathogens can only be resolved by isolating the *B. distachyon* genes that confer differential rust infection phenotypes. The microscopic phenotypic differences we have reported in this manuscript provide an entry point for isolating these genes and, based upon this information, two *B. distachyon* mapping families have been produced from parents that show differential NHR responses to rust pathogen infection. Analysis of *P. striiformis* f. sp. *tritici* infection of progeny from these families demonstrates that the different NHR phenotypes present in the parents are clearly heritable. Remarkably, these phenotypic differences do not appear to be under complex, additive genetic effects, because only parental phenotypes were observed to segregate in progeny upon *P. striiformis* f. sp. *tritici* infection. Preliminary genetic analyses indicate potentially simple inheritance of these phenotypes, with apparent single gene segregation observed in one family. These data demonstrate that *B. distachyon* will be a powerful model system for elucidating the molecular basis of NHR to rust pathogens of important cereal crop species.

MATERIALS AND METHODS

Plant and fungal material.

Seed of *B. distachyon* plants were provided by J. Vogel via I. Wilson. These accessions, most of which are described by Vogel and associates (2009), are all diploid and hence bona fide members of the *B. distachyon* species (Catalan et al. 2012). Plants were grown in a soil/compost (1:1) mixture at 21°C for 16 h of light and 16°C for 8 h of darkness in a glasshouse supplemented with additional lighting. For some accessions, seed was stratified at 4°C for 1 week to enhance germination. For rust inoculations, plants were placed in a humid chamber and rust spores applied as an aerosol. Spores were mixed with an equal volume of talc powder to assist in obtaining a more uniform inoculation. Tissue from rust-infected *B. distachyon* plants were harvested 3 to 4 weeks postinfection for microscopy analyses. Rust isolates were propagated on hosts by inoculation of seedlings at the three-leaf stage with an aqueous suspension of rust urediniospores. Inoculated seed-

lings were incubated overnight at 16°C in a humid chamber and, subsequently, transferred to the glasshouse for postinoculation rust development. The five rust pathogens used in this study were *P. graminis* f. sp. *tritici* Plant Breeding Institute (PBI) accession number 690822; *P. graminis* f. sp. *avenae* PBI accession number 962508; *P. graminis* f. sp. *phalaridis* PBI accession number 01197; *P. striiformis* f. sp. *tritici* PBI accession number 821559, and *P. triticina* PBI accession number 89172. All of these isolates are maintained in liquid nitrogen at the PBI Cobbitty.

Microscopy of rust infected plant tissue.

All rust-infected plant tissues were stained as described by Ayliffe and associates (2011). Briefly, harvested leaf tissue was autoclaved in 1 M KOH, then neutralized in 50 mM Tris, pH 7.0. Tissue was then stained with wheat germ agglutinin conjugated to fluorescein isothiocyanate (WGA-FITC) at 20 µg/ml (Sigma-Aldrich, St. Louis) and viewed under blue light. Autofluorescence was examined in the same tissue under UV light.

Analysis of callose deposition.

Leaf tissue that had been cleared and stained with WGA-FITC as described above was subsequently stained with aniline blue fluorochrome (Biosupplies, Bundoora, Australia) and examined under UV light to enable callose-producing cells to be detected. This double staining enabled rust infection structures to be observed under blue light and callose-producing plant cells at the same site to be observed under UV light. Each rust infection site was photographed and the number of callose-producing cells was counted. The area of rust infection was calculated for each photograph by measuring the maximum width (W) and length (L) of rust growth using the polyline facility of the AnalySIS Life Science Professional program. The rust infection was then assumed to be oval in shape and the area calculated as $L/2 \times W/2 \times 3.14$. Each infection site area was then divided by the number of callose-producing cells at the site to generate a value that represents the infection site area per callose-producing cell. A large value indicates that relatively few mesophyll cells are producing callose at the infection site whereas a small value indicates extensive callose deposition.

SA analysis.

B. distachyon seedlings were grown to the fifth-leaf stage and then infected with *P. graminis* f. sp. *tritici*. Three days postinfection, tissue was harvested from infected pots and uninfected control pots and SA content determined per gram fresh weight of tissue, essentially as previously described (Ayliffe et al. 2011). A minimum of three replicates was used for each *B. distachyon* genotype.

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