Combinative effects of a bacterial type-III effector and a biocontrol bacterium on rice growth and disease resistance

HAIYING REN[a,*], GANYU GU[a,*], JUYING LONG[a], QIAN YIN[a], TINGQUAN WU[a], TAO SONG[a], SHUJIAN ZHANG[a], ZHIYI CHEN[b] and HANSONG DONG[a,**]

[a]*Key Laboratory of Monitoring and Management of Plant Pathogens and Insect Pests, Ministry of Agriculture of China, and Department of Plant Pathology, Nanjing Agricultural University, 1 Weigang, Nanjing, 210095, China*
[b]*Institute of Plant Protection, Jiangsu-Provincial Academy of Agricultural Sciences, 1 Zhongling Street, Nanjing, 210014, China*

**These authors contributed equally to this paper*
***Corresponding author (Fax, 86-25-84395325; E-mail: hsdong@njau.edu.cn)*

Expression of $HpaG_{Xoo}$, a bacterial type-III effector, in transgenic plants induces disease resistance. Resistance also can be elicited by biocontrol bacteria. In both cases, plant growth is often promoted. Here we address whether biocontrol bacteria and $HpaG_{Xoo}$ can act together to provide better results in crop improvement. We studied effects of *Pseudomonas cepacia* on the rice variety R109 and the *hpaG*$_{Xoo}$-expressing rice line HER1. Compared to R109, HER1 showed increased growth, grain yield, and defense responses toward diseases and salinity stress. Colonization of roots by *P. cepacia* caused 20% and 13% increase, in contrast to controls, in root growth of R109 and HER1. Growth of leaves and stems also increased in R109 but that of HER1 was inhibited. When *P. cepacia* colonization was subsequent to plant inoculation with *Rhizoctonia solani*, a pathogen that causes sheath blight, the disease was less severe than controls in both R109 and HER1; HER1, nevertheless, was more resistant, suggesting that *P. cepacia* and $HpaG_{Xoo}$ cooperate in inducing disease resistance. Several genes that critically regulate growth and defense behaved differentially in HER1 and R109 while responding to *P. cepacia*. In R109 leaves, the *OsARF1* gene, which regulates plant growth, was expressed in consistence with growth promotion by *P. cepacia*. Inversely, *OsARF1* expression was coincident with inhibition in growth of HER1 leaves. In both plants, the expression of *OsEXP1*, which encodes an expansin protein involved in plant growth, was concomitant with growth promotion in leaves instead of roots, in response to *P. cepacia*. We also studied *OsMAPK*, a gene that encodes a mitogen-activated protein kinase and controls defense responses toward salinity and infection by pathogens in rice. In response to *P. cepacia*, an early expression of *OsMAPK* was coincident with R109 resistance to the disease, while HER1 expressed the gene similarly whether *P. cepacia* was present or not. Evidently, *P. cepacia* and G_{Xoo}-gene mediated resistance may act differently in rice growth and resistance. Whereas combinative effects of *P. cepacia* and $HpaG_{Xoo}$ in disease resistance have a great potential in agricultural use, it is interesting to study mechanisms that underlie interactions involving biocontrol bacteria, type-III effectors and pathogens.

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1. Introduction

Due to public concerns on pesticide use in crops, exploration on alternative methods has been a global effort for secure crop protection. Biocontrol bacteria and natural products from various sources show a great potential of agricultural use in disease control and improvement of crop productivity (Dixon 2001; Stuiver and Custers 2001; Zasloff 2001).

Keywords. Biocontrol bacteria; disease resistance; growth promotion; *hpaG*$_{Xoo}$-expressing rice line 1 (HER1); rice

Abbreviations used: EVTR, Empty vector-transformed rice; HER1, *hpaG*$_{Xoo}$-expressing rice line 1; ISR, induced systemic resistance; LB Luria-Bertani; PCR, polymerase chain reaction; Rif, rifampicin; RT-PCR, reverse transcriptase-PCR; SAR, systemic acquired resistance.

Pseudomonas species is an important class of biocontrol bacteria; they protect plants by number of mechanisms. When applied to rice, *P. fluorescens* produces oxalic acid to inhibit *Rhizoctonia solani*, the pathogen that causes rice sheath blight, one of the devastating diseases of rice in many rice production areas (Nagarajkumar *et al* 2005). *P. fluorescens* also induces defense compounds, like chitinase and peroxidase, which protect rice by action on the pathogen (Nandakumar *et al* 2001). Moreover, biocontrol bacteria can promote seed germination and plant growth, indirectly increasing resistance (Romanenko and Alimov 2000). Plant responses are induced as a result of colonization of plant roots by biocontrol bacteria and play a role in disease control depending on the response speed and magnitude in contrast to infection of plants by pathogens (Bostock 2005). Thus, facilitating plant responses is important.

Natural products that have a potential in crop improvement are various in nature and sources. Proteins of harpin group produced by many plant pathogenic bacteria belong to an important class of type-III effectors, which elicit multiple plant responses when secreted by pathogens during infection (Staskawicz *et al* 2001; Alfano and Collmer 2004), and which cause multiple beneficial effects when applied to plants (Dong *et al* 1999, 2004, 2005). $HpaG_{Xoo}$, a harpin produced by *Xanthomonas oryzae* pv. *oryzae*, stimulates plant growth and defense toward pathogens and insects (Noel *et al* 2002; Kim *et al* 2003; Peng *et al* 2003). In transgenic tobacco (Peng *et al* 2004b) plants expressing $HpaG_{Xoo}$, systemic acquired resistance (SAR) is induced. HrpNEa from *Erwinia amylovora* stimulates abscisic acid signalling to induce drought tolerance (Dong *et al* 2005). In response to HrpNEa, insect defense and plant growth enhancement occur coordinately under mediation by ethylene (Dong *et al* 2004). Thus, signalling pathways are distinct for different type-III effectors (Kim and Beer 2000; Lee *et al* 2001), leading to production of various defense compounds, such as chitinase and peroxidase, which also can be induced by biocontrol bacteria (Nandakumar *et al* 2001). Moreover, colonization of plant roots by biocontrol bacteria activates induced systemic resistance (ISR), and provides plants with a distinct battery of defense arsenal (Pieterse *et al* 1996; Dong *et al* 2001). Therefore, a combinative use of biocontrol bacteria and the type-III effectors could be more effective than use of either of them alone in crop improvement.

This study was aimed to determine how a biocontrol bacterium and a type-III effector interact to affect plant growth and disease resistance. Previously, we used *P. cepacia* P6854 to control rice sheath blight with desired results (Chen and Mew 1998). We have introduced $HpaG_{Xoo}$ into tobacco (variety Xanthi) and rice (variety R109), generating several transgenic lines. They were improved in disease resistance, as has been studied in tobacco (Peng *et al* 2004b). Here we show that the $HpaG_{Xoo}$-expressing rice line 1 (HER1) inhibited increased growth and activate defense toward salinity and pathogens. We present evidence that *P. cepacia* P6854 differentially affects growth and disease resistance in R109 and HER1.

2. Materials and methods

2.1 *Microbes*

The strain JXOIII of *Xanthomonas oryzae* pv. *oryzae* was used to clone the $hpaG_{Xoo}$ gene. The *Agrobacterium tumefaciens* strain EHA105 containing plant transformation and expression vector pBI121 were lyophilized and maintained at –80°C, and multiplied as described (Gerhardt *et al* 1981; Klement *et al* 1990) before use during manipulation of $hpaG_{Xoo}$. The *Pseudomonas cepacia* strain P6854 possesses rifampicin (Rif) resistance; it was multiplied for 48 h in Luria-Bertani (LB) medium supplied with 100 μg Rif/ml in a shaker under 28°C and 250 rpm. The *Rhizoctonia solani* strain RH-2 was stored under –86°C; before inoculation, it was incubated in potato dextrose agar medium at 28°C for 7 d.

2.2 *Engineering and analysis growth prameters of rice*

Cloning, sequencing, prokaryotic expression and product assay of a full length $hpaG_{Xoo}$ gene from JXOIII were done as described (Wen and Wang 2001; Peng *et al* 2004a). Ultimately, $hpaG_{Xoo}$ was inserted into pBI121 between the 35S promoter and the β-glucuronidase gene *uidA* by digestion with restriction enzymes *Xba*I and *Bam*HI and ligated with T4 DNA ligase (Clark 1997). The resulting pBI-hpaG construct (figure 1A) was sequenced to verify correct orientation of genes and transferred into *A. tumefaciens* EHA105 by electroporation. The rice (*Oryza sativa*) variety R109 was transformed with pBI-hpaG or pBI121 by soaking immature embryos with appropriate *A. tumefaciens* suspensions. Tissue culture and plant regeneration were done as described (Clark 1997). Seeds from T1 and T2 generations were screened on an MS agar medium (Gerhardt *et al* 1981) in the presence of kanamycin at 150 µg/ml (Jefferson *et al* 1987; Lazo *et al* 1991), and facilitated for reproduction by planting in the field at Yuanjiang County (Yunnan Province, China), where rice can be grown three times in an year. Genomic integration and expression of the transgene in plants of each generation were analysed by polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR). Five lines of $hpaG_{Xoo}$-expressing rice (HER) were obtained. The control vector without $hpaG_{Xoo}$ insert was also used to transform rice (EVTR) line. Production of the $HpaG_{Xoo}$ protein was determined using a previous method (Peng *et al* 2004b).

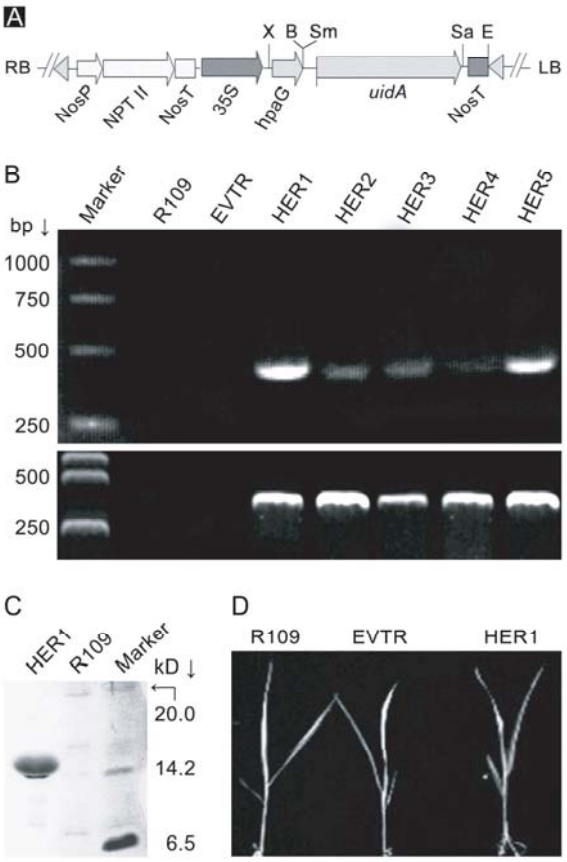

Figure 1. Generation and characterization of $hpaG_{Xoo}$-expressing rice (HER) lines. **(A)** The $hpaG_{Xoo}$-transformation unit pBI-hpaG constructed in the vector pBI121. Labels on top refer to restriction sites X (*Xba*I), B (*Bam*HI), Sm (*Sma*I), Sa (*Sac*I), and E (*Eco*RI). Labels on bottom indicate *NPTII* (kanamycin-resistant gene), 35S (promoter), and $hpaG_{Xoo}$ (a full-length, 420 bp). **(B)** PCR (top gel) and RT-PCR (bottom) analyses for the transgene. Lines HER1 through HER5 were compared with untransformed variety R109 and vector-transformed R109 (EVTR). HER1 lines and EVTR were tested for T1-T3 generation with similar results; shown here are results for T3 plants of HER lines and EVTR compared to R109. Gel pattern and size of markers are placed on left. **(C)** SDS-PAGE visualization of $HpaG_{Xoo}$ protein from HER1 compared with R109. Proteins were extracted from leaves of 20 days old plants and were subject to electrophoresis after purification. **(D)** R109, EVTR and HER1 seedlings at 20 days old. In **(A)** through **(D)** experiments were done 5 times, each involving 15 plants, with similar results.

To produce plants for assays, seeds were germinated by incubation for 2 d in Petri-dishes at 28°C in dark and transferred into plastic cups containing quartz sands saturated with rice nutrition solution (The International Rice Research Institute, Makati City, Metro Manila, Philippines). Subsequently, germinal seedlings were grown in a chamber under 25°C and a 16-h-light/8-h-dark cycle. Plants of 14-d old were used for experiments in lab.

2.3 *Plant and bacterial growth scoring*

To evaluate effects of *P. cepacia* P6854 on rice growth, seeds were soaked in water (control) or a P6854 suspension (5×10^8 cfu/ml) for 2 h and incubated on a wet filter paper in a Petri-dish. Root growth was monitored for a group of treated seeds. In another group, juvenile seedlings were transferred into nutrition solutions and incubated as described. At 14 d after seed treatment, plant weight was determined. P6854 population in root tissues was determined at intervals post-treatment. Roots were excised, weighted, sterilized in 70% ethanol and 33% sodium hypochlorite, respectively. Sterilized roots were washed in sterile water, homogenized in 10 ml sterile water, and placed in a –20°C refrigerator during operation to avoid bacterial multiplication in homogenates. Thawed homogenates were spun briefly and diluted properly; 5 μl of each dilution was placed on LB-Rif agar medium in a plate; one plate was used for one sample with different dilutions. Plates were incubated under 28°C for 2 d; bacterial numbers were counted and given as cfu/g fresh root.

2.4 *Disease evaluation*

To compare HER1 in T3 generation and R109 for productivity and disease resistance, field trial was done in 2004 and 2005 at Yuanjiang (Yunnan Province, China) situated at a geographical zone that favours epidemic development of various diseases. A trial involved 3 plots randomly distributed for each genotype; a plot was 20 m^2 in area. Panicle development and grain yield were investigated. Natural occurrence of blast caused by *Magnaporthe grisea*, leaf blight caused by *X. oryzae* pv. *oryzae*, and sheath blight was surveyed.

In lab assays, HER1 in T3 generation were compared with EVTR or R109 for salinity tolerance by observing plant response to 2% NaCl supplied to the nutrition solution (Xu *et al* 1996). Infection of plants by *R. solani* RH-2 and effect of *P. cepacia* P6854 were tested. The basal second sheaths of plants were inoculated with uniform RH-2 sclerotia placed between sheaths and stems. Inoculated sites were covered with wet absorbent cotton tied to stem by transparent pastern. Inoculated plants were incubated in the chamber. Sixteen h later, roots were soaked in water or a P6854 suspension (5×10^8 cfu/ml) for 2 h, followed by regular growth of plants. Sheath blight symptoms were monitored at 24, 48 and 72 h posttreatment (hpt) with P6854. Disease severity was evaluated based on length of blight lesions.

2.5 *Gene expression analysis*

Total RNA was isolated from whole plants, roots or leaves at intervals using the Tripure Isolution Reagent (Roche Diagnostics, Indianapolis, IN, USA) as per the manufacture's instruction. Gene expression was analysed by RT-PCR done with primers and programs shown in table 1. Reaction conditions were optimized and RT-PCR products were confirmed by sequencing as described (Peng *et al* 2003). The elongation translation factor gene *EF1α* was used as a control because it is expressed constitutively in eukaryotes (Berberich *et al* 1995). RT-PCR products were resolved by electrophoresis in 1% agarose gel and visualized by staining with ethidium bromide. Relative levels of gene expression were determined with a gel documentation system (Molecular Imager Gel Doc XR System and Quantity One 1-D analysis software, Alfred Nobel Drive Hercules, California, USA).

2.6 *Data analysis*

Results were presented when they were similar in replicate experiments. Each experiment was done 3 times and involved 3 replicates each time; a replicate contained 15 plants. Exceptions were specified elsewhere in the text or in figure legends. Quantitative data were subject to T and ANOVA tests using the SAS/STAT® tools (*http://www.sas.com*) to determine significantt differences between treatments.

3. Results

3.1 *Growth and defense in HER1 and R109*

Five transgenic rice lines were identified which contained *hpaG*$_{Xoo}$ in the chromosome (figure 1B, top). Based on levels of the transgene expression and seedling growth on medium and pots, HER1 showed desired properties. The line markedly accumulated the transgene transcript (figure 1B, bottom) and produced the HpaG$_{Xoo}$ protein (figure 1C) as well. HER1, EVTR and R109 were similar in morphology except that HER1 grew better apparently (figure 1D). Thus, HER1 was used in the further study.

In a lab assay, HER1 exceeded R109 to tolerate salinity stress caused by NaCl supplied to root system. The *OsLP* gene encodes an osmotin-like protein involved in salinity tolerance (Thomas and Bohnert 1993; Raghothama *et al* 1997; Zhang and Shih 2006). Expression of *OsLP* was not constitutive (data not shown) in R109 without induction

Table 1. PCR and RT-PCR primers and programs for genes tested in this study.

Gene	Genbank No.	Primers (product size in bp)	PCR programs
OsARF1	AJ306306	5'-gcagattactgcaggccatt-3', 5'-tcatccgctgaacctcttct-3' (899)	95°C 5 min; 95°C 30 s, 55°C 50 s, 72°C 50 s, 25 cycles; 72°C 10 min
OsEFα	AF030517	5'-ccgagcgtgagagaggtatc-3', 5'-gccaataccaccgatcttgt-3' (551)	95°C 5 min; 95°C 30 s, 55°C 45 s, 72°C 40 s, 25 cycles; 72°C 10 min
OsEXP1	Y07782	5'-taccggatcatgtgcgacta-3' 5'-gctgtgaggtcgagaaggtc-3' (517)	95°C 5 min; 95°C 30 s, 50°C 30 s, 72°C 45 s, 25 cycles; 72°C 5 min
hpaG$_{Xoo}$	AY139029	5'-gcggatttgttatcgattc-3' 5'-tattactgcattgatgcgcttcc-3' (420)	95°C 5 min; 95°C 30 s, 55°C 30 s, 72°C 30 s, 30 cycles; 72°C 10 min
OsMAPK5b	AF479884	5'-cgacatgatgacggagtacg-3', 5'-cagctgcttcatttggtcct-3' (496)	95°C 5 min; 95°C 30 s, 50°C 45 s, 72°C 40 s 25 cycles; 72°C 10 min
OsLP	L76377	5'-ctacttcgacatctcgctcgt-3' 5'-gaaaaactccgtgtccttgg-3' (657)	95°C 5 min; 94°C 45 s, 53°C 50 s, 72°C 45 s 35 cycles; 72°C 7 min

but became evident following NaCl treatment (figure 2A). When induced by salinity, expression level in HER1 was ca. 10-fold higher than that in R109 (figure 2A). Consistently, treated and untreated HER1 plants grew normally but R109 growth was inhibited evidently, compared to control (figure 2B).

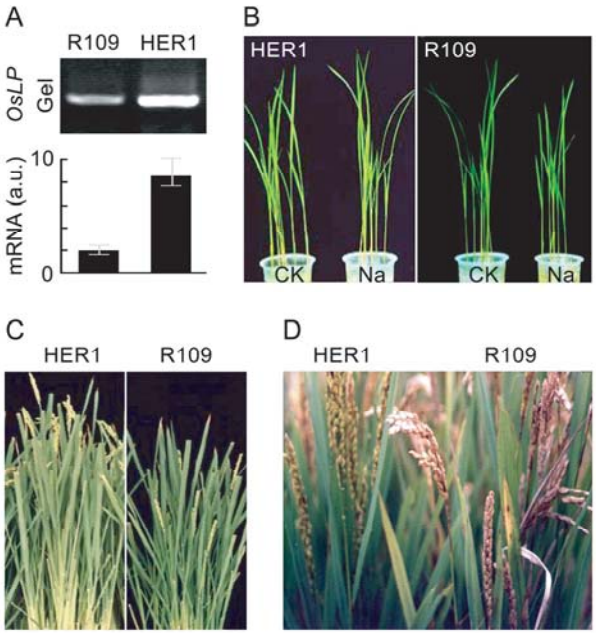

Figure 2. Several defense responses in the *hpaG*$_{Xoo}$-expressing rice line HER1 compared to untransformed rice variety R109. **(A)** Expression of the *OsLP* gene that encodes an osmotin-like protein in rice. Gene expression was analysed by RT-PCR conducted with RNA isolated from whole plants. RT-PCR products were resolved in agarose gel electrophoresis and visualized by staining with ethidium bromide. The constitutively expressed gene *OsEF1α* was used as a standard to verify uniform amplification of genes (gel not shown). Relative amount of *OsLP* mRNA was quantified as arbitrary units (a.u.) with a gel documentation system. **(B)** Growth of seedlings under regular condition (CK) and salinity stress (Na). Seedlings were grown in quartz sands saturated with nutrition solution. Salinity stress was made by supplying the nutrition solution with 2% NaCl at 10th day after seed germination. Seedlings were photographed 10 days later. (**C**, **D)** Plants showing panicle status and panicle blast severity in the field. The data shown are from plants in the field trial (Yuanjiang, Yunnan Province, China; 2004).

Field trial revealed that HER1 excelled R109 in productivity and resistance to several diseases. Results in 2004 and 2005 trials were similar; 2004 data are presented. HER1 apparently was growing better than R109 and produced greater numbers of grain (figure 2C). Blast, leaf blight and sheath blight all occurred less severe in HER1 vs R109 throughout the disease epidemics (figure 2D; table 2). In R109, panicle blast was spread over the plot; 47% spikes were damaged seriously, causing kraurosis of grains (figure 2D). In HER1, on the contrary, 87.2% panicles were free of disease (table 2) and developed normally (figure 2D). As a result, grain yield was markedly higher in HER1 relative to R109 (table 2). These differences were significant at $P = 0.05$ (ANOVA test). Clearly, HER1 was greater than R109 in productivity and disease resistance.

3.2 *Effects of* P. cepacia *P6854 on growth of R109 and HER1*

When applied to roots, the biocontrol bacterial strain P6854 differently affected growth of rootparts, stems, and leaves of both plants. As depicted in figure 3A, when compared to control, colonization of seeds by P6854 evidently increased root growth of both R109 and HER1. However, growth of HER1 stems and leaves apparently was inhibited.

To relate growth variations with P6854 multiplication, we determined bacterial population in rice tissues. As shown in figure 3B, bacterial numbers in roots of both R109 and HER1 increased with time during the course of study; they were greater in R109 relative to those in HER1 at each time point after 6 hpt. Difference in bacterial number between both genotypes was significant (ANOVA test, $P = 0.05$). Optimal population was found at 48 hpt; R109 supported bacterial growth to a number that was 5 times bacterial population in HER1.

The effects of P6854 on growth were quantified (figure 3C). Root growth increases for P6854 vs control were determined as 20.0% and 13.2% in R109 and HER1, respectively. However, growth of stems and leaves was different between R109 and HER1. For R109, 6.2% increase

Table 2. Severity of important diseases and rice grain yield in the field.

Genotype	Panicle blast (%)	Sheath blight (%)	Leaf blight (%)	Kg grain/20 m^2
R109	47.0 ± 5.0^a	75.5 ± 8.2^a	35.0 ± 4.6^a	26.4 ± 3.5^a
HER1	12.2 ± 1.5^b	30.2 ± 4.0^b	21.4 ± 2.7^b	32.5 ± 4.0^b

The field trial had 3 plots for a genotype. A plot, representing a replicate, was 20 m^2 in size. Observations were recorded on all plants. Results are shown as mean ± SD. Superscripts indicate significance in differences between vertical pairs of tested values (ANOVA test, $P = 0.05$).

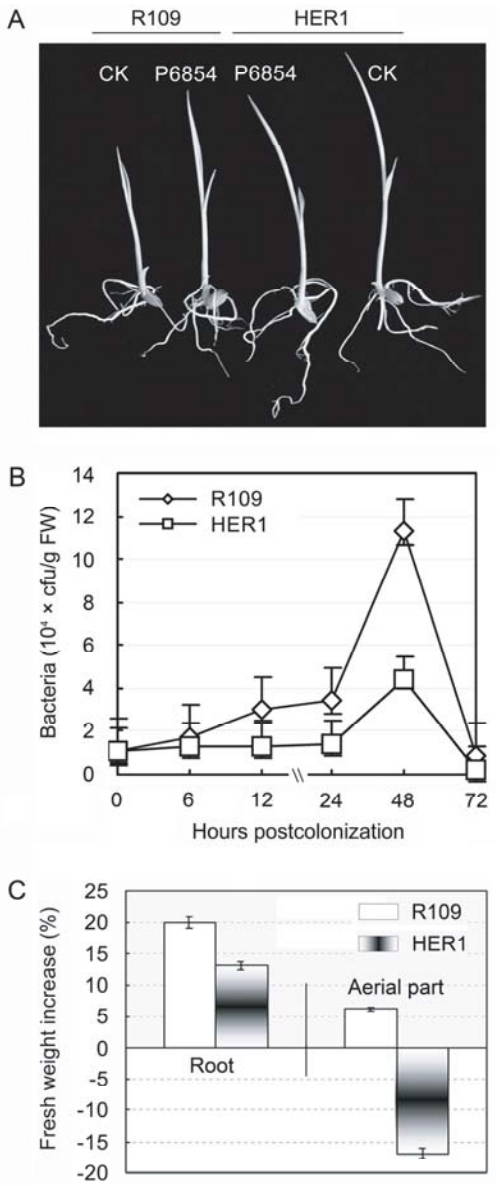

Figure 3. Effects of the biocontrol bacterial strain P6854 on growth of HER1 and R109. **(A)** Fourteen days old seedlings. Seeds were germinated on wet filter paper in Petri-dish for 2 days. Juvenile roots were soaked in sterile water (CK) or a P6854 suspension (5×10^8 cfu/ml). Two h later, juvenile seedlings were transferred to nutrition solution in plastic cups, and incubated as described. Photos represent 90 plants in each treatment subjected to assays below. (**B)** Bacterial numbers in root tissues of plants from (**A**). Bacteria were recovered from plants immediately after soaking roots in a P6854 suspension and at the indicated intervals. Bacterial population was determined as cfu/g fresh weight (FW). Curves represent mean ± standard deviation (SD) of results from 3 replicates ($n = 45$ plants). (**C)** Weight increase in P6854-colonized plants vs CK plants. Plants from (**A)** were grown for 14 days. Increase in plant weight was determined for P6854-colonized plants in contrast to CK plants. SD bars ($n = 45$ plants) are shown cross tops of histograms.

was detected. In contrast, HER1 showed an 16.9% decrease in growth of stems and leaves. The differences were significant between control and P6854 treatment (*T* test, $P = 0.05$) and among treatments in R109 and HER1 (ANOVA test, $P = 0.05$), suggesting a significant effect of P6854 on rice growth. These results indicated that stem and leaf growth in R109 and HER1 was not coincident with P6854 population.

3.3 *Effects of* P. cepacia *P6854 on sheath blight in R109 and HER1*

A lab assay for rice sheath blight revealed a cooperative effect of P6854 and HER1 in decreasing severity of the disease (figure 4). When plants were not treated with P6854, HER1 was less diseased than R109 (figure 4A). In R109, sheath blight symptoms appeared by 12 h after inoculation, when no symptom was seen in HER1. When observed at 24 h after inoculation, symptoms spread in R109 over the leaf and stem that contacted with sclerotia as inocula, but symptoms occurred only on the leaf in HER1 (figure 4A, CK). These results indicate that HER1 had acquired resistance. Following P6854 application to roots, on the other hand, sheath blight was alleviated markedly compared to control in both R109 and HER1. However, blight lesions were evidently shorter and less extensive on sheath of HER1 vs R109 (figure 4A, P6854). These results were confirmed by quantifying disease severity evaluated based on length of blight lesions (figure 4B). When determined at 24, 48 and 72 hpt and compared to control, length of blight lesions of R109 were decreased by 65.5%, 37.5% and 15.5%; the rate in THR109 was 100%, 55.0% and 39.4%. Clearly, P6854 and HER1 cooperated in alleviating sheath blight severity.

P6854 multiplication in *R. solani*-inoculated plants was consistent with the reduction of disease severity (figure 4C). During 6-24 hpt, bacterial population increased with time in R109 and HER1, but bacterial numbers were evidently smaller in HER1 than R109 at each point of times. At 48 hpt, bacterial amounts in root tissues of R109 were scored as ca. 5×10^5 cfu/g, which was 2.5-fold of that in HER1. These results represented significant differences between R109 and HER1, and between CK and P6854 treatment in both genotypes (ANOVA test, $P = 0.05$).

3.4 *Growth-related gene expression in R109 and HER1*

To seek a reason for the disparity between HER1 and R109 in growth promotion of roots, stems and leaves responding to P6854, as shown in figure 3, genes *OsARF1* (*Oryza sativa* auxin response factor 1) and *OsEXP1* (encoding a rice expansin) were studied by RT-PCR conducted with the *EF1α* gene used as a standard (figure 5A). OsARF1 regulates

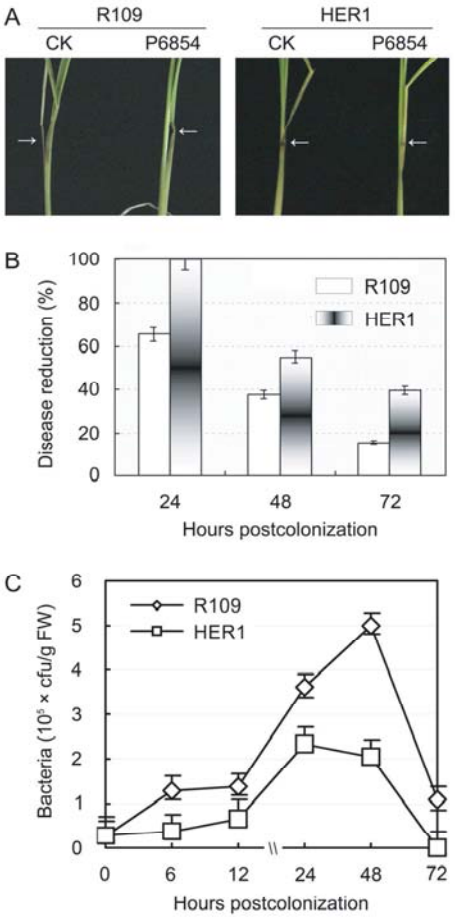

Figure 4. Effects of P6854 on sheath blight. (**A)** Disease symptoms on R109 and HER1. Plants at 14-d-old were inoculated with *R. solani* at the sites indicated by narrows. Sixteen h later, roots were soaked in sterile water (CK) or a P6854 suspension for 2 h. Plants were photographed at 24 h postinoculation. (**B)** Disease severity reduction in P6854-colonized plants vs CK plants. Length of sheath blight on plants equivalent to those in (**A)** was determined. Disease severity reduction was determined by comparing length of blight lesions on P6854-colonized plants with that on CK plants. SD bars (n = 45 plants) are indicated. (**C**) Bacterial numbers in tissues of plants equivalent to those in (**A)**. Assay was similar as in figure 3A. SD bars (n = 45 plants) are indicated on curves.

an early response to an auxin signal during plant growth and is most active in fast-growing parts of rice (Waller *et al* 2002), whereas expansins are required for cell expansion and plant growth in regular growth process and in response to a harping protein (Dong *et al* 2004). When plants were not colonized by P6854, *OsARF1* was not expressed in R109 and HER1. *OsEXP1*, however, was expressed conspicuously in leaves but feebly in roots of both plants; expression level in HER1 leaves was higher than that in R109 leaves (figure 5A). When tested at 12 h after root colonization by P6854, the two genes behaved differently in R109 and HER1. A great level of *OsARF1* transcript was detected in leaves of

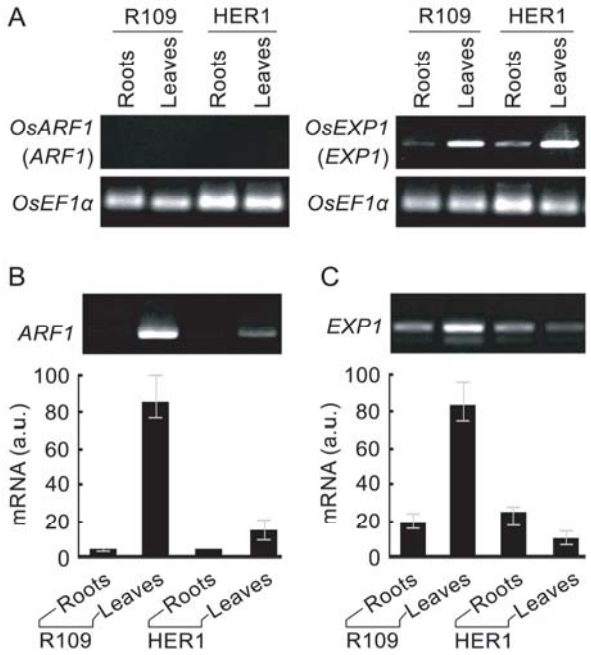

Figure 5. Differential expression of *OsARF1* and *OsEXP1* expression in roots and leaves. (**A)** Assay with plants in the absence of P6854. Observation on 14 days old plants. (**B, C)** Assay with plants in the presence of P6854. Root colonization by P6854 was executed 12 h prior to the assay. In (**A)** to (**C**), RNA was isolated from roots and leaves as indicated. RT-PCR was conducted and arbitrary units (a.u.) of mRNA amounts were determined similarly as in figure 2A.

R109 and not in HER1, while expression was not evident in R109 roots and HER1 roots and leaves (figure 5B). Unlike *OsARF1*, *OsEXP1* was expressed to varying extents in roots and leaves of both plants (figure 5C). The expression level was close in R109 and HER1 roots and optimal in R109 leaves, which accumulated the transcript to an extent that it was ca. 6-fold higher than the level in HER1 leaves (figure 5C). Apparently, *OsARF1* and *OsEXP1* were not quite related to root growth of R109 and HER1 in response to P6854. Instead, induced expression of both genes was coincident with growth of leaves of R109, and noticeably coincident with promotion in R109 growth but inhibition in HER1 growth.

3.5 *OsARF1 expression in the interaction complex*

We determined how *OsARF1* behaves in leaves when P6854, $HpaG_{Xoo}$ and *R. solani* are present together in rice plants. The gene expression was compared in leaves of plants after they were inoculated with *R. solani* RH-2 followed by P6854 colonization on roots. Leaves of plant not inoculated by the pathogen, and plants without the biocontrol colonization served as control. Figure 6A depicts gene expression pattern.

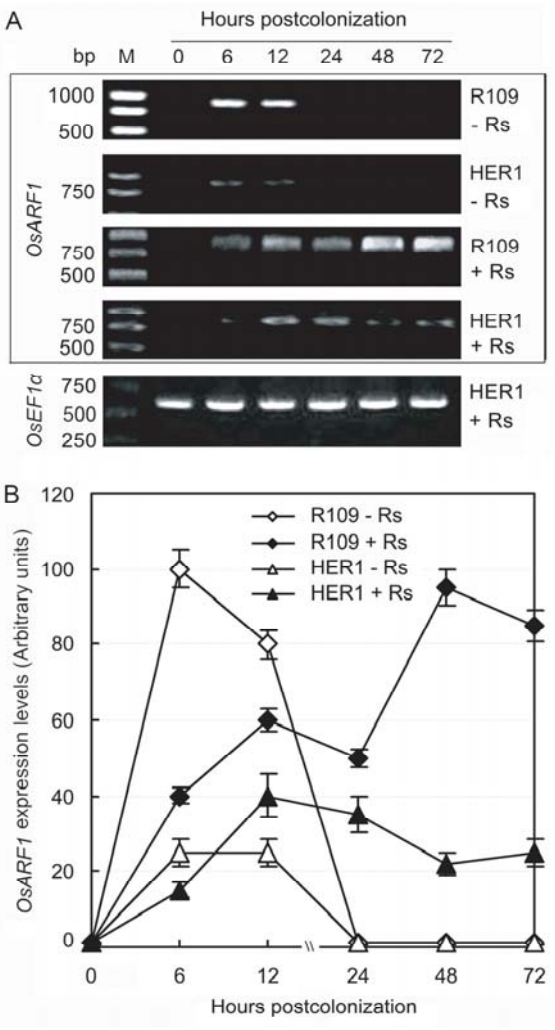

Figure 6. Time course (**A**) and level (**B**) of *OsARF1* expression in leaves of plants colonized by P6854 and inoculated with *R. solani* or without inoculation. Fourteen days old plants were inoculated; 16 h later, roots were soaked in a P6854 suspension for 2 h, followed by continuous incubation of plants. RNA was isolated at indicated times and analysed as in figure 2A. R109 - Rs meaned R09 plants colonized with P6854, but not inoculated with *R. solani* RH-2; R109 + Rs meaned R109 plants inoculated with RH-2 and subsequently colonized with P6854; HER1 - Rs meaned HER1 plants colonized with P6854, but not inoculated with RH-2; HER1 + Rs meaned HER1 plants inoculated with RH-2 and subsequently colonized with P6854.

When plants were not inoculated, *OsARF1* expression was detected at 6 h and 12 h but after that it was not detectable; expression levels were greater in R109 in contrast to HER1. When plants were inoculated, *OsARF1* was expressed in both plants, but stronger in R109 vs HER1; expression level also was increased with time. Quantifying gene expression level (figure 6B) and comparing it with growth of stems and leaves (figure 3B) revealed that time course of induced *OsARF1* expression was important to promotion of leaf and stem growth while responding to P6854 and *R. solani*. The earlier expression was coincident to plant growth promotion, whereas the increased expression after 12 h of colonization (28 h after inoculation) apparently was related to infection by the pathogen.

3.6 *OsMAPK5b expression in the interaction complex*

We studied *OsMAPK5b*, a gene that encodes a mitogen-activated protein kinase in rice. OsMAPK5b is required for salinity tolerance but inhibits SAR (Xiong and Yang 2003) and thus may be engaged in the role of biocontrol bacteria in R109 vs HER1, which shows defense to pathogens (Table 2) and salinity (figures 2A and 2B). *OsMAPK5b* had a constitutive expression but patterns (figure 7A) and

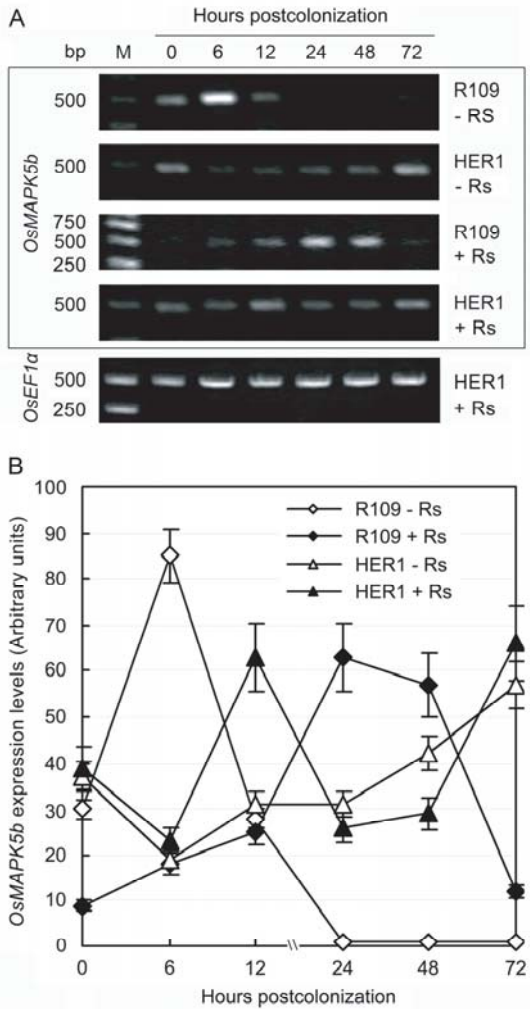

Figure 7. Time course (**A**) and level (**B**) of *OsMAPK5b* expression in leaves of plants colonized by P6854 and inoculated with *R. solani* or without inoculation. Plant treatments and assays were similar to those in figure 6.

levels (figure 7B) of expression varied with time and plant genotypes. In HER1, the gene expression was similar in the different combinations, indicating that it was not related with responses to P6854 or *R. solani*. In R109, however, *OsMAPK5b* expression was affected markedly by P6854 and *R. solani*. In R109 plants without inoculation, *OsMAPK5b* expression was evident during 0–12 h after P6854 colonization on roots and became feeble subsequently since 24 h. When R109 plants were inoculated with *R. solani*, time of *OsMAPK5b* expression was extended to 48 h, suggesting an effect by the pathogen.

4. Discussion

We have engineered the rice variety R109 with *hpaG*$_{Xoo}$, a bacterial gene encoding a type-III effector, generated HER lines, and compared HER1 and R109 for growth and disease resistance in response to *P. cepacia* P6854, a biocontrol bacterial strain. Based on results detailed here, P6854 and HpaG$_{Xoo}$ interacted differently during rice growth and resistance development. Both cooperate in inducing resistance, promising a great potential in agricul-tural use. Complicated mechanisms underlie interactions involving P6854, HpaG$_{Xoo}$ and the sheath blight pathogen *R. solani*.

Through replicate experiments we obtained several results. First, relative to R109, HER1 shows increase in growth, grain yield, disease resistance and salinity tolerance (figures 1 to 3; table 2), supporting an earlier finding that the application of a type-III effector of harpin group to various plants induces multiple beneficial effects (Dong *et al* 1999, 2004, 2005; Peng *et al* 2003). We have shown that HpaG$_{Xoo}$ induces disease resistance in transgenic tobacco plants (Peng *et al* 2004a, b). Here we show similar effects in rice, a staple food crop in the world. Thus, the engineering approach is effective to improve crops productivity. Second, application P6854 is more effective in HER1 than it is in R109 to achieve resistance. Evidence in supporting the conclusion was seen in the consistence of resistance with the application of P6854 (figure 4). Clearly, P6854 and HpaG$_{Xoo}$ act together to affect disease resistance, and could be integrated into a practice to provide better effect than applying either of them. Lastly, P6854 interferes with function of HpaG$_{Xoo}$ gene in promoting rice growth. The negative interaction was seen in differences between R109 and HER1 in growth of roots and, particularly, stems and leaves (figures 1 to 3). The antagonism also was evident in the disparity between rice growth and P6854 population (figure 3). It is important to test whether the antagonism diminishes rice productivity.

Results of studying *OsARF1*, *OsEXP1* and *OsMAPK5b* disclose complicatedness in molecular basis of interactions involving P6854, HpaG$_{Xoo}$ and *R. solani*. Different signals may be recruited to affect rice growth in response to P6854 and HpaG$_{Xoo}$. Transcription of *OsARF1* is an early response to an auxin signal in rice and is most active in parts of facilitated growth (Waller *et al* 2002). We found that *OsARF1* expression was induced in R109 leaves but was nullified in HER1 (figure 6). Consistently, P6854 promoted growth of R109 leaves and stems but inhibited HER1 growth at the equivalent parts (figure 3). Growth inhibition in HER1 apparently matched P6854-compromised proportion of the growth increase relative to R109. Thus, *OsARF1* may at on aerial parts of the plant to affect P6854-HpaG$_{Xoo}$ antagonism. Moreover, plant growth stimulated by a harpin involves expansins, which promote cell growth by losening the wall (Li *et al* 2003). Actions by expansins and harpin both are subjected to ethylene signalling (Dong *et al* 2004; Belfield *et al* 2005; Vreeburg *et al* 2005; Shi *et al* 2006). The behaviour of *OsEXP1* in R109 and HER1 (figure 6) suggests a role *OsEXP1* plays in the positive and negative effect of P6854 on R109 and HER1, respectively. Many studies are required to determine how the involved signals and genes modulate the antagonism found in HER1.

The pattern and time course of *OsMAPK5b* expression (figure 7) indicate that P6854-HpaG$_{Xoo}$ interaction may affect distinct signalling pathways. In rice, OsMAPK5b regulates tolerance to drought, salinity and coldness but acts to suppress salicylic acid-mediated SAR (Xiong and Yang 2003). SAR, however, serves as a pathway of type-III effectors in harpin group to induce disease resistance when the proteins are applied to plants (Strobel *et al* 1996; Dong *et al* 1999) or produced in transgenic plants (Peng *et al* 2004b). Nevertheless, plant growth-promoting biocontrol bacteria are believed to induce ISR, which interferes with SAR in signal transduction (Johri *et al* 2003; Gielen *et al* 2004; Tjamos 2005). Studying an individual factor, like OsMAPK5b, in the proposed signaling network merely is the first step toward studies on components critical to the interaction.

In HER1 vs R109, retarded infection is consistent with resistance (figure 4). After inoculated to rice plants, *R. solani* sclerotia produce tubes to infect the host usually within 24 h causing disease and causes disease in 24 h (Rohilla *et al* 2002; Wang and Tan 2005; figure 4A). The evident expression of *OsARF1* and *OsMAPK5b* within 12 h reasonably is attributed to P6854 colonization on roots (figures 6 and 7). Subsequent increases in gene expression levels may be caused by the pathogen. Plant basal defense (Dangl and Jones 2001) can be induced by pathogens but occurs rather later during infection process than required for protecting plants from diseases (Vijayan *et al* 1998; Maleck and Dietrich 1999). This may be the case for *OsMAPK5b*. An inverse mode was seen for *OsARF1*. The pathogen seems to affect plant growth by affecting *OsARF1* expression, which is concomitant with plant growth in response to an auxin signal (Waller *et al* 2002). The conspicuous expression of *OsARF1* after 28 h of inoculation (figure 6) indicates that the gene behaves

depending on challenges that the plant encounters (Waller *et al* 2002). The hypothesis remains to be tested.

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