Pathogenicity of Rice Blast Fungus Magnaporthe oryzae on Brachypodium distachyon

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Abstract: Inoculation methods for rice blast fungus Magnaporthe oryzae to Brachypodium distachyon were developed to investigate the infection process and symptom development in comparison with those on rice (Oryza sativa) and barley (Hordeum vulgare). M. oryzae could infect leaves, sheathes, stems and panicles of B. distachyon and cause blast disease. Spraying conidial suspension on either intact seedlings or leaf segments induced typical symptoms on B. distachyon. During the intact seedling inoculation, the symptom developed on B. distachyon leaves closely resembled that on rice; but the lesions on B. distachyon had better uniformity in shapes and sizes than those on rice or barley. In the leaf segments inoculation, only initial and low-developed lesions could be found on rice, while normal symptoms on B. distachyon and barley. Inoculated with low-virulent mutants of M. oryzae, B. distachyon produced low-level symptoms. The symptom level of each mutant on B. distachyon corresponded well to that on rice. In addition, typical infection processes presented on B. distachyon leaves: forming melanized appressoria, penetrating into host epidermis and then forming hyphae in epidermal cells. According to these results, B. distachyon can be used as a candidate for studying fungus-plant interactions and as a probable source of disease resistance.

Key words: Brachypodium distachyon; Magnaporthe oryzae; interaction; model plant; pathogenicity

Rice blast is the most destructive disease on rice (Oryza sativa). Plant-fungus interaction is a hot topic in plant pathology. In recent years, the rice blast fungus (Magnaporthe oryzae) is becoming a valuable model for such research. Besides rice, M. oryzae infects barley (Hordeum vulgare), wheat (Triticum aestivum), millet (Eleusine coracana) and other species of the Poaceae family (Igarashi et al, 1986; Ekwamu, 1991). Investigating interactions of this fungus on different hosts (even non-host) to reveal differences among them are important to both plant pathology research and disease control. Du et al (1995, 1996) investigated Pyricularia spp. derived from different grasses in Zhejiang Province, China and used the cross-protection among them to control the disease. Maeda et al (2009, 2010) and Park et al (2009) analyzed the interaction of M. oryzae on Arabidopsis and isolated related genes. However, the knowledge is still limited so far on mechanism and application of the interaction of M. oryzae to non-rice hosts. Therefore, establishing efficient interaction systems of the fungus on non-rice plants, especially on the Poaceae species, and clarifying their susceptibility, infection features and symptom development, are important for both phyto-pathologic research and new resistant resources discovery.

Brachypodium distachyon is a wild annual temperate grass classified in the Poaceae family and Pooideae subfamily. It has sister relationship to the ancestor of the four 'core pooid' tribes, i.e., Triticeae, Aveneae, Bromeeae and Pooae (Davis and Soreng, 1993; Kellogg, 2001), and closely relates to main cereal crops such as T. aestivum, H. vulgare, Avena sativa, O. sativa, Zea mays, Sorghum bicolor and the biofuel crop Panicum virgatum (Caetano-Anolles, 2005). B. distachyon has small physical stature, short lifecycle, simple growth requirement, self-fertility and strong reproduction ability (Chen et al, 2008). Various chromosome ploidies, such as diploid (2n = 10), tetraploid (4n = 20) and hexaploid (6n = 30), were found in B. distachyon (Shi et al, 1993). The genome of diploid Bd21 of B. distachyon has been sequenced. It spans 271.9 Mb (The International Brachypodium Initiative, 2010) and has similar component and structure to those of other Poaceae members (Wang et al, 2007). The biolistic bombardment and Agrobacterium-mediated transformation (AtMT) systems of B. distachyon have already been established and presented high regeneration rates (Li et al, 2008). By AtMT, T1 generation could be obtained within eight months (Alves et al, 2009). Therefore, B. distachyon was regarded as an ideal experimental
model plant for functional genomics of Poaceae plants and biofuel crops (Li et al., 2008).

*B. distachyon* is susceptible to a variety of important plant fungal pathogens. For example, the leaf rust fungus *Puccinia triticina* and the stripe rust fungus *P. striiformis* could penetrate and colonize on *B. distachyon* leaves. The powdery mildew fungus *B. graminis* induced papilla response on *B. distachyon*. Thus, *B. distachyon* can be used to research the plant-fungus interactions (Draper et al., 2001; Parker et al., 2008). However, such research is still very limited and efficient methods are required at present. In this study, we developed inoculation methods for rice blast fungus to *B. distachyon*, investigated the disease development and symptom characteristics, and compared them with those on rice and barley. Our work provided a tool for the research of plant-fungus interaction and discovery of resistance genes.

**MATERIALS AND METHODS**

**Materials**

*Fungal strains, host cultivars and cultivation methods*

The wild type of *M. oryzae* used was Guy11. The mutant strains *Δcpka* (Mitchel and Dean, 1995), *Δmac1* (Choi and Dean, 1997), *Δmpg1* (Talbot et al., 1993) and *Δpmk1* (Xu and Hamer, 1996) were kindly provided by Professor Lin Fucheng at the Institute of Biotechnology, Zhejiang University, China (Table 1). All the strains were cultured on complete media (CM) using routine procedures described by Talbot et al. (1993). The *B. distachyon* variety used was Bd21, a kind gift from Professor An Hailong at the Faculty of Life Sciences, Shandong Agricultural University, China. The barley variety was ZJ-8 and the rice variety was CO-39.

**Instruments and reagents**

Leica DM2500 (Germany) was used for fluorescence microscopy. Aniline blue was purchased from Sigma (Germany) and other reagents were from Huadong Medicine Ltd. Co., China.

**Inoculation**

**Inoculum preparation**

The spores of *M. oryzae* were harvested from 10 d colonies on complete media and suspended in sterilized water to $2 \times 10^5$ conidia/mL.

**Smearing inoculation on seedlings**

The spore suspension of *M. oryzae* was smeared on leaves, sheaths, stems and panicles of *B. distachyon* seedlings by using a painting brush. The inoculated seedlings were kept in dark for 12 h light/12 h dark, and then inoculated and kept moist in a sterilized plastic bag. The symptoms were checked at 9 d post-inoculation (dpi).

**Spray inoculation on seedlings**

The seedlings of 10-day-old barley, 21-day-old rice and 15- to 90-day-old *B. distachyon* were inoculated with the spore suspension of *M. oryzae* by using a mini sprayer. The inoculated plants were kept in dark for 24 h and then transferred to 28 °C, 12 h/12 h alternating light/dark for 9 d.

**Spray inoculation on detached leaves**

The young leaves (the second top leaves of tillers) of 10-day-old barley, 21-day-old rice and 15- to 90-day-old *B. distachyon* were removed and cut into 5 cm segments, and then spray inoculated. The inoculated leaves were kept in dark for 24 h and then incubated at 28 °C, 24 h continuous light for 9 d.

**Droplet inoculation on detached leaves**

The young leaves of *B. distachyon* were collected, cut into 5 cm segments and inoculated with 20 μL droplets of the spore suspension of *M. oryzae* by using a pipette. The inoculated leaves were kept in dark for 24 h and then incubated at 28 °C, 24 h continuous light for 9 d.

**Microscopy of fungal structure and plant surface**

The droplet-inoculated *B. distachyon* leaf segments were treated with ethanol for 3–4 times to remove the chlorophyll, dissociated with 5% KOH for 0.5 h at 65 °C and then stained with aniline blue. The fungal structures

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**Table 1. M. oryzae mutants used in this study.**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutant phenotype</th>
<th>Related gene</th>
<th>Full name of gene</th>
<th>Possible gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δcpka</td>
<td>Host surface sensing; appressorium formation defective; abolished pathogenicity</td>
<td>CPKA</td>
<td>cAMP-dependent protein kinases</td>
<td>Kinase; activating PMK1 signaling of appressorium initiation</td>
</tr>
<tr>
<td>Δmac1</td>
<td>Appressorium formation defective; reduced pathogenicity</td>
<td>MAc1</td>
<td>Magnaporthe adenylate cyclase</td>
<td>Activating the CAMP signaling pathway</td>
</tr>
<tr>
<td>Δmpg1</td>
<td>Appressorium formation defective; reduced pathogenicity</td>
<td>MPG1</td>
<td>Magnaporthe pathogenicity gene 1</td>
<td>Host surface hydrophobin protein assembly; surface thigmotrophic signaling</td>
</tr>
<tr>
<td>Δpmk1</td>
<td>Lack of melanization in appressorium; inability to cease nuclear division in appressorium; abolished pathogenicity</td>
<td>PMK1</td>
<td>Pathogenicity MAP-Kinase 1</td>
<td>Signaling in appressorium maturation; regulation of melanin biosynthesis; cession of nuclear division inside appressorium</td>
</tr>
</tbody>
</table>
on the treated leaf segments were observed under the Leica DM2500 fluorescence microscope. Similar manipulation was used to observe the epidermal structures of the plants.

RESULTS

Rice blast fungus infects different parts of *B. distachyon*

To clarify the pathogenicity of the rice blast fungus on different parts of *B. distachyon*, the *M. oryzae* strain Guy11 was inoculated on leaves, sheaths, stems and panicles using the smearing inoculation method. At 3 dpi, the inoculated leaves produced small brown dots, which expanded gradually and developed into typical diamond-shaped lesions. The sheaths were also infected, producing small brown dots and then developing into lesions. Some of the lesions could spread to the linked leaves. Symptoms on the stems also developed from small brown dots to spindle-shaped lesions. Some lesions on the stems covered 1 to 2 nodes, leading the diseased tiller and the related panicles to wilt. The panicles could also be infected. The lesions presented first on the glumes, and then extended inside. Afterwards, the diseased grains turned dark gray. Finally, the diseased panicles were covered completely with a layer of gray mycelia (Fig. 1). The results indicate that the rice blast fungus can infect different parts of *B. distachyon* and produce typical symptoms. In addition, at 3-5 d after the mycelia layers emerged on the inoculated sites, new lesions formed on non-inoculated parts, indicating that the fungus could re-infect *B. distachyon*.

Symptoms on intact seedlings and detached leaves of *B. distachyon*, rice and barley

To compare the disease development of the rice blast fungus on the leaves of *B. distachyon*, rice and barley, the intact seedlings and the detached leaves of the three species were spray-inoculated under the same conditions. When intact seedlings of the three species were spray-inoculated, the symptoms on *B. distachyon* and rice developed in similar speeds, whereas those on barley developed faster (Fig. 2-A). At 3 dpi, only small brown dots were observed on the leaves of *B. distachyon* and rice, whereas the lesions had already expanded to 1−2 mm with dark green centers and yellow borders on the barley leaves. At 5 dpi, lesions on the leaves of rice and *B. distachyon* expanded and turned gray-brown, and lesions on the barley leaves enlarged continuously and almost covered the entire leaves, and the centers of the lesions turned gray and the borders became brown. At 7 dpi, the lesions on the

*Fig. 1. Symptoms caused by *M. oryzae* Guy11 on different parts of *B. distachyon*. A, Leaf; B, Sheath; C, Stem; D, Panicle.*
No seta but only few short spines was found along the veins (Fig. 3-C). The *B. distachyon* leaves had the epidermal structure seemingly between rice and barley: the epidermal cells were larger than those of rice but smaller than those of barley; the shape and arrangement of the epidermal cells and the stomas distribution were similar to those of barley, whereas the setae were similar to those of rice in shape and distribution (Fig. 3-B). Considering that the disease development on *B. distachyon* was also between rice and barley, namely, the symptom morphology and development resembled those of rice whereas the inoculation requirements were similar to those of barley, we deduced that the differences of leaf surface structures were the reason for the differences in disease development.

**M. oryzae mutants have different virulence to *B. distachyon***

To further understand the pathogenicity of the rice blast fungus to *B. distachyon*, four virulence-changed mutants (Δmpg1, Δmac1, Δcplka and Δpmk1) and the wild type Guy11 were spray-inoculated on the detached leaves. Rice was also inoculated as a control. Because the detached rice leaves were difficult to produce...
symptoms, we used intact seedlings in rice inoculation. At 5 dpi, the *B. distachyon* leaves inoculated with the wild type isolate formed numerous of gray-brown lesions. The leaves inoculated with Δmpg1 or Δmac1 produced typical lesions, but fewer and smaller, indicating that the virulence of these two mutants was partially reduced. On the leaves inoculated with Δpmk1 or Δcpka, only 1 to 2 (or even no) lesions were found, indicating that the pathogenicity of these two mutants decreased greatly (or completely lost) (Fig. 4). The pathogenic phenotypes of each mutant showed on *B. distachyon* leaves corresponded well with those on rice. The results suggest that *B. distachyon* can be used to differentiate the virulence altering of mutants.

**Cytology of *M. oryzae* infection on *B. distachyon* leaves**

To clarify the cytological infection process of *M. oryzae* on *B. distachyon*, the inoculated leaves were observed by using fluorescence microscopy combined with biochemical staining. At 24 h post-inoculation (hpi), the fungus formed normal mature appressoria, spherical and melanized well. Some of the appressoria already penetrated into the epidermal cells and differentiated into infection hyphae (Fig. 5-A). The infection hyphae grew continually and filled the first infected cells at 36 hpi (Fig. 5-B), then punched the cell walls and invaded the adjacent cells at 48 hpi (Fig. 5-C). At 72 hpi, the fungus broke through the host epidermis and produced a number of aerial hyphae, conidiophores and spores (Fig. 5-D). These characteristics, including the shape, the presenting time and the development of the appressoria and the infection hyphae, closely resembled those on rice. The results indicate that the rice blast fungus was able to penetrate the cuticles of *B. distachyon* leaves and invade the...
epidermal cells, then expand in the tissue and form propagules by using the nutrition from the host and finally produce the typical symptoms.

**DISCUSSION**

In this study, we investigated the pathogenicity and infection processes of the rice blast fungus on *B. distachyon* by different inoculation methods. Under the artificial conditions, the fungus could cause blast disease on different parts of *B. distachyon*, form typical symptoms and complete the life cycle. Similarly to that on rice leaves, the fungus presented typical infection processes on *B. distachyon* leaves, such as spor germination, appressorium formation, penetration and colonization. In recent years, some work has been done on the interaction of the rice blast fungus on *B. distachyon*. Routledge et al (2004) reported that the different ecotypes of *B. distachyon* responded differently to the fungus. The responses occurred in the resistant ecotypes resembled in resistant rice cultivars. Allwood et al (2006) found that the membrane phosphatidic acids of *B. distachyon* were possibly important to be susceptible or resistant to the fungus. These findings will benefit the further study on this topic.

Rice is a routine model plant for plant-fungus interaction but has many disadvantages, such as the long life cycle, reproductive difficulty in artificial conditions and complicated manipulation in inoculation. Barley is thus often used to study the infection cytology of the rice blast fungus. Here, we revealed that the rice blast fungus was able to invade *B. distachyon* leaves, form typical symptoms and present an intact infection process. The virulence changes of mutants on *B. distachyon* corresponded very well to those on rice. Therefore, *B. distachyon* could be an alternative experimental host besides rice and barley to analyze the pathogenicity changes and observe the infection cytology of mutants. For this usage, *B. distachyon* has many advantages. First, *B. distachyon* resembles more to rice, the natural host of the fungus in symptom development, than barley. Parker et al (2009) suggested that the differences in disease development among species were due to different epidermal structures. Our microscopic observation of the leaf surfaces gave evidence to this viewpoint. Second, *B. distachyon* is easier to be sampled than rice, because its leaves from any developmental stages are suitable for inoculation. Third, *B. distachyon* is easier to be manipulated in inoculation. Either intact seedlings or detached leaf segments are inoculable and present the same lesions and symptom development, whereas the lesions on leaf segments of rice are difficult to develop. One of the reasons why the detached leaves of *B. distachyon* could generate the ideal symptoms was likely to be the capacity to keep fresh-green, which benefited the survival of the fungus and the lesions expansion. And fourth, the symptoms on *B. distachyon* leaves have better uniformity than those on rice.

Along with the development of bioscience and application of biotechnology, the experimental model organisms are getting more important. The related statistics indicated that more than 80% of the research on life process and life mechanism published in the top journals such as Nature, Science and Cell, were performed by using model organisms (Zhu, 2006). As a new model plant, *B. distachyon* is benefiting greatly the functional genomics of grass. The pathogen stressed functional genomics is an important part of plant functional genomics, but it is still very limited in *B. distachyon*. The establishment of the interaction methods between *B. distachyon* and the rice blast fungus will provide a useful tool for such research. We will use it to further analyze the interaction-related genes. This work also gives a probable model for studying Pryculria on weeds and a genetic resource of disease resistance.

**REFERENCES**


