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Construction of overexpression vectors of *Magnaporthe oryzae* genes *BAS1* and *BAS4* fusion to mCherry and screening of overexpression strains

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ABSTRACT. The aim of this study was to construct overexpression vectors and selecting strains of the *Magnaporthe oryzae* effectors BAS1 and BAS4. Primer pairs of BAS1, BAS4, and mCherry were designed based on their known nucleotide sequences. The coding sequences of BAS1 and BAS4 were amplified, and the pXY201 plasmid was selected as a template to amplify the mCherry sequence. Fragments of BAS1 and mCherry, and BAS4 and mCherry were ligated into the pCAMBIA1302 vector. The recombinant pCAMBIA-BAS1-mCherry and pCAMBIA-BAS4-mCherry plasmids were transformed into *E. coli* DH5α competent cells. Transformants were screened by PCR, and plasmids from the positive transformants were extracted by enzymatic digestion to obtain pCAMBIA-BAS1-mCherry and pCAMBIA-BAS4-mCherry. The pCAMBIA-BAS1-mCherry and pCAMBIA-BAS4-mCherry plasmids were transformed into protoplasts of rice blast strains and the transformed

strains were screened by PCR using primer pairs against the hygromycin gene. The result showed that the PCR products corresponded with the theoretical sizes. RT-PCR was used to analyze the expression of *BAS1* and *BAS4* in five transformed strains of *BAS1* and *BAS4*, and the result showed that the higher expression level of the two genes was occurred in five transformant strains comparing to wild-type strain A3467-40 (the strain containing *BAS1* and *BAS4*), but there was no difference among the five overexpression strains. The sporulation and spore germination of transformed strains was higher than in wild type strain, and there was no difference in the germination time. Construction of overexpression vectors and strains of *M. oryzae* effectors BAS1 and BAS4 provide reference material for other new effectors.

Key words: *Magnaporthe oryzae*; Effector protein; Overexpression vector; PEG-mediated transformation

INTRODUCTION

Magnaporthe oryzae (Ascomycotina) infection causes severe diseases in rice that affects yield, and can also infect many other gramineous plants (Kato et al., 2000; Couch and Kohn, 2002). Rice varieties that contain resistance genes can easily lose their resistance after years of consecutive large-scale cultivating, because the resistance is quickly circumvented by the emergence of virulent strains, which are caused by deletion of the *Avr* gene (Yoshida et al., 2009), expression modification (Kang et al., 2001; Fudal et al., 2007) and point mutation (Orbach et al., 2000).

Many variety-specific or species-specific *Avr* genes have been cloned and identified. For example, BAS1-BAS4 are biotrophy-associated secreted proteins, which are secreted and enter rice cells through a special method during compatible interaction of rice and *M. oryzae* (Khang et al., 2010). During the compatible interaction, BAS1 is translocated into the rice cell cytoplasm but BAS4 enters the apoplast. Many novel effector genes have been discovered since genome sequencing of *M. oryzae* have been completed. However, their functions have not been identified, to date.

Therefore, we can consider the function of identified *M. oryzae* effector proteins as a reference to identify new proteins in pathogenic bacteria.

In this study, we constructed overexpression vectors of *M. oryzae* effector gene (BAS1 and BAS4) fusion to mCherry to provide a significant reference for subsequent research, in which we will study the function and location of the cloned novel genes of the *M. oryzae*.

MATERIAL AND METHODS

Materials and reagent

The cloning vector pMD®19-T, DNA purification kit, and plasmid extraction kit were purchased from Tiangen Biotech (Beijing) Co., Ltd., China; the overexpression vector pCAMBIA1302 was preserved in our laboratory; the binary expression vector pXY201 was obtained from the State Key Laboratory of Crop Stress Biology for Arid Areas of Northwest Agriculture and Forest University, China. *M. oryzae* strain A3467-40 was preserved in our

laboratory and results from PCR amplification confirmed the presence of BAS1 and BAS4 fragments in the strain of A3467-40. Wild *M. oryzae* strains 95234I-1b and A1343R-7 were preserved in our laboratory, results from PCR amplification confirmed that there was no BAS1 fragment in 95234I-1b and no BAS4 fragment in A1343R-7. Competent cells of *Escherichia coli* DH5α were purchased from Tiangen Biotech (Beijing) Co., Ltd., China.

Taq DNA polymerase, DL2000 Maker, and Ex Taq enzyme were purchased from Tiangen Biotech (Beijing) Co., Ltd., China. A gel recycling kit, plasmid extraction kit, reverse transcription kit, and restriction enzyme were purchased from Kunming Diangongtech., Ltd., China. TRIZOL extraction kit and reverse transcription kit were purchased from Tiangen Biotech (Beijing) Co., Ltd., China. PCR machine (9700, Applied Biosystems ABI, America), gel imaging analysis system (HR410, American UVP company, America), and electrophoresis apparatus (DYY-7C, Beijing Liuyi, China) were also utilized in this study. Antibiotics (chloramphenicol, benzylpenicillin, rifampicin, and kanamycin), tryptone, yeast extract, and powdered agar were purchased from Kunming Diangongtech., Ltd., China.

Mycelium culture, total RNA extraction of mycelia, and cDNA synthesis

Based on the polymorphical analysis of BAS1 and BAS4 in *M. oryzae* strains, we selected strains in which target genes were detected and the mycelia were harvested when they were cultured in liquid potato sugar agar for 7 days. And mycelia were then collected on sterilized filter paper and the total RNA was extracted with TRIZOL and cDNA was synthesized using TransScript II First-Strand cDNA synthesis kit (TransGen Biotech., Ltd., China). The cDNAs were stored at -20°C.

Primer design and PCR amplification

Primers for BAS1 and BAS4 genes were designed according to their nucleotide sequence. The primer sequences were as follows: BAS1-F (5'-GCCACTAGTATGCACGTTTT CAA-3'), BAS1-R (5'-GCCCTCGAGCGGGTAATAATTCT-3'), BAS4-F (5'-GCCACTAGTA TGCAGCTCTCATT-3'), and BAS4-R: (5'-GCCCTCGAGAGCAGGGGGGATAG-3'), and *Spe*I and *Xho*I restriction enzyme sites were added to the ends of each primer (underlined). Primers for mCherry, mCherry-F (5'-GCCCTCGAGATGGTGAGCAAGGGCGAGG-3') and mCherry-R (5'-GCCACTAGTTCACTTGTACAGTTCGTCC-3'), were designed according to its nucleotide sequence, and *Spe*I and *Xho*I restriction enzyme sites were added to the ends of each primer (underlined). cDNA from the hypha of *M. oryzae* strain A3467-40 was taken as a template, and primers against BAS1-F/BAS1-R and BAS4-F/BAS4-R were used to obtain BAS1 and BAS4 sequences. Considering binary expression vector pXY201 as complete, the mCherry gene sequence was obtained using the mCherry-F/mCherry-R primers. PCR products, the target fragments of BAS1, BAS4, and mCherry, were isolated from gel after electrophoresis in 1.0% agarose gel. The 25-μL PCR system included 2.5 μL 10X buffer [200 mM Tris-HCl (pH 8.0, 200 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$], 1.5 μL, 2.0 μL dNTP, 0.8 μL primers, and 0.2 μL PFU Taq enzyme, 0.8 μL cDNA supplemented with an appropriate amount ddH_2O. The PCR was performed using the following conditions: pre-denaturation at 95°C for 5 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1min, and a final extension at 72°C for 10 min. The products were stored at 4°C.

Cloning *BAS1* and *BAS4* genes

DNA fragments obtained from gel recovery were ligated to the pMD®19-T vector and transfected into *E. coli* DH5α competent cells. The cells were cultured on a LB plate with 50 μg/mL Amp and Xgal and maintained at 37°C in an incubator overnight for blue-white selection. We selected white bacterial colony to use for a PCR assay and the positive clone was sent to Shanghai Yingjun for sequencing. The BioEdit software was utilized for sequence alignment to analyze the positive clone with the correct sequence.

Construction of overexpression vector

A positive strain was selected from which to extract plasmids. The plasmids were validated by the enzyme cutting method. Target genes fragments were recovered using gel recovery kit for the next linkage. We fused BAS1 to mCherry and BAS4 to mCherry, toligate into a pCAMBIA1302 overexpression vector digested using corresponding enzymes of *Spe*I and *Xho*I. A total of 10 μL enzyme cutting system including 8.4 μL plasmid DNA, 1 μL 10X H buffer, 0.3 μL digestion enzyme of *Spe*I and *Xho*I, the reaction system were incubated in a 37°C water bath for at least 4 h.

PEG-mediated protoplast transformation

M. oryzae protoplast preparation

Cultured mycelia were collected with three layer of lens paper, washed three times with 20% sucrose solution and resuspended in 5 mL 20% sucrose solution. A sample of 200 mg lysase from *Trichoderma harzianum* was dissolved in 20 mL 20% sucrose solution and then cultured at room temperature by slowly shaking for 1.5 h after filtration sterilization. At the same time, we ensured digestion of the cell wall and appropriately extended the digestion time to 0.5 h or 1 h until the hyphal cell transformed into spheroplasts. The protoplasts were filtered using three layer of lens paper and washed 2-3 times with 0.7 M NaCl, they were then centrifuged at 4°C, 3000 rpm for 10 min. The precipitates were resuspended with 5 M 1X STC, re-centrifugated, and resuspended. We amended the protoplast concentration to 5 x 10^7 cell/mL, placed 200 μL of the suspension into a 1.5-mL EP tube and stored at -80°C.

M. oryzae protoplast transfection

About 5 μg plasmid DNA mix with 2-fold volume 2X STC (100 μL) and transferred them into 15-mL screw centrifuge tubes, in which 200 μL thawed protoplasts were added (5 x 10^7 cell/mL), and the solution were gently shaken and cultured at room temperature for 10 min. The 1-mL PTC (2X STC and 2X PEG) were slowly added along the tubal wall, and samples were cultured at room temperature for 20 min. Finally, 3 mL TB3 liquid medium was added and the samples were cultured with light shaking at room temperature for 6 h. The regenerated protoplasts were spread on a TB3 plate (8% agar, 200 μg/mL hygromycin B) and cultured at 22°C in the dark for 1 week. The colonies were transferred into TB3 solid medium with hygromycin.

PCR screen transformants of *M. oryzae*

The transformants grew on TB3 solid medium (containing 200 μg/mL hygromycin) for 3-4 days and were then transferred onto PSA solid medium with hygromycin. Following culture for 1 week, we extracted the DNA of transformants with a mini-preparation DNA extraction method (Lan et al., 2012). Primer pairs for the nucleotide sequence of hygromycin were designed to screen transformants using PCR. We utilized a 20-μL PCR system including 1 μL (50-100 ng) genomic DNA, 8 μL 2X Easy PCR SuperMix (Quanshijin company), 0.8 μL primer HPH-F (5'-ATCCTTCGCAAGACCCTTCCT-3'), and 0.8 μL HPH-R (5'-GGTGTCGTCCATCACAGTTTG-3') and the reaction condition was as follows: predenaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 3 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

RESULTS

Cloning and identification of the *BAS1*, *BAS4*, and *mCherry* genes

Using primer pairs for BAS1-F/BAS1-R and BAS4-F/BAS4-R and cDNA extracted created from mycelia as a template, the target genes *BAS1* and *BAS4* were got using PCR amplification. The *mCherry* coding sequence were got when plasmid of pXY201 were selected as template and primer pairs of mCherry-F/mCherry-R. The PCR products were determined through agarose gel electrophoresis, and the results showed that the fragment sizes of the target genes *BAS1*, *BAS4*, and *mCherry* were 348, 309, and 711 bp respectively, which were corresponded to the theoretical value as predicted (Figure 1).

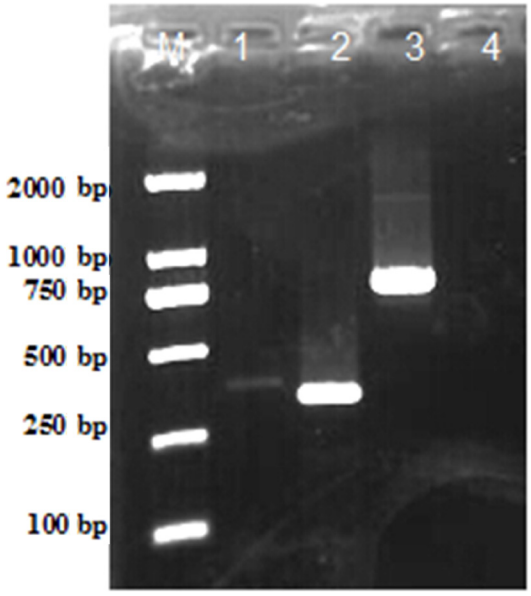

Figure 1. Amplification of *BAS1* and *BAS4* from *Magnaporthe oryzae* and *mCherry*. *Lane M* = DNA marker (DL2000), *lanes 1-3* = *BAS1*, *BAS4*, and *mCherry*. Sizes were 348, 309, and 711 bp, respectively.

Fragments of the target genes *BAS1*, *BAS4*, and *mCherry* were each ligated to the pMD®19-T vector and then transfected into competent DH5α cells. We selected positive transformants by PCR and identified the correct transformants by sequencing method. Recombinant plasmids pMD19-BAS1 and pMD19-BAS4 were extracted from the positive transformants and identified by double restriction enzyme digestion with *Spe*I and *Xho*I (Figure 2).

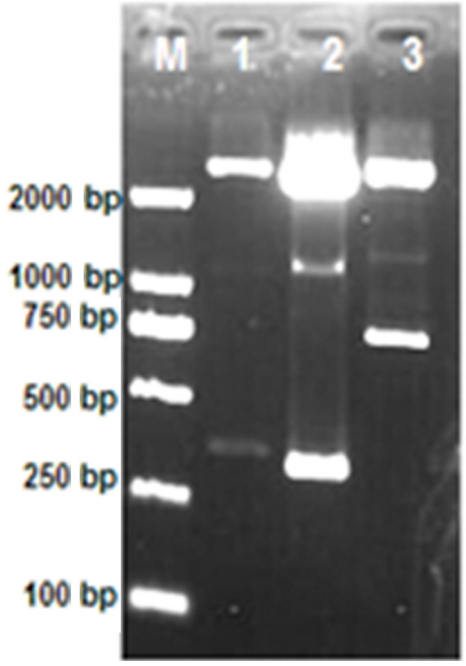

Figure 2. Identification of the pMD19-T-BAS1 and pMD19-T-BAS4 plasmids with *Spe*I and *Xho*I, and pMD19-T-mCherry with *Xho*I and *Spe*I. *Lane M* = DNA marker (DL2000), *lanes 1-2* = identification of the recombinant plasmids of pMD19-T-BAS1, pMD19-T-BAS4 was cleaved with *Spe*I and *Xho*I, *lane 3* = recombinant plasmid of pMD19-T-mCherry was cleaved with *Xho*I and *Spe*I.

Construction and identification of overexpression vectors of *BAS1* and *BAS4* fused to *mCherry*

The plasmids of pMD19-BAS1, pMD19-BAS4, and pMD19-mCherry plasmids were digested using two restriction enzymes, and the fragments (*BAS1* and *mCherry*, *BAS4* and *mCherry*) were ligated into a *Spe*I digested binary expression vector (pCAMBIA1302) and transfected into competent DH5α cells. Positive transformants were screened by PCR. Recombinant plasmids were extracted and identified by *Spe*I restriction enzyme digestion. The results showed that their size was as predicted and that the target fragments and pCAMBIA1302 were ligated correctly (Figure 3). The positive plasmids identified by double restriction enzyme digestion were further confirmed through sequencing method. Finally, we obtained the overexpression plasmid with the correct sequence (pCAMBIA-BAS1-mCherry and pCAMBIA-BAS4-mCherry).

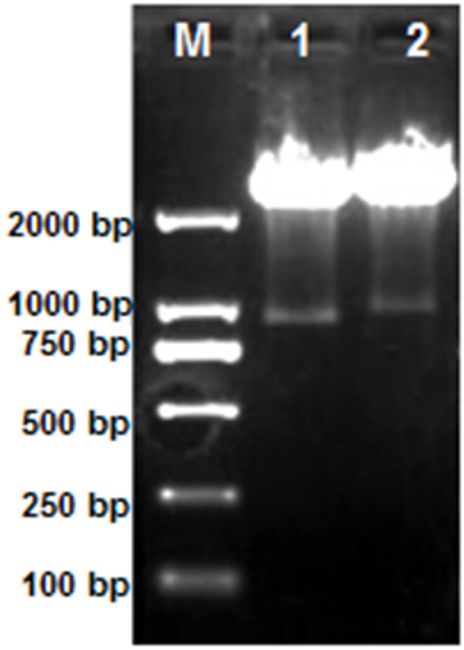

Figure 3. Identification of the pGEX-4T-BAS4-mCherry and pGEX-4T-BAS1-mCherry plasmids with *Spe*I. *Lane M* = DNA marker (DL2000), *lanes 1-2* = identification of the recombinant pGEX-4T-BAS1-mCherry and pGEX-4T-BAS4-mCherry plasmids were cleaved with *Spe*I.

PEG-mediated protoplast transfection, screening, and identification of transformants

We utilized PEG-mediated protoplast transfection to transfer the overexpression plasmids of pCAMBIA-BAS1-mCherry and pCAMBIA-BAS4-mCherry into protoplasts of rice blast strain. The quality and quantity of the plasmids developed here were used in subsequent studies. The overexpression vector and prepared protoplasts were co-incubated for 6 h, and were then spread on TB3 culture medium and placed in an incubator at 25°C until the transformants appeared. In this study, the protoplast transfection presented higher efficiency. The transformants were cultured in PDA culture media with 200 µg/mL hygromycin and the transformants DNA were extracted using mini-preparation DNA extraction method, then hygromycin gene primers were used to screen transformants. The size of the amplification fragments was as predicted result (Figure 4). The transformant strains identified by PCR were cultured to produce spores. Finally, we selected single spore to perform subsequent experiments such as a pathogenicity assay and infection behavior analysis.

Expression of *BAS1* and *BAS4* in five overexpression strains using RT-PCR

The expression level of *BAS1* in the five 35S:BAS1/Mo overexpression stains were analyzed using semiquantitative RT-PCR. The results showed that *BAS1* was expressed at almost the same level in these strains (Figure 5). The intensities of the amplification bands were nearly the same. Expression of the *BAS4* gene in the five 35S:BAS4/Mo transformant strains

was analyzed using RT-PCR, and the results showed that *BAS4* was expressed at almost the same level in all these strains (Figure 5). The intensities of the bands were nearly the same.

And we also determined the expression of *BAS1* and *BAS4* in wild-type strain of A3467-40. The result showed that the lower expression level occurred in wild-type strain than one in five 35S:BAS1/Mo and 35S:BAS4/Mo overexpression strains (data not shown), which indicated the expression level of *BAS1* and *BAS4* appeared significantly higher when the two genes promoted under 35S promoter.

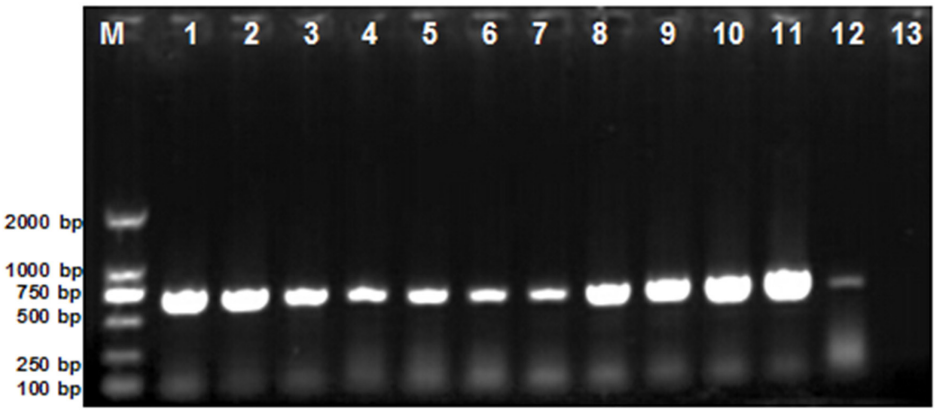

Figure 4. PCR screening of overexpressing transformants harboring pCAMBIA-BAS1-mCherry and pCAMBIA-BAS4-mCherry. *Lane M* = DNA marker (DL2000), *lanes 1-7* = PCR screening of transformants with pCAMBIA-BAS1-mcherry, *lanes 8-12* = PCR screening of transformants with pCAMBIA-BAS4-mcherry.

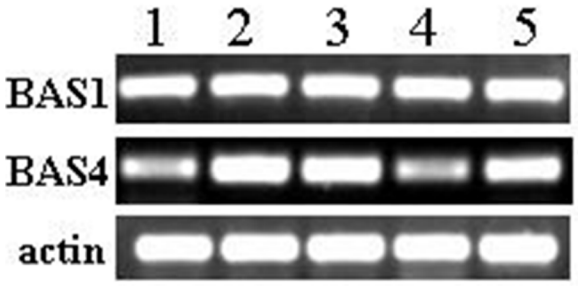

Figure 5. Expression of BAS1 and BAS4 in mycelia of five overexpressing transformant strains carrying BAS1 and BAS4, respectively.

Spore number and germination rates of 35S:BAS1/Mo and 35S:BAS4/Mo overexpression strains

In order to analyze the spore production rate and the germination rate of 35S:BAS1/Mo and 35S:BAS4/Mo overexpressing strains, we analyzed the spores, adhesion spores, hypha, spore number, germination rate, and hypha growth rate of the five overexpressing strains.

The results showed that there were no significant differences among the five overexpressing strains. But the spore number and germination rate of overexpressing strains showed higher than those of wild-type strains.Therefore, only the results of the statistical analyses on spore-number and germination rate in one of the five overexpressing strains are shown (Figure 6A-D). We could see that the spore number (Figure 6A and B) and germination rate of overexpressing strains (Figure 6C and D) were significantly higher than those of wild-type strains, but the spore germination time showed no difference between overexpressing and wild-type strains.

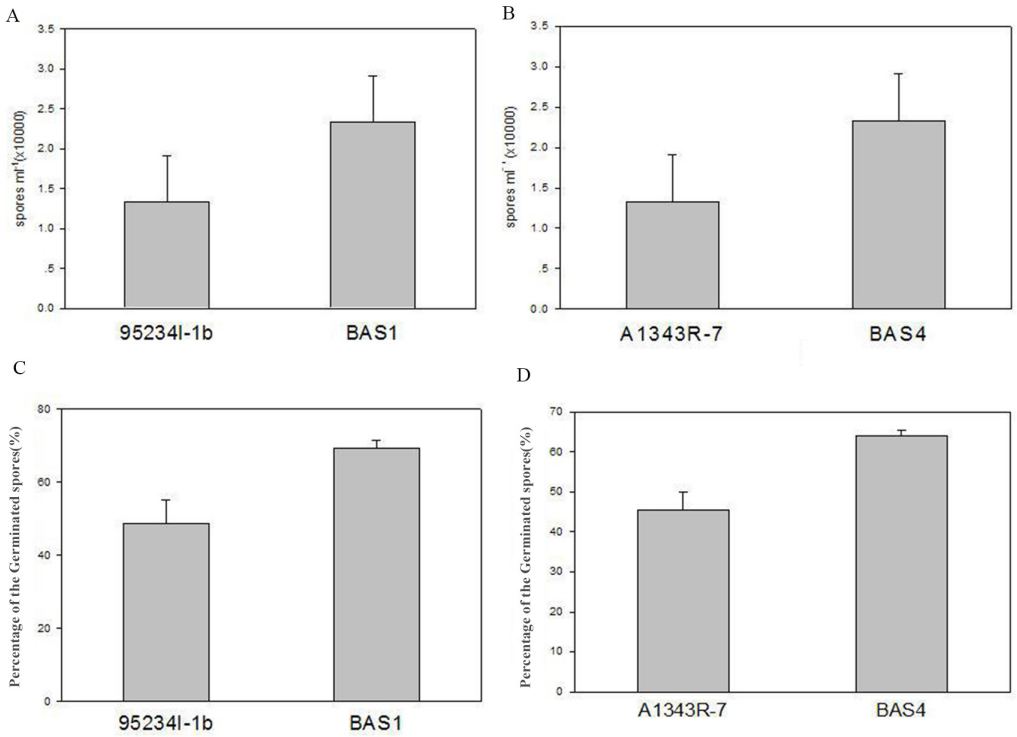

Figure 6. Sporulation and spore germination of transformants. Sporulation of BAS1 transformants and wild-type strain of 95234I-1b (**A**), sporulation of BAS4 transformants and wild-type strain of A1343R-7 (**B**), spore germination of BAS1 transformants and wild-type strain of 95234I-1b (**C**), spore germination of BAS4 transformants and wild-type strain of A1343R-7 (**D**). The experiments were repeated three times, 100 spores were used to statistic each repetition.

DISCUSSION

Although we have invested a lot of manpower and material resource to control rice blast for decades, the disease still threatens global food security (Wang and Valent, 2009). *M. oryzae* infects rice through secreting effector proteins into the host cytoplasm to destroy the defense system. Many cytoplasmic effector proteins have been identified as virulence factors, and they can be recognized through the corresponding resistance gene and further caused host hypersensitive reaction (HR) and resistance. Many studies have shown that the effector proteins secreted through infected hyphae play important roles in the early stage of host penetration,

which successfully complete parasitic invasion at the same time. The effector proteins may be recognized by the host and can cause HR or escape host recognition to infect host. Khang et al. (2010) observed that the secreted AVR effector protein accumulated in the biotrophic interfacial complex (BIC; Khang et al., 2010). BIC formation is associated with the differentiation of filamentous hypha to global infected hyphae, whose growth is necessary in disease development (Veses and Gow, 2009). Pathogenic secreted proteins PWL2 and BAS1 are first located in BICs and are then transferred into rice cytoplasm. However, BAS4, is not located in BICs and is not further transferred into rice cytoplasm. Khang et al. (2010) observed the secretion, transport, and cell-cell movement (Khang et al., 2010) of the effector proteins BAS1 and BAS4 during the infected rice, which provides an important reference for observing the secretion, location, transport, and movement of other effector proteins. We clone and constructing overexpression vectors of *BAS1* and *BAS4* fused to *mCherry* and obtain the corresponding overexpression strains of 35S:BAS1/Mo and 35S:BAS4/Mo, which provides important reference to study the effect to *M. oryzae* strain virulence, the expression, secretion, location, and function in the infected rice by overexpressing novel genes encoding effector proteins in *M. oryzae*.

We utilized PEG-mediated protoplast transfection to transfer plasmids carrying BAS1: mCherry and BAS4:mCherry into wild-type strains and obtain 5 35S:BAS1/Mo and 35S:BAS4/Mo for each gene. We found that the mycelia that were global could be used to prepare protoplast, and transparent mycelia liquid is beneficial to protoplast preparation. However, small mycelia and brown mycelium liquid suggest that the mycelia are ageing and cannot be utilized for protoplast preparation. Many factors such as the amount of mycelia, cell age, enzyme concentration, enzymolysis temperature and time, and centrifugation speed could influence the protoplast products.

We also found that both the spore production and germination rate of 35S:BAS1/Mo and 35S:BAS4/Mo overexpressing strains were higher than those of the wild type strains. The expression level of *BAS1* and *BAS4* in the overexpressing strains showed no obvious difference, but appeared higher expression level than those in wild-type strain of A3467-40, which indicated that BAS1 and BAS4 were promoted overexpressing under 35S promoter. To further study the virulence change of 35S:BAS1/Mo and 35S:BAS4/Mo overexpression strains, we will investigate the pathogenicity of the overexpressing strains and to observe the secretion, movement, and location of BAS1 and BAS4 during the overexpressing strains infecting rice. Our study will provide a reference for the study of novel effector protein secretion, location, and function in *M. oryzae*.

This study shows that the construction of overexpression vectors of *BAS1* and *BAS4* fusion to *mCherry* will provide a reference for research on other effector protein genes that can be overexpressed in *M. oryzae* or other fungi.

Conflicts of interest

The authors declare they have no conflict of interest.

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