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Determining the basis of nonhost resistance in rice to cereal rusts

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Abstract Cereal rusts are a constant disease threat that limits the production of almost all agricultural cereals. Rice is atypical in that it is an intensively grown agricultural cereal that is immune to rust pathogens. This immunity is manifested by nonhost resistance (NHR), the mechanisms of which are poorly understood. As part of the Borlaug Global Rust Initiative (BGRI), studies are being undertaken to dissect the molecular mechanisms that provide rust immunity in rice and determine if they can be transferred to wheat via transgenesis. Microscopic analyses showed that cereal rusts are capable of entering the rice leaf via formation of an appressorium over a stomate and subsequent infection of underlying mesophyll cells. However, there is considerable variation in the extent of colonization at each infection site. Our research effort has focused on screening for increased growth of cereal rust using natural and induced variants of rice. Two collections of rice mutants, T-DNA insertional mutants and chemical/irradiation-induced mutants, and diverse germplasm accessions are being screened for compromised NHR to cereal rusts. Preliminary screening with stripe rust identified several potential mutants that allow increased fungal growth. The confirmation of these lines will serve as the foundation for the isolation of gene(s) responsible for this compromised resistance. Details of the strategies being undertaken and progress to date are provided.

Keywords Host–pathogen interaction · Nonadapted pathogen · Disease · Wheat · *Puccinia*

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Introduction

Nonhost resistance (NHR) prevents plants from being infected by most potential microbial pathogens. Specialized plant pathogens infrequently cross

nonhost barriers and infect new crop plant species, providing testimony to the durability of NHR mechanisms. The molecular basis of NHR is of obvious interest given the durability of this resistance and the potential to transfer this resistance mechanism(s) by transgenesis to crop plants that are hosts of a given pathogen species. The transfer of the maize *Rxo1* gene, which confers resistance to the adapted bacterial pathogen *Burkholderia andropogonis*, into rice where it confers resistance to *Xanthomonas oryzae* pv. *oryzicola*, a nonpathogen of maize is the first example of transfer of NHR between species (Zhao et al. 2005).

Rust pathogens reduce the production of nearly all agricultural cereals and grasses including wheat, maize, barley, sorghum, oats, triticale, rye, sugarcane, sorghum, and millet. The emergence of a new group of wheat stem rust (*Puccinia graminis* f. sp. *tritici*) (*Pgt*) races in the Ug99 (TTKSK) lineage that threaten global wheat production emphasises the need for durable rust resistance in most cereals (Pretorius et al. 2000; Singh et al. 2006, 2007; Stokstad 2007). In contrast, rice is the only intensively grown cereal crop for which no known rust has been identified. As part of the Borlaug Global Rust Initiative (BGRI), research is being undertaken to elucidate the molecular basis of immunity in rice to cereal rusts.

NHR mechanisms—what is known

Although the mechanisms of NHR are not well defined, current models describe layers of plant defense that must be circumvented by a potential pathogen for successful plant infection (Fig. 1). An obvious initial requirement is basic compatibility in that appropriate physical and chemical signals are required for microbial recognition of a potential host. Preformed plant physical and chemical (anticipins) barriers must be circumvented by an invading microbe which requires appropriate infection structures and anticipin immunity. Microbes produce a suite of highly conserved molecules (e.g. flagellin, translation elongation factors and liposaccharides) collectively known as *p*athogen *a*ssociated *m*olecular *p*atterns (PAMPs) that are recognised by plant receptor proteins (reviewed by Zipfel 2008) leading to the induction of a plant defense response. A successful plant pathogen must suppress this PAMP-triggered immunity (PTI) which is achieved by introducing an array of pathogen effector proteins and products directly into plant cells that target specific plant molecules involved in the defense process (reviewed by Hogenhout et al. 2009). Having circumvented PTI a successful pathogen infection is established. This microbe species is therefore an adapted pathogen of the particular host

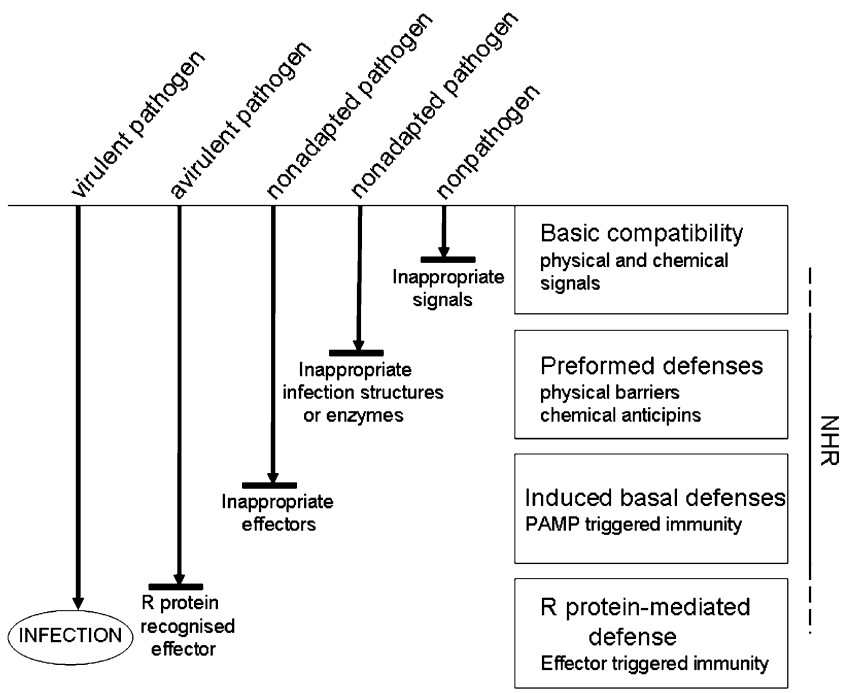


Fig. 1 Summary of plant defense mechanisms that must be circumvented by a pathogen for successful colonisation. Adapted from Ayliffe et al. (2009)

plant species. For those plant species that the microbe is incapable of infecting due to an inability to circumvent the plant NHR response it is considered a nonadapted pathogen.

The successful parasitism by a plant pathogen leads to plant host and pathogen co-evolution whereby the host plant evolves recognition mechanisms of the adapted pathogen and subsequent activation of a defense response. This recognition is most commonly, but not exclusively, mediated by NBS-LRR resistance proteins that recognise pathogen effector products either directly or more commonly by effector-mediated modification of host plant proteins. This effector triggered immunity (ETI) is the underlying basis of gene-for-gene resistance (Jones and Dangl 2006). The pathogen in turn alters or loses recognized effectors or effector functions thereby reinstating virulence.

NHR resistance is therefore multifaceted and the result of physical, chemical, preformed and active recognition processes.

A number of studies investigated the interaction of rust pathogens on nonhost plants. These include the growth of the cowpea rust pathogen (*Uromyces vignae*), wheat leaf rust pathogen (*Puccinia triticina*) and Asian soybean rust pathogen (*Phakopsora pachyrizi*) on Arabidopsis (Mellersch and Heath 2003; Shafiei et al. 2007; Loehrer et al. 2008); the growth of the barley leaf rust pathogen (*P. hordei*) on wheat (Prats et al. 2007) and *P. triticina*, *P. hordei-murini, P. hordei-secalini* and *P. persistens* growth on barley (Jafary et al. 2008). In the latter interaction barley is considered an occasional host for these pathogens with most, but not all, barley accessions being resistant to these rusts (Jafary et al. 2008). Collectively these studies demonstrated that resistance to nonadapted rust pathogens is polygenically inherited and is an active response involving salicylic acid signalling and the production of reactive oxygen species. These latter biochemical events are often also common to host resistance responses.

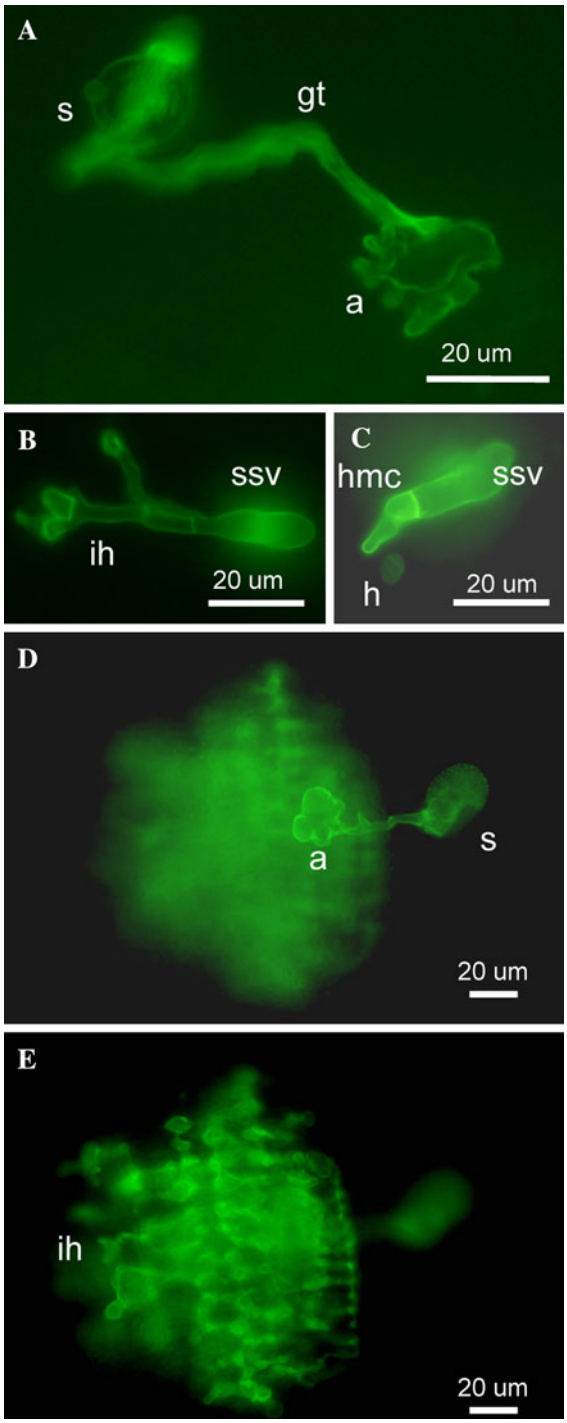
Infection of rice by cereal rusts

A prerequisite for examining rice NHR to cereal rust pathogens was to demonstrate that these fungi are capable of infecting rice. That is, to determine if these pathogens can recognise rice as a potential plant host for colonisation and that the immunity of rice is not a consequence of a basic incompatibility. Microscopic analyses of *Pgt* infection of rice demonstrated that this pathogen is capable of producing all the infection structures necessary for successful plant colonisation including haustoria, the specialised cells used by the fungus for nutrient acquisition (Fig. 2). At some infection sites the extent of *Pgt* growth was very large (i.e. in excess of 100 μm in diameter) with several hundred rice mesophyll cells colonised, arguing for nutrient acquisition from the host (Fig. 2d, e; Ayliffe et al. 2008). Similar patterns of infection were observed with *P. striiformis* f. sp. *tritici* and *P. triticina*. Rice responded to *Pgt* challenge with an active recognition response that involved hydrogen peroxide production, callose deposition and in some instances plant cell death. To dissect the molecular basis of rice immunity to cereal rusts, several approaches are being investigated.

Analysis of genetic variation in the NHR response of rice to stem rust

Potential phenotypic variation in NHR to stem rust is being investigated both macroscopically and microscopically. Diverse rice germplasm was macrosopically screened by co-inoculation with isolates of *P. graminis* f. sp. *avenae*, race TJS and *P. graminis* f. sp. *secalis*, races BBBB and 92-MN-90. To date, 9,000 rice lines have been screened, including accessions from Africa, Asia, Australia, Europe, North America and South America, in addition to accessions of wild rice and *Oryza glaberrima* (Fig. 3). In no accession did infection develop to the stage of sporulation thus demonstrating conservation of rice NHR to stem rust across this entire range of plant diversity. However, 34 rice lines showed macroscopic symptoms (lesions) to stem rust infection indicating phenotypic variation in the NHR response to these nonadapted pathogens. Microscopic analysis of the 34 lines indicated that increased fungal growth was not associated with this lesion formation. All lesions were only ever associated with small infection sites that had little rust development beyond substomatal vesicles. These 34 accessions were derived from Africa (12%), Asia (76%), Europe (9%) and North America (3%).

Microscopic analysis of a number of *Pgt* infected rice lines was undertaken and average infection site



◀ **Fig. 2** Infection of rice leaf tissue by *P. graminis* f. sp. *tritici* (*Pgt*). **a** Germination of a *Pgt* urediniospore (s) to produce a germ tube (gt) and appressorium (a) on the surface of a rice leaf; **b** *Pgt* substomatal vesicle (ssv) and infection hyphae (ih) within a rice leaf; **c** production of a *Pgt* haustorium mother cell (hmc) and haustorium (h) within a rice leaf; **d** a large *Pgt* infection site on a rice leaf. The urediniospore (s), germ tube (gt) and appressorium (a) can be seen on the surface of the rice leaf. The large amount of fungal material underneath the appressorium is shown in **e** and consists of infection hyphae ramifying throughout the mesophyll tissue. Amongst the infection hyphae are distinct non-staining circular structures which are mesophyll cells in juxtaposition to infection hyphae. In all images fungal material was stained with wheat germ agglutinin conjugated to the fluorophore alexa 488 and visualised under blue light following the clearing of tissue. Adapted from Ayliffe et al. (2009)

areas determined by microscopic measurement. Two rice cultivars (Kyeema and Namaga) were identified that reproducibly showed the largest infection site sizes when compared with the remaining lines. An F_2 family derived from a cross between Kyeema and a highly restrictive line (IR64) typical of the remaining rice cultivars, was assessed for average *Pgt* infection site size over two replicate experiments. These datasets were shown to be correlated ($P = 0.008$), but with only a moderate correlation coefficient ($r = 0.49$). From these analyses we conclude that genetic factors do affect the NHR response in this cross but it is impractical to identify loci segregating in this material by QTL analyses.

Mutagenesis

The identification of genes conferring NHR by mutagenesis and microscopic screening was demonstrated in Arabidopsis by isolation of the *PEN* genes (reviewed by Lipka et al. 2008). Screening of EMS-mutagenized Arabidopsis plants with a barley powdery mildew fungus identified a number of plants that showed enhanced epidermal penetration by the non-adapted pathogen. Mutations that allowed enhanced penetration were identified microscopically with a mutation frequency of approximately 0.1% (Stein et al. 2006). These mutant plants were in three complementation groups that led to the isolation of three genes involved in NHR, namely *PEN1-3*, that encode a syntaxin protein involved in vesicle targeting to mildew infection sites (Collins et al. 2003), a peroxisome-localised glycoside hydrolase (Lipka et al. 2005; Bednarek et al. 2009; Clay et al. 2009) and an ATP-binding cassette transporter (Stein et al. 2006; Kim et al. 2007), respectively. Both *PEN2* and *PEN3* contribute to a signalling pathway leading to callose formation following PAMP recognition (Clay et al.

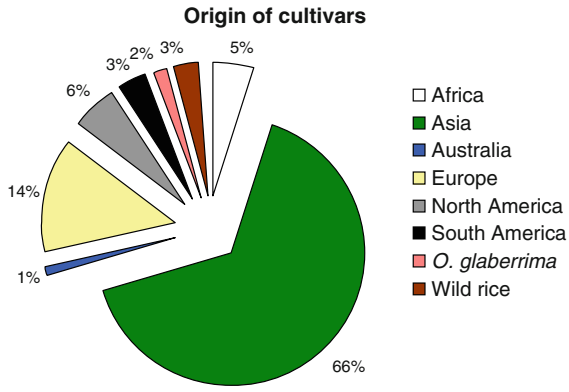


Fig. 3 Origin of 9,000 rice accession from diverse geographical locations screened with oat and rye stem rust isolates

2009). The identification of the *PEN* genes confirms that mutagenesis is a reasonable approach for dissecting NHR mechanisms.

Unlike nonadapted powdery mildew pathogens on Arabidopsis, microscopic analyses showed that cereal rust fungi have little difficulty in entering the rice leaf via formation of an appressorium over a stomate and subsequent infection of underlying mesophyll cells (Fig. 2d). Given the relative absence of rust penetration barriers a logical first step is therefore to screen for rice mutants that allow increased fungal growth relative to the wild type. We assembled two large collections of rice mutants for screening against rusts. The first collection consists of approximately 60,000 M4 lines of the indica variety IR64 produced by chemical and irradiation mutagenesis at IRRI (Wu et al. 2005). The second collection consists of >20,000 mutants of the japonica variety Zhonghua 11, made by T-DNA insertional mutagenesis at Huazhong Agricultural University, Wuhan, China (Zhang et al. 2007). We are currently undertaking a systematic screen of these mutants using stem rust and stripe rust pathogens.

Screening for increased wheat stem rust growth on rice

Approximately 450 EMS mutagenized IR64 rice lines were screened microscopically for altered *Pgt* infection phenotypes. The first 300 rice lines were identified in previous genetic screens as showing increased susceptibility to compatible races of *Magnaporthe grisea* (rice blast pathogen), i.e. these plants showed reduced basal resistance to an adapted pathogen. None of these lines showed increased growth of the nonadapted *Pgt* pathogen suggesting little overlap between NHR to *Pgt* and basal resistance to rice blast.

Screening for increased stripe rust growth on rice

Two mutagenized rice populations are currently being screened microscopically for altered response to *P. striiformis* infection. We have so far screened 5,229 rice T-DNA lines using a local Chinese *Pst* isolate of race CYR-32. Three potential mutant plants appear to show increased fungal growth compared with

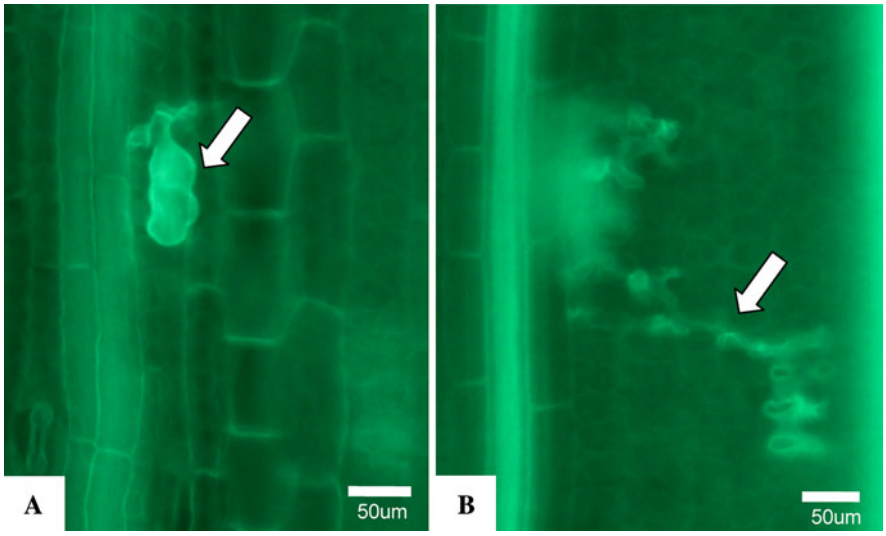


Fig. 4 A T-DNA insertion rice mutant (2611-E-2, in Zhonghua 11 background) showing increased growth of *Puccinia striiformis* f. sp. *tritici* (*Pst*). **a** Substomatal vesicle (*arrow*) in inoculated leaf tissue 20 days after inoculation. **b** Growth of hypha in inoculated leaf tissue 20 days after inoculation (*arrow*). Tissues were stained by Calcofluor White M2R and observed under blue light

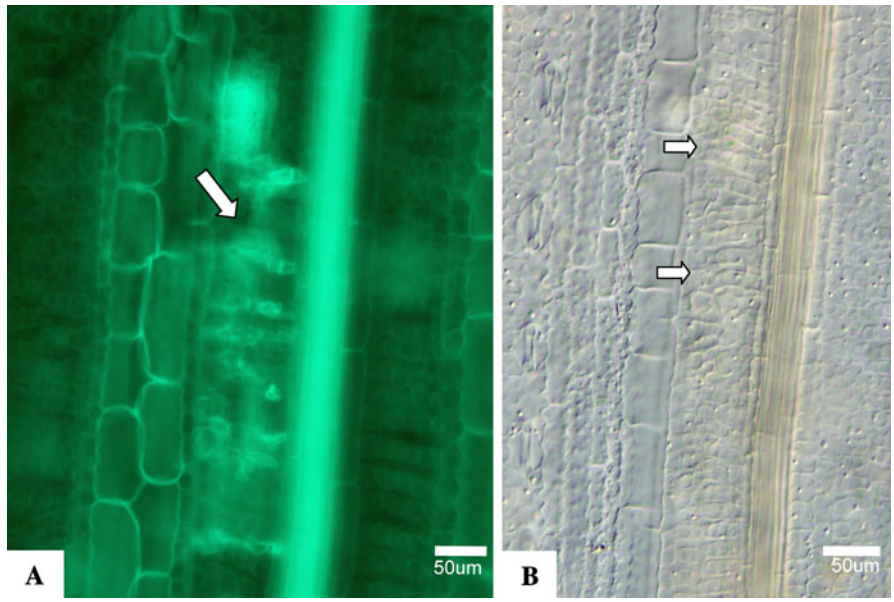


Fig. 5 IR64 mutant (B12-2) showing increased growth of *Puccinia striiformis* f. sp. *tritici* (*Pst*). **a** Colonization by *Pst* hypha (*arrow*) 20 days after inoculation. Leaf tissue was stained with Calcofluor White M2R and observed under UV light. **b** Same leaf tissue as (**a**) shown by Nomarski interference optics indicating plant cell structure. The area of rust infected cells is highlighted with *arrows*

wild type (Fig. 4). A second population consisting of 5,000 EMS-mutagenized M4 lines was also screened with the same isolate. A pooling strategy of 100 pools of 50 lines was employed to screen the population. Several potential mutant plants showing increased levels of stripe rust growth were identified in two pools (Fig. 5). Potential mutant plants were grown for progeny testing and, if the mutant phenotypes are confirmed, for genetic analysis. Genomic sequences that flank T-DNA insertion sites are also being isolated.

Candidate gene approach

Combining mutations in the *PEN2* and *PEN3* genes with mutations in genes (Wiermer et al. 2005) involved in basal defense, salicylic acid signalling and ETI to adapted pathogens (*PAD4* and *EDS1*) generated Arabidopsis plants that showed dramatic increases in the growth of nonadapted mildew pathogens. These additive mutation affects were further enhanced in a triple mutant background (*pen2pad4-sag101*) whereby mutant plants became virtual hosts of nonadapted powdery mildew pathogens, allowing the formation of condiophores on this nonhost plant species (Lipka et al. 2005, 2008; Stein et al. 2006).

Unlike mildew pathogens which directly penetrate the plant epidermis, the infection structures produced by urediniospores enter the leaf through stomates. Hence *PEN*-like genes may not be of relevance in the interaction between rice plants and infection by cereal rust urediniospores (in contrast the sexual phase of cereal rust infection does involve direct epidermal penetration). We have therefore begun microscopic screening of rice lines that are deficient in genes known to be involved in basal resistance and ETI, for compromised NHR to *Pgt* upon inoculation with urediniospores. Preliminary evidence suggests that rice plants deficient for the *RAR1* gene, *EDS1* gene or *CeBip* gene do not show increased growth of *Pgt*.

From these data the following conclusions can be drawn:

(1) Extensive screening of diverse rice gemplasm demonstrates that rice is a true nonhost of stem rust and not an occasional host.
(2) *Pgt* is capable of infecting rice and producing all the infection structures necessary for colonisation.
(3) Some infection sites encompass many (hundreds of) mesophyll cells suggesting nutrient uptake from the pathogen.
(4) Rice responds with an active NHR response to *Pgt* that involves the production of reactive oxygen species and callose deposition.
(5) Phenotypic variation in the NHR response can be observed amongst rice cultivars both macroscopically and microscopically.

(6) Loss of basal resistance to rice blast does not affect the NHR response to cereal rust.
(7) Perturbation of several defense pathway molecules did not alter the NHR response to *Pgt* suggesting a great deal of redundancy in this resistance.
(8) Multiple potential mutants showing increased growth of *P. striiformis* were recovered from rice mutant collections.

Future work

We will continue screening for potential diversity in NHR response to cereal rusts. Diverse rice germplasm previously screened with the oat and rye stem rust pathogens will be rescreened with wheat stem rust isolates. In addition 10,000 EMS mutagenized rice lines will be screened for macroscopic *Pgt* symptoms. Microscopic screening for altered *Pgt* infection phenotypes on mutagenized rice lines will also continue. Molecular analyses will be used to confirm the identity of T-DNA insertion mutants in rice and demonstrate that these insertions perturb the genes of interest. The genetic analyses of rice mutants that potentially allow increased growth by *P. striiformis* will continue. Demonstrating stable inheritance of increased rust growth phenotypes will be a high priority in these lines. It will also be of great interest to determine if these rice lines are also perturbed in NHR to other rust pathogens such as *Pgt*.

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