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Original article

Overexpression of *BAS1* in rice blast fungus can promote blast fungus growth, sporulation and virulence in *planta*

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ABSTRACT

Background: BAS1 is biotrophy-associated secreted protein of rice blast strain (*Magnaporthe oryzae*). In order to study the effect of BAS1 on virulence of rice blast strain, we characterized function of BAS1 using a purified prokaryotic expression product of *BAS1* and its overexpression strain. *Results:* Our results showed *in vitro* the purified expression product caused rapid callose deposition and ROS production in rice leaves and calli, indicated it triggered transient basal defense. When the purified expression product of BAS1 was sprayed onto rice leaves, and 24 h later the leaves were inoculated with blast strain, the results showed the size and number of lesions, on purified BAS1 product-pretreated leaves of the Lijiangxintuanheigu (LTH) challenged with blast strain, was higher than those in BAS1-untreated leaves directly challenged with the same strain, which suggested the defense response trigged by BAS1 can be overcome by other effectors of the fungus. More severe symptoms, higher sporulation, higher relative fungal growth and more lower expression level of defense-related genes appeared in LTH leaves challenged with overexpression strain 35S:BAS1/Mo-2 than those in LTH inoculated with wild-type strain. *Conclusions:* These data suggest both *in vitro* pretreatment with *BAS1* prokaryotic expression products and overexpression in blast strains can increase virulence of blast fungus.

1. Introduction

The immune response capabilities of plants have evolved to respond to and resist pathogen infection. Host plants recognize pathogen associated molecular patterns (PAMPs) such as flagellin, lipopolysaccharide, glycoprotein, and chitin as the first line of the defense response (Dangl and Jones, 2001). These receptors encode transmembrane receptor-like kinases. Once the receptor recognizes the PAMP, it triggers a series of immune responses in the host plant. These responses include stomatal closure, MAPK activation, ROS generation, differential gene expression, callose deposition, and other physiological processes, which lead to a basic immune response or PAMP-triggered immunity (PTI) (Melotto et al., 2006; Navarro et al.,2004; Schwessinger and Zipfel, 2008; Zipfel et al., 2004). In contrast, the pathogen secretes a large number of effector proteins that inhibit PTI to invade and colonize the host (Göhre and Robatzek, 2008; Lindeberg et al., 2009).

Pathogenic bacteria infect plants, and produce type III effector proteins in order to suppress the immune response (Büttner and He, 2009). Bacterial type III secretary effector (T3SE) has multiple functions, which can transport into the host cell and reprogram multiple metabolic pathways, such as the induction of defense-related gene expression, downstream defense signal activation, specific protein modification, as well as production of SA, JA, and Et signaling molecules, etc. (Stulemeijer and Joosten, 2008). Overexpression of the type III effector protein in plants can alter the plant response to the pathogen, either resulting in plant infection or inducing the plant defense response. AvtaBsT, identified from bacterial scab disease, is the first type III effector protein of the YopJ family. Effector proteins in the YopJ family mainly inhibit host ETI response (Büttner and He, 2009) while AvrBsT is required for HR induction of resistance in response to pathogen infection (Büttner and He, 2009). When bacterial scab pathogens infect host plants, AvrBsT is transported into plant cells to initiate defense responses (Kirik and Mudgett, 2009). GST (Glutathione S transferase)-AvrBsT fusion protein induces leaf cell death in *Arabidopsis* seedlings (Hwang et al., 2012), and its transient expression causes hypersensitive cell death in tobacco and pepper leaves (Kim et al., 2010), However, certain concentrations of

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GST-AvrBsT fusion protein apparently decrease fungi infection in young leaves of Arabidopsis (Hwang et al., 2012). The effector protein AvrBsT mitigates pathogen infections *in vitro*, but the underlying mechanism for the induction of the basal defense response(s) remains unclear.

The pathogenicity of *Magnaporthe oryzae* to rice, is well studied, yet rice blast disease still threatens global rice production and food security in many rice growing regions (Wang and Valent, 2009). *M. oryzae* secretes large amounts of effector proteins during the infection of rice tissue, which facilitates the penetration of the fungus into the host epidermal cells, evading host recognition, and reprogramming host defense genes, to create a favorable environment for the growth and reproduction of the pathogen. The interaction between this hemibiotrophic fungus and its host is a complex system involving infection hyphae and the host membrane system termed the BIC (biotrophic interface complex). Both the host and the pathogen regulate the structure and function of this complex system. The fungi secrete a large amount of the effector proteins, including PWL2 and Avr-Pita in the biotrophic phase. A large number of genes were up-regulated in the biotrophic phase of fungal growth have been designated as biotrophy-associated secreted (BAS) proteins (Mosquera et al., 2009). BAS1-4 have been widely studied, but their function within the fungus and the host tissues is still not clear, and whether the function of BAS effectors are limited to the biotrophic stage of infection is of particular interest. The expression of BAS1 is upregulated 100-fold in the fungal infection hyphae (IH) (Mosquera et al., 2009), which can enter the rice cytoplasm (Ribot et al., 2013; Gao et al., 2017). In the present study, we investigated the effect of a BAS1-overexpression strain and the prokaryotic expression product of GST-BAS1-mCherry on the susceptible rice variety LHT. We also tested the effect of BAS1 on the infection of *M. oryzae* strains *in vitro* by pretreating rice leaves for 24 h using GST-BAS1-mCherry before inoculating the leaves with a conidial suspension. Moreover, we tested the pathogenicity, hyphal growth and sporulation *in planta* of the BAS1 overexpressed transformants and the expression levels of defense-related genes in rice leaves challenged with the BAS1 overexpression strain. The objective of which was to determine the role of BAS1 in the blast fungus infection process.

2. Materials and methods

2.1. Rice blast strain and rice cultivar

We used the rice variety, Lijiangxintuanheigu (LTH) that is highly susceptible to *M. oryzae* strains. The *M. oryzae* strains used in this study was 66b (strong pathogenic strain), BAS1 overexpressing strain under 35S promoter (35S:BAS1/Mo-2) (the overexpression strain was got previously), and wild-type strain 95234I-2b (PCR analysis showed that this strain did not harbor the *BAS1* gene). All these strains were preserved in our laboratory. GST-BAS1-mCherry was the prokaryotic expression product used for spraying rice seedlings.

2.2. Activation of M. oryzae and preparation of spore suspension

Mycelia of *M. oryzae* were inoculated on petri plates containing PDA solid medium (potato 200 g, glucose 20 g, agar 15 g, and water 1000 ml), which was cultured in a 28 °C incubator until the mycelia covered the entire agar surface. Mycelium blocks were transferred to a flask, which was cultured in a 28 °C shaker for 5–7 d, and then stored in 4 °C refrigerator prior to use. The mycelium liquid of *M. oryzae* was spread out evenly on petri plates containing tomato-oat medium (tomato-oat medium: tomato juice 300–400 ml, oats 40 g, $CaCO_3$ 0.6 g, agar 20 g, and water 1000 ml). The plates were incubated at 25 °C for 7–10 days to sporulate. Approximately, 20 ml of sterile water was added into the dish, and then the plates were gently scraped, washed, and filtered to obtain the spore suspension. The concentration was adjusted to 1×10^5 cells/ml.

2.3. Cultivation of rice seedlings and leaf inoculation

Rice seeds were sterilized with 1.5% sodium hypochlorite and incubated at 28 °C for germination. The germinated seedlings were sown in a seedling tray. When the rice grew to the 3–4 leaf stage, it was moved to an inoculation box. Spore suspension of *M. oryzae* was sprayed on the rice and sufficient moisture was provided for 24 h after which the seedlings were transferred to a greenhouse. Disease incidence was investigated at six days, and leaf samples were collected at different times. Three repeats were performed for each treatment, and 15 seedlings were surveyed for each repeat. Four seedlings were sampled for each repeat at each time point.

2.4. Real-time RT-PCR analysis of defense-related genes in rice leaves

Total RNA was extracted using the TRIZOL (Invitrogen) extraction kit. Total RNA was reverse transcribed using Superscript III (Invitrogen) to obtain cDNA. Real-time RT-PCR primer sequences for the defense-related genes in rice a shown in Table 1.

Real-time PCR (Bio-Rad) 25.0 μl reaction system: 2.0 μl template cDNA, 0.5 μl forward primer, 0.5 μl reverse primer, 12.50 μl 2 × EasyTaq PCR SuperMix, and 9.5 μl sterilized ddH_2O. Amplification cycle parameters: pre-denaturation at 95 °C for 3 min, denaturation at 95 °C for 20 s, annealing extension at 59 °C for 20 s, and a collection of fluorescence signals at 65 °C; a total of 44 cycles were performed. Dissolution curve parameters: the temperature was increased starting from 59 °C; fluorescent signals were collected at each cycle with the temperature increased by 0.5 °C, and a total of 80 cycles were performed. Three repeats were performed for each sample. Ct values were recorded to calculate the relative expression levels. Real-time PCR data was analyzed with the $2^{-\triangle\triangle Ct}$ method. Expression levels of the resistance genes in rice were calculated. The relative gene expression level = treated sample (target gene Ct – actin Ct) – blank sample (target gene Ct – actin Ct).

2.5. Callose and ROS observation

LTH blades were selected at the two-leaf stage, and shortened to 4 cm length. The blades were then immersed in clear water for 2 h and then placed on wet filter paper in a petri dish. The

Table 1
Disease incidence on leaves inoculated with blast strain.

Treatment	Disease incidence (%)
BAS1/66b	39.81 ± 2.49
PBS/66b	28.24 ± 2.33
66b	25.67 ± 1.68
BAS1/Guy11	26.19 ± 1.52
PBS/Guy11	20.28 ± 2.15
Guy11	17.05 ± 1.01

Note: BAS1/66b indicated leaves that treated with BAS1 solution were challenged with blast strain of 66b; PBS/66b as control indicated leaves that treated with PBS solution were challenged with 66b; 66b as control indicated leaves were challenged with 66b. BAS1/Guy11 mean leaves that treated with BAS1 solution were challenged with blast strain of Guy11; PBS/Guy11 as control indicated leaves that treated with PBS solution were challenged with Guy11; Guy11 as control indicated leaves were challenged with Guy11.

purified BAS1 prokaryotic expression products were sprayed on the leaves, which were then put in an incubator with 26 °C and humidity for 24 h. The leaves were stained with aniline blue and DAB respectively.

Callose observation: The leaves were soaked in ethanol lactophenol solution (12.5 g phenol, 12.5 ml glycerol, 12.5 ml lactic acid and 12.5 ml water to mix well) and kept in a 65 °C water bath until the chloroplasts were cleared. The treated blades were rinsed with 50% ethanol followed by rinsing with sterile water. The blades were then stained with 0.1% aniline blue (dissolved in 150 mM K_2HPO_4, pH 9.5) for 0.5 h. The stained samples were placed in 50% glycerol. The callose deposition was observed with UV light under a fluorescent microscope (Leica).

ROS observation: After the leaves soaked with protein solution had been rinsed with sterile water, they were wiped gently with filter paper and stained with 1 mg/ml DAB solution for 24 h. The stained leaves were then soaked in ethanol lactophenol solution and kept in a 65 °C water bath until the chloroplasts were cleared. ROS was observed under a fluorescent microscope (Leica).

2.6. In vitro punch inoculation of 35S:BAS1-mCherry overexpression strains and real-time PCR analysis of the relative growth of the fungus

We washed the spores cultured in tomato-oat medium and observed under a microscope (Leica, DM750), LTH blades at the two-leaf stage were cut into blades of 4 cm length and placed on a wet filter paper in a petri dish. Two wounded punch spots (spaced 1 cm apart) were made in the 4-cm long leaf (not to penetrate the leaves), punch spots spacing of 1 cm. Spore suspension was applied on the holes of the blade with a pipette. The leaf blades pretreated with BAS1 challenged with strain of 66b or Guy11 (BAS1/66b or BAS1/Guy11), and the leaf blades pretreated with PBS challenged with strain of 66b or Guy11 (PBS/66b or PBS/Guy11) and the leaf blades directly challenged with strain of 66b or Guy11 (66b or Guy11), the last two was as controls. The blades were kept for 24 h in a 28 °C dark incubator with a relative humidity of 90% and then cultured for seven days in a 28 °C light incubator with uninterrupted moisturizing spray. The length and width of the lesions were measured seven days after onset of disease. Genomic DNA and total RNA were extracted from the lesions (2 × 1 cm). Real-time PCR (Bio-rad) 25.0 μl reaction system: 1.5 μl template cDNA, 0.5 μl forward primer, 0.5 μl reverse primer; 12.50 μl 2 × Easy Taq PCR SuperMix, and 10 μl ddH_2O. Amplification cycle parameters: pre-denaturation at 95 °C for 3 min, denaturation at 95 °C for 20 s, annealing extension at 58 °C for 20 s, and collection of fluorescence signals at 65 °C; 44 cycles were performed. Dissolution curve parameters: the temperature was increased starting from 59 °C; fluorescent signals were collected at each cycle with the temperature increased by 0.5 °C, for 80 cycles. Three repeats were performed for each sample. Ct values were recorded to calculate the absolute quantitative expression levels. The relative growth of the fungus was calculated as $2^{[Ct(MoPot2) - Ct(OsUBQ)]} \times 100$, where MoPot2 is the *Pot2* gene of *M. oryzae*, OsUBQ is the rice gene *ubiquitin*, and 2 is the number of primer pairs of the two detected genes of *Pot2* and *OsUBQ*.

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3. Results

3.1. Callose deposition and ROS production in susceptible rice leaves in vitro treated with BAS1

We observed callose deposition on LTH rice leaf blades sprayed with BAS1 (Fig. 1a), whilst no callose was deposited on rice leaf blades sprayed with PBS (Fig. 1b). Callose deposition is triggered by the PAMP immune response, indicating that BAS1 induces the basal defense response in rice. Rice leaf blades sprayed with

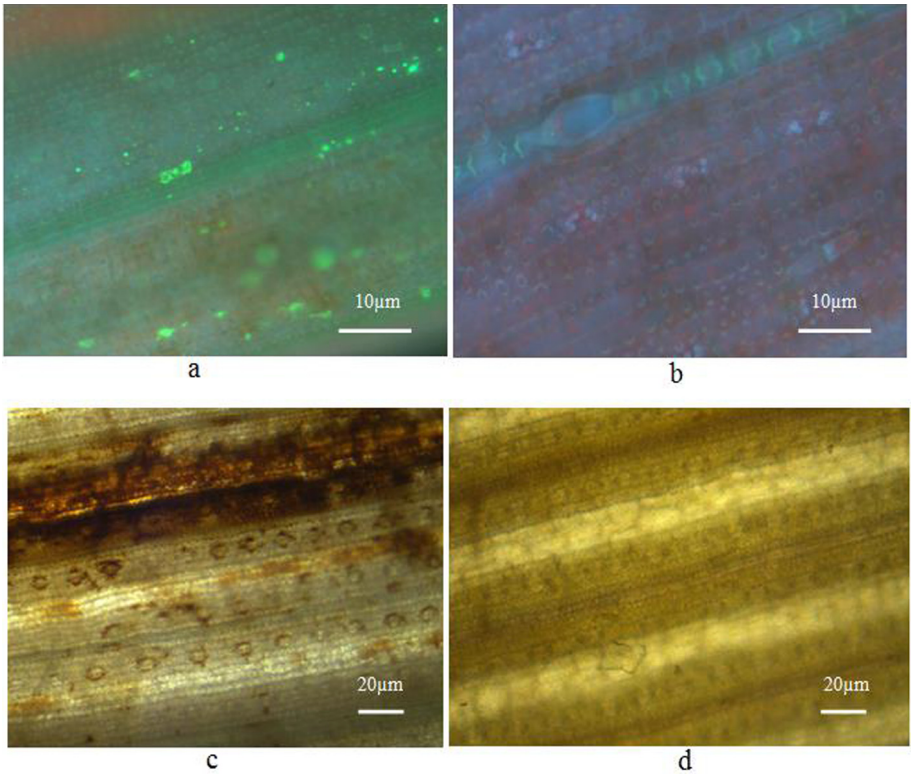

Fig. 1. Callose deposition and ROS generation in rice leaves sprayed with purified *BAS1* prokaryotic expression products at 24 h. Callose deposition in rice leaves sprayed with purified *BAS1* prokaryotic expression products at 24 h (a); Callose deposition in rice leaves sprayed with PBS at 24 h (b); ROS generation in rice leaves sprayed with purified *BAS1* prokaryotic expression products at 24 h (c); ROS generation in rice leaves sprayed with PBS at 24 h (d). Each experiment had three biological replicates.

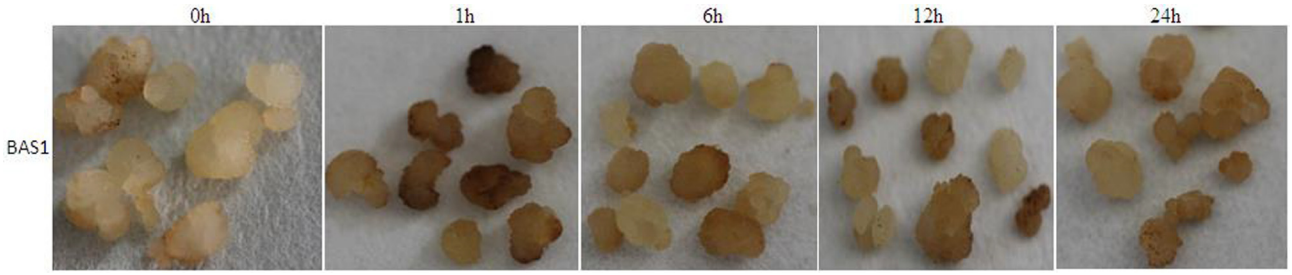

Fig. 2. BAS1 prokaryotic expression production induce ROS production at different time points.

BAS1 produced high levels of ROS (Fig. 1c), while none was observed on control rice leaf blades sprayed with PBS (Fig. 1d). Therefore, our results indicate that BAS1 induces ROS production and callose deposition in susceptible rice leaf blades suggesting that BAS1 can induce an early basic defense response in rice tissues *in vitro*, but that the defense induced is transient in nature.

3.2. BAS1 induced ROS production in rice calli

In order to further analyze whether BAS1 could rapidly induce ROS production in rice, rice calli were treated with BAS1 fusion protein and ROS production in the treated rice calli was observed at 0 h, 1 h, 6 h, 12 h and 24 h post treatment. The results obtained demonstrated that the ROS production in the treated rice calli occurred from 1 h to 24 h post treatment. But, that large quantities of ROS were produced after 1 h and that the ROS production then decreased at 6 h and remained at a similar level until 24 h after treatment with the fusion protein (Fig. 2). These data demonstrated that *BAS1* could apparently induce ROS production in 1 h, at the stages of 12 h and 24 h, the ROS production decreased, which indicated BAS1 *in vitro* induce an almost immediate strong basal defense response, but that its effect rapidly decreased then plateaued over time.

3.3. Effect of BAS1 on the infection of the M. oryzae strain 66b in vitro

In order to further analyze whether or not the defense response triggered by BAS1 can be overcome, we pretreated rice leaves using

Fig. 3. Blast disease symptoms on leaves BAS1-pretreated challenged with blast strain (BAS1/66b, BAS1/Guy11). Rice seedlings were pretreated using GST-BAS1-mCherry (4μg/ml) or PBS as a control for 24 h before being inoculated by blast strain of 66b and Guy11.The leaves of symptoms were photographed seven days post-inoculation, symptom on BAS1-pretreated rice seedlings of LTH challenged with 66b (A), Symptoms on PBS-pretreated rice seedlings of LTH challenged with 66b (A, control) and symptoms on rice seedlings of LTH directly challenged with 66b (A, control). Symptom on BAS1-pretreated rice seedlings of LTH challenged with Guy11 (B), Symptoms on PBS-pretreated rice seedlings of LTH challenged with Guy11 (B, control), and symptoms on rice seedlings of LTH directly challenged with Guy11 (B, control). Lesion number on leaves of the BAS1-treated and control rice seedlings were counted, the results show averages from three triplicate samples in one experiment, and each sample was 60 rice seedlings.

Table 2
Lesion area on leaves inoculated with blast strain.

Treatment	Lesion area (mm)
BAS1/66b	13.2 ± 0.14
PBS/66b	8.6 ± 0.32
66b	9.9 ± 0.07
BAS1/Guy11	15.3 ± 0.26
PBS/Guy11	8.9 ± 0.37
Guy11	9.6 ± 0.73

Note: BAS1/66b indicated leaves pretreated with GST-BAS1-mCherry were punch inoculated with 66b, 66b indicated leaves were punch inoculated with 66b, PBS/66b indicated leaves treated with PBS were punch inoculation with 66b. h: hours post-inoculation. Error bars represent ± SD of the mean. The data are from three independent experiments.

BAS1 prokaryotic expression products, then the pretreated-leaves were challenged with the blast strain. Rice leaf blades pretreated with 4 μg/ml fusion protein GST-BAS1-mCherry challenged with 66b and Guy11 showed an increased number and size of lesions as compared to leaf blades pretreated with PBS challenged with 66b/Guy11 and too leaf blades directly challenged with 66b/Guy11 (Fig. 3). For GST-BAS1-mCherry pretreated leaf blades challenged with blast strains, the incidence of lesions was 39.81% after inoculation with 66b; whereas it was 26.19% after inoculation with Guy11. In the control (PBS pretreatment), the incidence of lesions was 28.24% and 20.28% respectively in leaf blades inoculated with 66b and Guy11 and the leaf blades directly challenged with blast strains of 66b and Guy11 showed 25.67% and 17.05% incidence of lesions, respectively (Table 1). These results suggest that BAS1 caused more severe symptoms in rice leaves challenged with both strong and weak pathogenic strains (66b and Guy11) of *M. oryzae* as compared with the two control groups.

Table 3
Effect of BAS1 on weight of mycelia of blast strain.

Treatment	Weight (g)
BAS1 + 66b	0.30 ± 0.02
PBS + 66b	0.02 ± 0.01
H_2O + 66b	0.03 ± 0.00
BAS1 + Guy11	0.26 ± 0.01
PBS + Guy11	0.04 ± 0.00
H_2O + Guy11	0.02 ± 0.01

Note: The weight of mycelia in 20 ml culture, the experiment repeats three times, three bottles.
BAS1 + 66b indicated 66b mycelia grew in PSA medium adding BAS1 solution; PBS + 66b as control indicated 66b mycelia grew in PSA medium adding PBS solution; H_2O + 66b as control indicated 66b mycelia grew in PSA medium adding water volume equal to PBS or BAS solution. BAS1 + Guy11 indicated Guy11 mycelia grew in PSA medium adding BAS1 solution; PBS + Guy11 as control indicated Guy11 mycelia grew in PSA medium adding PBS solution; H_2O + Guy11 as control indicated Guy11 mycelia grew in PSA medium adding water volume equal to PBS or BAS solution.

3.4. BAS1 increased the lesion biomass of infected rice leaves

To further clarify the mechanism by which BAS1 enhanced the virulence of *M. oryzae* strains *in vitro*, we analyzed the lesion area, the number of spores per lesion, and the fungal growth rate in infected 6-cm-long rice leaves at 6 days after being sprayed with 4 μg/ml GST-BAS1-mCherry before *in vitro* inoculation of 66b and Guy11. The results showed that the lesion area was significantly larger (13.2 ± 0.14 mm) in BAS1/66b as compared to PBS/66b (8.6 ± 0.32 mm) and 66b (9.9 ± 0.07 mm) (Table 2). Similarly, the lesion area was significantly larger (15.3 ± 0.26 mm) in BAS1/Guy11 as compared with the lesion area on PBS/Guy11 (8.9 ± 0.37 mm) and Guy11 (9.6 ± 0.73 mm) samples (Table 2).

BAS1/66b treatment resulted in a significantly higher number of spores (2.5×10^5/ml) on blade lesions than on leaf blades treated with PBS/66b (1.1×10^5/ml) and 66b (1.3×10^5/ml). Moreover, the number of spores recorded on BAS1/Guy11 blade lesions was significantly higher (9.5×10^4/ml) than PBS/Guy11 (1×10^4/ml) and Guy11 (1.5×10^4/ml) (Fig. 4) (see Table 4).

BAS1/66b treatment also resulted in a significantly higher fungal growth rate (5667.61) in rice leaves as compared to PBS/66b (1702.21) and 66b (1294.68). The fungal growth rate in BAS1/Guy11-treated leaves was significantly higher (2710.71) than in PBS/Guy11-treated (940.07) and Guy11-treated leaves (778.90) (Fig. 5).

Based on the above results, BAS1 was shown to increase the lesion area, spore count and fungal relative growth rate in rice leaves infected with strong/weak pathogenic strains of *M. oryzae*, i.e., BAS1 enhanced the lesion biomass of rice leaves infected with strong/weak virulence strains of *M. oryzae*.

3.5. BAS1 promoted sporulation and mycelial growth of M. oryzae

The mycelium weight of 66b and Guy11 (0.3 g, and 0.26 g) was highest in the PSA medium containing BAS1 protein solution. The mycelium weight of 66b and Guy11 in the PSA medium containing PBS and water was 0.03 g, 0.17 g, 0.04 g and 0.02 g, respectively (Table 3 and Fig. 6). These results indicated that overexpression of *BAS1* in blast strains facilitated mycelia growth.

Moreover, we also found that the spore count of 66b and Guy11 was the highest in the PSA medium containing BAS1 protein solution, 1.9×10^6 and 1.65×10^6 respectively. However, the spore

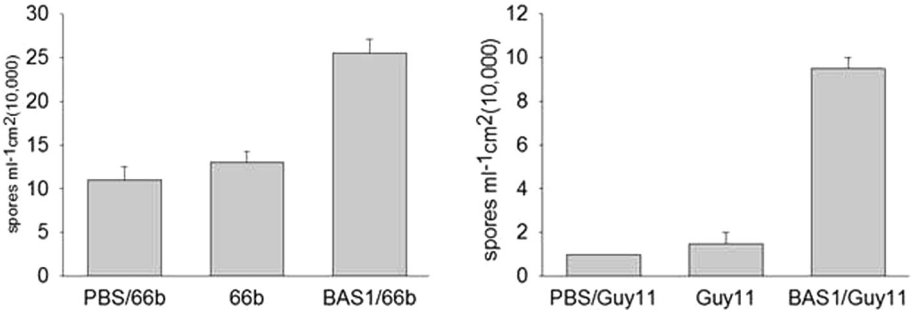

Fig. 4. Spores on leaves pretreated with BAS1 were challenged with 66b and Guy11 at 6d after punch inoculation. BAS1/66b mean leaves pretreated with GST-BAS1-mCherry were punch inoculated with 66b; 66b mean leaves were punch inoculated with 66b, PBS/66b mean leaves pretreated with PBS were punch inoculation with 66b. h: hours post-inoculation. Error bars represent ± SD of the mean. The data is from three independent experiments. Values are the means of three replications, and error bars represent the $_{SE}$ (n = 6).

Table 4
Effect of BAS1 on sporulation of blast strain.

Treatment	Sporulation (spores/ml)
BAS1 + 66b	1.9×10^6
PBS + 66b	1.75×10^5
H_2O + 66b	1.9×10^5
BAS1 + Guy11	1.65×10^6
PBS + Guy11	1.9×10^5
H_2O + Guy11	1.22×10^5

Note: BAS1 + 66b indicated spores were treated with BAS1 solution; PBS + 66b as control indicated spores were treated with PBS solution; H_2O + 66b as control indicated 66b spores were treated with volume equal water to PBS or BAS solution. BAS1 + Guy11 indicated spores were treated with BAS1 solution; PBS + Guy11 as control indicated spores were treated with PBS solution; H_2O + Guy11 as control indicated spores were treated with volume equal water to PBS or BAS solution.

count of 66b and Guy11 decreased in the PSA medium containing water, PBS, and denatured protein solution, 1.9×10^5, 1.75×10^5, 1.53×10^5 and 1.22×10^5, respectively (Table 4).

These data suggest that overexpression of *BAS1* promoted both sporulation and mycelial growth of *M. oryzae in vivo*.

3.6. Overexpression of BAS1 in M. oryzae strains inhibited expression of defense-related genes in infected rice

Based on the findings that *in vitro* treatment of BAS1 solution on susceptible rice leaves could significantly enhance the infection of strong/weak virulence strains of *M. oryzae*, we further investigated the effect of *BAS1* overexpression on the infection by *M. oryzae* strains. Based on our previous results from a real-time PCR analysis of overexpression of *BAS1* in five 35S:BAS1/Mo strains, we selected the 35S:BAS1/Mo-2 overexpression strain for our follow-up experiments. The expression level of *BAS1*, in 35S:BAS1/Mo-2 overexpression strains, was 418.82-fold, in contrast to 152.6-fold,

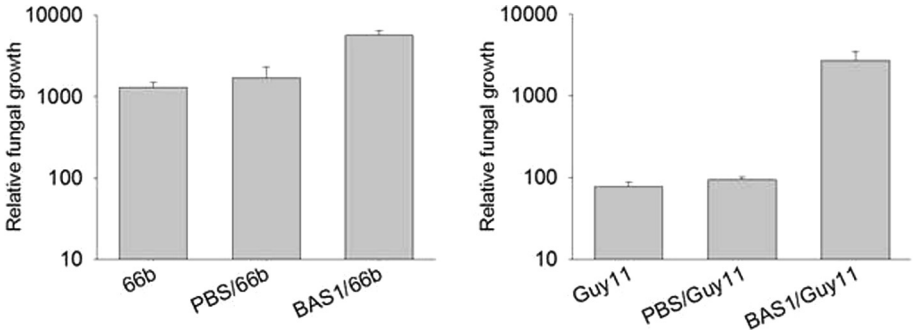

Fig. 5. Relative fungal growth on leaves pretreated with BAS1 was challenged with 66b and Guy11 at 6d after punch inoculation. BAS1/66b mean leaves treated with GST-BAS1-mCherry were punch inoculation with 66b, 66b mean leaves were punch inoculated with 66b, PBS/66b mean leaves treated with PBS were punch inoculation with 66b. BAS1/Guy11 mean leaves treated with GST-BAS1-mCherry were punch inoculation with Guy11, Guy11 mean leaves were punch inoculated with Guy11, PBS/Guy11 mean leaves treated with PBS were punch inoculation with Guy11. h: hours post-inoculation. Error bars represent ± SD of the mean. The data is from three independent experiments. Values are the means of three replications, and error bars represent the $_{SE}$ (n = 6).

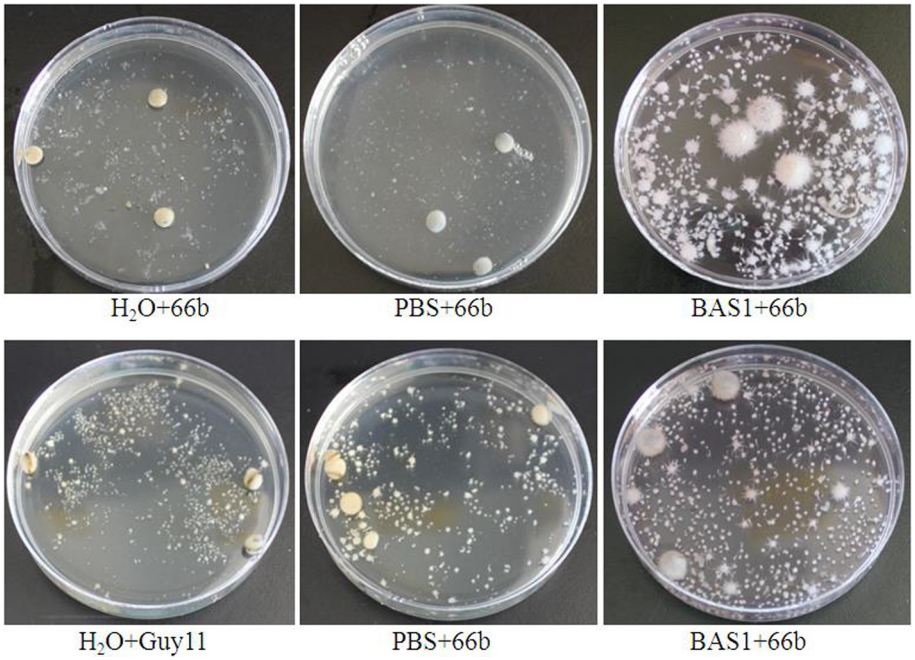

Fig. 6. BAS1 protein solution promoted the mycelial growth of *M. oryzae* strains of 66b and Guy11. The experiment repeats three times, three bottles. BAS1 + 66b mean 66b mycelia grew in PSA medium adding BAS1 solution; PBS + 66b as control mean 66b mycelia grew in PSA medium adding PBS solution; H_2O + 66b as control mean 66b mycelia grew in PSA medium adding water volume equal to PBS or BAS solution. BAS1 + Guy11 mean Guy11 mycelia grew in PSA medium adding BAS1 solution; PBS + Guy11 as control mean Guy11 mycelia grew in PSA medium adding PBS solution; H_2O + Guy11 as control mean Guy11 mycelia grew in PSA medium adding water volume equal to PBS or BAS solution.

Table 5
Incidence of rice leaves inoculated with *M. oryzae* strains.

Strain	Incidence (%)
95234I-1b	26.77 ± 1.597
35S:BAS1/Mo-2	33.75 ± 0.7542

Note: 95234I-1b is wild-type strain, 35S:BAS1/Mo-2 is BAS1 overexpression strain.

285.7-fold, 198.8-fold and 296.1-fold in the remaining four strains of 35S:BAS1/Mo-1, 35S:BAS1/Mo-3 and 35S:BAS1/Mo-4.

Using real-time RT-PCR, we analyzed the expression of defense-related genes in the susceptible rice variety (LTH) infected with the 35S:BAS1/Mo-2 overexpression strain at different time points post inoculation. Rice leaves inoculated with this overexpression strain exhibited blast disease symptoms that were more severe than those inoculated with the wild-type strain. The incidence of rice blast in rice leaves inoculated with the 35S:BAS1/Mo-2 overexpression strain was 22.75%, while the incidence in rice leaves inoculated with the wild-type strain was 16.77% suggesting that the overexpression strain may cause more serious rice blast symptoms than the wild-type strain (Table 5).

We further analyzed the expression levels of rice pathogenesis-related protein genes (*OsPR1a*, *OsPR10a* and *OsRPR10b*), transcription factor genes (*WRKY53* and *WRKY71*), and two defense-related genes (*Chit1* and *MPK12*) in rice inoculated with the 35S:BAS1/Mo-2 overexpression strain at 0 h, 24 h, 48 h and 72 h. *PR1a*, *PR10a,* and *RPR10b* were expressed at each time point in rice infected with the 35S:BAS1/Mo-2 overexpression strain, but the expression levels were less than onefold as compared to the 95234I-1b wild-type strain expression levels (Fig. 7). The expression levels of *PR1a* in rice infected with the overexpression strain at 24 h, 48 h and 72 h (0.3-fold, 0.8-fold and 0.6-fold) were significantly lower than those in rice infected with the 95234I-1b wild-type strain (0.8-fold, 0.95-fold and 9.6-fold). The expression levels of *PR10a* in rice infected with the 35S:BAS1/Mo-2 overexpression strain at 24 h, 48 h and 72 h (all less than onefold) were significantly lower than those in rice infected with the 95234I-1b wild-type strain (0.8-fold, 0.9-fold and 9.5-fold). The expression levels of *RPR10b* in rice infected with the 35S:BAS1/Mo-2 overexpression strain at 24 h, 48 h and 72 h (0.5-fold, 0.4-fold and 1.2-fold) were significantly lower than those in rice infected with the 95234I-1b wild-type strain (105-fold, 98-fold and 100-fold). These results demonstrated that overexpression of *BAS1* in *M. oryzae* strains inhibited the expression of rice disease-resistance genes.

The expression levels of transcription factor genes *WRKY53* and *WRKY71* in rice infected with the 35S:BAS1/Mo-2 overexpression strain at all three time points were significantly lower than in rice infected with the wild-type strain of 95234I-1b (Fig. 7). *WRKY53* is up-regulated in rice in response to the induction of rice blast. In

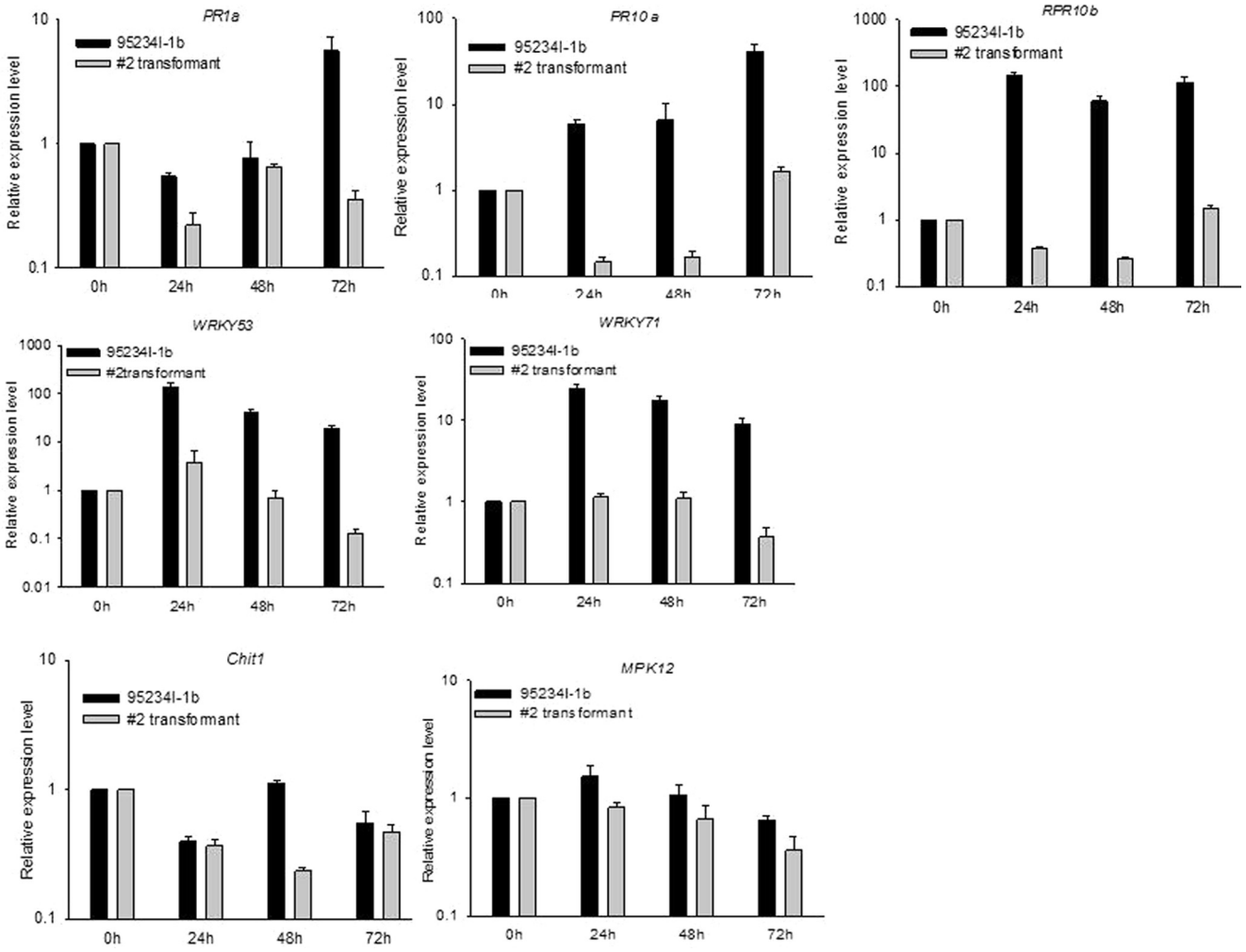

Fig. 7. Expression levels of defense-related genes *OsPR-1a*, *OsPR-10a*, *OsPR-10b*, *ORK10*, *SK2*, *WRKY53*, *WRKY71*, *Chit1* and *MPK12* in rice infected with *M. oryzae* overexpression strains at different time points.

this study, the expression levels of *WRKY53* in rice infected with the 35S:BAS1/Mo-2 overexpression strain at 24 h, 48 h and 72 h (8.5-fold, 1.5-fold and 0.2-fold) were significantly lower than those in rice infected with the wild-type strain (101-fold, 80-fold, and 50-fold). *WRKY71* is up-regulated in rice in response to the induction of rice bacterial blight. The expression levels of *WRKY71* in rice infected with the 35S:BAS1/Mo-2 overexpression strain at 24 h, 48 h and 72 h (1.2-fold, 1.1-fold and 0.6-fold) were significantly lower than those in rice infected with the wild-type strain (60-fold, 45-fold and 10-fold). Thus, *WRKY53* and *WRKY71* were slightly up-regulated in the early stage of rice infected with the 35S:BAS1/Mo-2 over-expression strain (24 h), but the expression level of *WRKY53* dropped at 48 h and was close to 0.1-fold at 72 h. Overall, the expression of *WRKY53* in the 35S:BAS1/Mo-2 overexpression strain was also suppressed.

We also studied the expression of *Chit1* and *MPK12* in rice infected with the 35S:BAS1/Mo-2 overexpression strain and the 95234I-1b wild-type strain at various time points (Fig. 7). The expression levels of *Chit1* in rice infected with the overexpression strain at 24 h, 48 h, and 72 h were lower than 0.6-fold. The expression levels of the *MPK12* gene in rice infected with the overexpression strain at 24 h, 48 h and 72 h were lower than 1.0-fold, and their expression levels with very similar expression levels at the three time points. These results showed that overexpression of *BAS1* in rice infected with *M. oryzae* strains significantly inhibited the expression of defense-related genes in rice.

3.7. Overexpression of BAS1 in M. oryzae strains increased the strain virulence

To further clarify the role of BAS1 in the infection of *M. oryzae* strains, we inoculated the leaves of the susceptible rice variety, Lijiangxintuanheigu (LTH) with the 35S:BAS1/Mo-2 overexpression strain. The overexpression strain caused the development of lesions that were significantly larger than the wild-type strain. The lesion area in rice leaves inoculated with the 35S:BAS1/Mo-2 overexpression strain ($10.1 \pm 0.9\ mm^2$) was greater than that in those inoculated with the wild-type strain ($6.9 \pm 0.1\ mm^2$) (Table 6). Moreover, the spore count and fungal relative growth rate were also higher in 35S:BAS1/Mo-2 leaves (Fig. 8) than in those infected with the wild-type strain.

Table 6
Lesion area of rice leaves *in vitro* inoculated with *M. oryzae* strains.

Strain	Lesion area (mm^2)
95234I-1b	6.9 ± 0.1
35S:BAS1/Mo-2	10.1 ± 0.9

Note: 95234I-1b is wild-type strain, 35S:BAS1/Mo-2 is BAS1 overexpression strain.

4. Discussion

Plant pathogens can secrete effector proteins directly into the host cell to inhibit or promote plant defense system. As a result of which, plants have evolved their own complex defense network against infection by pathogens, such as ROS generation, MAPK cascade induction, host *PR* gene reprogrammed transcriptional, and lignin and callose deposition in plant cell walls (Khang et al., 2010; Chisholm et al., 2006).

Mosquera and coworkers identified the effector protein genes (*BAS1-BAS4*) specifically expressed in the infection hypha (IH) based on their developed method of RNA extraction sample preparation (Mosquera et al., 2009). Most of these effector proteins are small secretory proteins (Mosquera et al., 2009). Among these, BAS1-4 are specifically expressed in IH, and 100-fold upregulated in IH, the high upregulation in IH suggest they play an important role in the biotrophic phase of the *M. oryzae* life cycle (Mosquera et al., 2009), and the functional studies of these genes contribute to effectively discover the mechanism on interaction during infection. However, knockout mutants of these genes encoding effector proteins have similar infectivity as wild types, suggesting complex regulatory pathways and functional redundancy. In the present study, we analyzed the function of BAS1 in the host-pathogen interaction. To further understand the function of BAS1 during fungal infection, we sprayed purified prokaryotic expression fusion protein GST-BAS1-mCherry onto the leaves of a susceptible rice cultivar LTH. Our results showed that this treatment resulted in callose deposition and ROS production, indicating that BAS1 can stimulate the basal defense response in the rice plant. Because callose deposition and ROS are features of the basal defense response generated by host plants when plants response to external environmental stimuli such as pathogen infection, PAMPs recognition and exciton induction. Our data indicate that BAS1 induced an early, basal defense response in susceptible rice, but that the level of the defense initiated is very limited. The most probable explanation for which, is that the defence pathway mediated by BAS1 in the susceptible variety LTH is very weak. So, future studies comparing the response to BAS1 in different rice cultivars would be of interest and could reveal functional variation among rice varieties. Our study also showed that spraying BAS1 solution on rice leaves significantly increases their susceptibility to *M. oryzae* infection. Compared to the symptom and the expression levels reported by Mosquera et al. (2009), the symptoms observed on BAS1 pretreated-leaves challenged with blast strain were more severe and *BAS1* expression level also increased, which imply that BAS1 is translocated into rice cytoplasm independently of a fungal-derived machinery and the amount of the protein contributes to the virulence of *M. oryzae*. AVR1-CO39 is the only confirmed effector which can translocation into rice cells independently of fungal-derived machinery up to now (Jones and Dangl, 2006). These data show that the biotrophic effector BAS1, like many reported

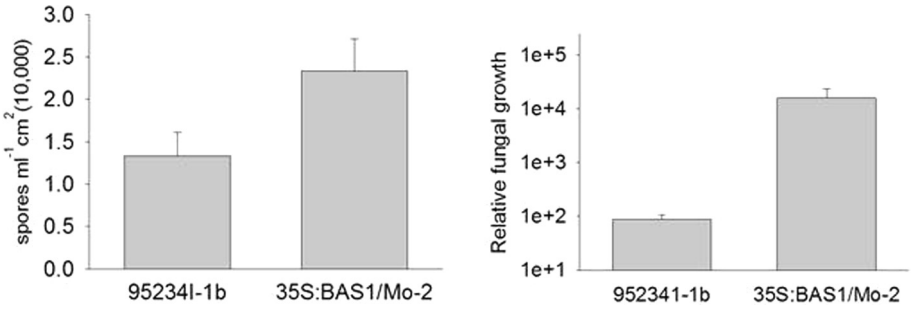

Fig. 8. Fungal sporulation rate and relative growth rate in lesion on leaves inoculated with overexpression strain.

effectors, can both increase the virulence of the pathogen and enhance pathogen virulence. More importantly, these results suggest that probably some effectors of blast fungus can translocated into rice cytoplasm dependent on host machinery.

Some AVR proteins have an important role in the virulence of strains. For example, heterologous expression of AVR2 and AVR4 (*Cladosporium fulvum*) in *Arabidopsis thaliana* can enhance the susceptibility of plants to *Cladosporium fulvum*, *Botrytis cinerea,* and *Verticillium dahlia*, and they are representative as a class of non-toxic factors only when the corresponding resistance gene *Cf-2* is present in the host plant (van Esse et al., 2008). The effector protein Avr1b of *Phytophthora sojae* can increase the toxicity of pathogen, inhibit the programmed cell death of yeast, soybean and *Nicotiana benthamiana* induced by pro-apoptotic BAX protein (Dou et al., 2008), indicating that effector proteins have a function to suppress the host defense response. For example, the effector protein AVR3a of *Phytophthora infestans* can not only cause R3a-mediated allergic reactions, but can also suppress cell death of *Nicotiana benthamiana* induced by INF1 (Bos et al., 2006). Both stimulation of host defense responses and enhancement of pathogen virulence are characteristics of effectors, suggesting that this class of effector proteins is multifunctional effector proteins. Although we observed ROS generation and callose deposition induced by prokaryotic expressed BAS1 in rice, there were severe symptoms in rice by the seventh day after inoculation with pathogenic strains. These studies indicate that BAS1 has a greater role in promoting virulence of pathogen and inhibiting the rice defense system rather than inducing the early basic defense responses in rice. Moreover, BAS1 promotes *M. oryzae* mycelium colonization, development of infection hyphae in the host tissue and on media in *Petri-dish*, suggest the role of BAS1 is essential for fungus, beyond the biotrophic stage of infection host.

The *PR* family of genes confers systemic acquired resistance (SAR) when plants are subjected to pathogens or abiotic stresses. These genes are also programmed cell death (PCD) marker genes, of which, *PR10a* and *RPR10b* are involved in the plant defense response. Previous studies have shown that in the early stages of pathogen infection (12hpi), *PR10a* and *RPR10b* have relatively high transcription levels (Mosquera et al., 2009). Our study shows that the expression of three pathogenicity-related genes of *PR1a*, *PR10a* and *RPR10b* was downregulated in leaves inoculated BAS1 overexpression strain than wild-type. This result indicates that rice defense was suppressed by overexpression of BAS1. The lesion size, fungal infection hyphae growth rate and sporulation are increased in rice leaves of overexpressed BAS1 in isolates of 66b Guy11, indicate that serious symptoms on rice plants are the comprehensive results of improvement of virulence in blast fungus and suppression the rice defense. Moreover, BAS1 promoted sporulation and mycelia growth suggesting that a role across the whole process of interaction, from the biotrophic phase to sporulation. Both the fungal growth rate and the spore count of lesions were increased in the susceptible rice variety LTH inoculated with overexpression strains compared to those in rice leaves inoculated with wild-type strains, implying its virulence function is based on the accumulated amount of protein. Therefore, overexpression of *BAS1* enhances infection of blast strain, hyphae growth *in planta*, suppression of host defence leading to severe disease symptoms.

Since deletion mutants of BAS1-BAS4 have no great impact on pathogen virulence, it may be possible that these genes only play roles in a transient, dynamic process, or that they need to interact with other proteins to exert their function(s). The minor impact of a single gene on fungal and host phenotype might be a common feature of many effector proteins such as BAS1-BAS4 (Mosquera et al., 2009). Actually, many secreted proteins of different pathogens have been investigated. However, only a few of them were functional characterized. In case of *M. oryzae*, 1120 fungal genes were detected higher expression in IH relative to mycelium, it is reasonable to predicted that many effectors similar to BAS1, are difficult to investigate their phenotype by deletion mutation and expression with their native promoter, because only minor effect were detection (Mosquera et al., 2009). Over-expression and ectopic expression should be alternative strategy to show their function.

This study investigated the dual role of the rice blast biotrophic effector BAS1 in both increasing the virulence of blast strains and triggering the basal defense response. The functional site of which is most likely beyond the BIC and its function only can detected till accumulating to a certain level. This suggests that extra-invasive hyphal membrane (EIHM) probably limits the location of BAS1. Overexpression of *BAS1* can promote fungal infection, mycelia growth and the sporulation of *M. oryzae in planta*, which suggesting that the roles of BAS1 is beyond the biotrophic phase.

5. Conclusions

Based on what above-mentioned results, it is concludes that prokaryotically expressed BAS1 *in vitro* caused callose deposition and ROS production in rice leaves and calli, which suggests that BAS1 is translocated into rice cytoplasm independently of a fungal-derived machinery, pretreating with ectopic expression of BAS1 increased the virulence of fungus suggest that defense response was overcome by other effectors of the same fungus. In addition, our results also imply that overexpression and ectopic expression of effector proteins can be used as a strategy for the quantitative analysis of such kind of effectors.

Author contributions

LL, JY and CW carried out the experiment work. JY, CL, CL, CW and YZ helped in drafting the paper and interpreting the data. All authors read and approved the final manuscript.

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