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Expression of *hpa1* Gene Encoding a Bacterial Harpin Protein in *Xanthomonas oryzae* pv. *oryzae* Enhances Disease Resistance to Both Fungal and Bacterial Pathogens in Rice and *Arabidopsis*

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Xanthomonas oryzae pv. *oryzae* causing bacterial leaf blight disease in rice produces and secretes Hpa1 protein that belongs to harpin protein family. Previously it was reported that Hpa1 induced defense responses when it was produced in tobacco. In this study, we expressed *hpa1* gene in rice and *Arabidopsis* to examine the effects of Hpa1 expression on disease resistance to both fungal and bacterial pathogens. Expression of *hpa1* gene in rice enhanced disease resistance to both *X. oryzae* pv. *oryzae* and *Magnaporthe grisea*. Interestingly, individual transgenic rice plants could be divided into four groups, depending on responses to both pathogens. *hpa1* expression in *Arabidopsis* also enhanced disease resistance to both *Botrytis cineria* and *Xanthomonas campestris* pv. *campestris*. To examine genes that are up-regulated in the transgenic rice plants after inoculation with *X. oryzae* pv. *oryzae*, known defense-related genes were assessed, and also microarray analysis with the Rice 5 K DNA chip was performed. Interestingly, expression of *OsACSI* gene, which was found as the gene that showed the highest induction, was induced earlier and stronger than that in the wild type plant. These results indicate that *hpa1* expression in the diverse plant species, including monocot and dicot, can enhance disease resistance to both fungal and bacterial plant pathogens.

Keywords : bacterial leaf blight pathogen, defense response, harpin, *OsACSI* gene, rice blast pathogen

Harpins are heat-stable and glycine-rich proteins, but have few cysteine and aromatic amino acids (Beer et al., 1993). They are secreted from bacteria through type III secretion system and are known to be located at intercellular space of plant tissues (Alfano and Collmer, 1997). Harpin proteins have been identified from diverse Gram-negative plant-pathogenic bacteria such as *Erwinia amylovora* (Kim and Beer, 1998; Wei et al., 1992), *Pseudomonas syringae* (Charkowski et al., 1998; He et al., 1993; Kvitko et al., 2007), *Ralstonia solanacearum* (Arlat et al., 1994), and *Xanthomonas* species (Kim et al., 2003; Noel et al., 2002; Zhu et al., 2000). For example, Hpa1 is the harpin protein that is produced and secreted from *X. oryzae* pv. *oryzae* causing bacterial leaf blight in rice (Zhu et al., 2000). It is a 13-kDa glycine-rich protein carrying two coiled-coil regions in its N- and C-termini (Ji et al., 2011). Its expression is regulated by HrpB, a regulatory protein that controls expression of other *hrp* (hypersensitive response and pathogenicity) genes in *X. oryzae* pv. *oryzae* by directly binding their promoters with PIP boxes (Zhu et al., 2000). Hpa1 orthologs have been identified from other *Xanthomonas* species such as HpaG from *X. axonopodis* pv. *glycines* and XopA from *X. campestris* pv. *vesicatoria* (Kim et al., 2003; Noel et al., 2002).

Originally harpin proteins, including HrpN of *E. amylovora* were identified as elicitors of hypersensitive response (HR) in tobacco (Wei et al., 1992). HR represents the rapid localized cell death that occurs at the infection sites in plants (Keen, 1990). This is one of strong disease resistance phenotypes to plant pathogens. It has been reported that several harpin proteins, including Hpa1 of *X. oryzae* pv. *oryzae* and its ortholog HpaG of *X. axonopodis* pv. *glycines*, but not XopA of *X. campestris* pv. *vesicatoria*, induce strong HR phenotypes, when they are partially purified and infiltrated into tobacco leaves (Kim et al., 2003). This

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implies that harpin proteins function outside the plant cells to trigger HR, consistent with evidence that harpin proteins are secreted from bacterial pathogens and targeted outside the plant cells (Perino et al., 1999).

In addition to HR, defense responses without HR induction in plants can be initiated by recognition of pathogen factors, and this recognition triggers increase of the internal level of signaling molecules like salicylic acid (SA), nitric oxide (NO), reactive oxygen species (ROS), jasmonic acid (JA) and ethylene (Hammond-Kosack and Parker, 2003). These signaling molecules activate downstream defense signal transduction pathways, resulting in induction of diverse defense-related genes such as pathogenesis-related (PR) genes. SA is the key molecule for induction of SAR, which confers disease resistance to biotrophic pathogens such as *Hyaloperonospora arabidopsidis* (formerly *Peronospora parasitica*) and *P. syringae* pv. *tomato* in *Arabidopsis* (Durrant and Dong, 2004). The SA signaling cascade for systemic acquired resistance (SAR) induction requires NPR1 (also known as NIM1) to activate the expression of *PR1* gene (Cao et al., 1994; Delaney et al., 1995). On the other hands, JA and ethylene are crucial molecules for SA-independent defense responses, which are important for resistance to necrotrophic pathogens like *X. campestris* pv. *campestris* and *Botrytis cinerea* in *Arabidopsis* (Thomma et al., 1998; Thomma et al., 1999). JA is also related with plant's wound responses and defense against insects (Reymond et al., 2000).

Interestingly, when harpin genes are expressed and may be located inside plant cells, they do not seem to trigger HR, but instead confer defense responses to diverse plant pathogens including fungi, oomycetes, bacteria, and viruses. For examples, it was previously shown that *hrpN* expression under Cauliflower mosaic virus (CaMV) 35S promoter in *Arabidopsis* induces expression of *PR1* gene by activating SAR (Dong et al., 1999), and also that *hpa1* expression under 35S promoter in tobacco induces expression of defense-related genes such as *PR-1a* and *PR-1b* in a NPR1-dependent manner without HR phenotype (Peng et al., 2004). Recently, transcriptome analysis after *hpa1* expression in cotton showed that genes responsible for multiple defense signaling pathways are constitutively expressed (Miao et al., 2010).

In this study, *hpa1* gene of *X. oryzae* pv. *oryzae* strain KXO85 was transformed in rice and *Arabidopsis* and was expressed under a constitutive promoter (*actin1* promoter in rice or CaMV 35S promoter in *Arabidopsis*). In these transgenic rice and *Arabidopsis*, the effect of *hpa1* expression on disease resistance to both bacterial and fungal pathogens was examined. Here, we show that expression of *hpa1* gene increases disease resistance to both bacterial and fungal pathogens in both rice and *Arabidopsis*, and ex-

pression of rice *OsACS1* gene was induced in the *hpa1*-transformed rice plant.

Materials and Methods

Bacterial and fungal strains and growth conditions.

Escherichia coli strains, DH5 α and Top10 (Invitrogen, USA) and *Agrobacterium tumefaciens* strains GV3010 and LBA4404 were grown in LB medium at 37°C and 28°C, respectively. *X. oryzae* pv. *oryzae* strain KXO85 and *X. campestris* pv. *campestris* strain 1366R were cultivated at 28°C in peptone-sucrose agar (PSA; 10 g Bacto-peptone, 10 g sucrose, 1 g Na-glutamate, 15 g agar/l) or LB broth. Antibiotics were used as follows: rifampicin (Rif), 50 μ g/ml; kanamycin (Km), 50 μ g/ml; ampicillin (Ap), 50 μ g/ml. The fungal pathogens, *Magnaporthe grisea* and *Borytis cineria* were grown at 28°C on rice sucrose agar medium (20 g rice bran, 20 g sucrose, 20 g agar/l) and potato dextrose agar medium (39 g/l, Difco, USA), respectively.

Gene constructs for plant transformation. To generate *hpa1* gene constructs for plant transformation, 0.42-kb DNA fragment carrying the full length *hpa1* gene was amplified by PCR with the cosmid pJG3 containing 26-kb genomic DNA of *X. oryzae* pv. *oryzae* strain KXO85 as a template and two gene-specific primers, the forward primer (5'-GGATCCATGAATTCTTTGAACACAC-3' with *Bam*HI site) and the reverse primer (5'-TCTAGATGGCCGGTAGGGGCG-3' with *Xba*I site). The PCR product was inserted into pCR2.1 (Invitrogen, USA) to produce pMS-*hpa*TOPO. The *hpa1* gene from pMS-*hpa*TOPO was transferred into the *Bam*HI-*Xba*I digested pBluescriptII KS(+) vector to produce pMS-*hpa1*.

For rice transformation, a 1.3-kb *act1* promoter was cut out from the parent plasmid (McElroy et al., 1990) and the fragment was blunt-ended by the Klenow reaction. This fragment was ligated into *Sma*I-digested pMS-*hpa1* plasmid to produce pMS-pact1-*hpa1*. After the pSBG-MAR vector (Jang et al., 1999) was digested with *Xho*I and blunt-ended by the Klenow reaction, it was digested with *Xba*I again and ligated with the 1.75-kb *Eco*RV-*Xba*I DNA fragment, including the *act1* promoter and *hpa1* gene from pMS-pact1-*hpa1*, to produce pSBG-pact1-*hpa1*. (Fig. 1A). The pSBG-pact1-*hpa1* was introduced to *A. tumefaciens* strain LBA4404 for rice transformation.

For *Arabidopsis* transformation, a 0.85-kb *Hind*III-*Bam*HI DNA fragment of pBI221 (Clontech, USA), including the 35S promoter, was ligated into *Hind*III-*Bam*HI digested pMS-*hpa1* vector to produce pMS-p35S::*hpa1*. Then, p35S::*hpa1* in this plasmid was transferred into pCAMBIA2301 vector (CAMBIA, Australia) to generate pCAM-p35S::*hpa1* (Fig. 3A). The pCAM-p35S::*hpa1* was introduced to *A. tume-*

faciens strain GV3101.

Agrobacterium-mediated transformation in rice and *Arabidopsis*. For rice transformation, *Agrobacterium* strain LBA4404 containing pSBG-pact1-*hpaI* was used to transform Nagdong rice calli, according to the method described by Hiei et al. (1994). Transgenic plants overexpressing *hpaI* gene were regenerated from transformed calli showing resistance to phosphinothricin. Then, they were transplanted to soil in greenhouse and their herbicide resistance was tested with 0.6% solution of Basta® (Misung chemicals, Korea).

For *Arabidopsis* transformation, *A. tumefaciens* strain GV3010 containing pCAM-p35S::*hpaI* construct was grown for 24 h at 28°C in LB medium supplemented with kanamycin. 100 µl of pre-culture was used to inoculate fresh LB media (100 ml) with the same antibiotic. Cells grown for 2 days were collected by centrifugation and resuspended with transformation medium (5% sucrose and 0.05% Silwet L-77) (Clough and Bent, 1998), and its concentration was adjusted to OD₆₀₀=1.0. Transformation was done by floral dip method in *A. thaliana* ecotype Columbia (Col-0), which was grown at the greenhouse (Clough and Bent, 1998). After dipping, the plants were placed on a plastic flat and covered with a plastic dome to maintain humidity. The dome was removed next day. The putative transgenic *Arabidopsis* plants were selected by planting the seeds on a 1/2 MS (half MS salts and vitamins, B5 vitamin, 2 g Pytagel/l) medium containing kanamycin (100 mg/l). After 3 weeks on the medium, the kanamycin-resistant T1 plants were transferred to soil and grown at the greenhouse, and they were used for disease assay and for generating T2 plants.

Pathogen inoculation. For diseases caused by *Xanthomonas* pathogens, *X. oryzae* pv. *oryzae* strain KXO18 in rice and *X. campestris* pv. *campestris* strain 1366R in *Arabidopsis*, bacteria were grown on PSA medium for 2–3 days at 28°C, and inoculum was prepared by suspending bacterial cells in 10 mM MgCl₂. Their final concentration was adjusted to 5 × 10⁸ cfu/ml and 3 × 10⁶ cfu/ml, respectively. Bacterial inoculation was performed by the leaf clipping method (Kauffman et al., 1973) in rice at the maximum tillering stage and *Arabidopsis* at rosette growth complete stage, and lesion length was measured on three leaves 14 days after inoculation. These experiments were repeated at least twice, and 3–5 plants were used per each replicate.

For disease caused by fungal pathogen, *M. grisea*, the mycelium was grown on rice sucrose agar medium at 28°C in dark for 7 days. For spore formation, it was stressed by scraping and placed under continuous lights for 2 days. For gray mold disease caused by *B. cineria* in *Arabidopsis*, the

fungus was grown on potato dextrose agar medium. The spores were collected by scraping with 0.05% Tween 20 and the concentration of both fungal pathogens was adjusted to 10⁵ spores/ml. Inoculation was carried out by spraying the spore suspension with an air compressor onto 3rd to 4th leaf stage rice plants and *Arabidopsis* at growth stage 3.7 (Boyes et al., 2001) until leaves were covered with fine droplets. Plants were incubated in a dew chamber at 28°C for 24 hr and then transferred to greenhouse. Disease was measured after 6 days by estimating the relative leaf area covered with lesions. These experiments were repeated at least twice, and 3–5 plants were used per each replicate.

Total RNA isolation and RT-PCR. Total RNAs were extracted from rice leaves sampled from rice plants inoculated with *X. oryzae* pv. *oryzae* strain KXO18 by using the TRIzol according to the manufacturer's manual (Invitrogen, USA). 2 µg of total RNA, oligo dT₁₅ primer, and AMV reverse transcriptase (Roche, USA) were used to synthesize cDNAs for the semi-quantitative RT-PCR. Gene-specific primers used to check expression of *hpaI*, *OsPRI* (GenBank: AF306651), *OsPAL* (GenBank: X16099), *OsCHS* (GenBank: AB058397), *OsPOX* (GenBank: AF014467), *OsPRI0* (GenBank: AF395880) or actin gene (GenBank: AC092557.7) as an internal control were listed in Table 1.

Microarray analysis. Microarray experiments were performed using the Rice 5 K DNA chip generated at the National Institute of Agricultural Sciences and Technology (NIAST, Korea). The 4,800 unique rice cDNA clones selected from cold-treated rice cDNA library were mounted on Rice 5 K DNA chip by Generation III ArraySpotter (Amersham, USA). Cy3 and Cy5-labeled probes were prepared

Table 1. Primers used to assess expression of genes shown in Fig. 4

Target genes	DNA sequences
<i>hpaI</i>	Forward: 5'-TGAATTCTTTGAACACACAATTCG-3' Reverse: 5'-CTCGAGAGGTTGGTGCCGAGG-3'
<i>OsPRI</i>	Forward: 5'-ATGGCAACCTCCAGCTTGCTAG-3' Reverse: 5'-GAAGACGCCGAGGCTGTTGTC-3'
<i>OsPAL</i>	Forward: 5'-GCGACATCTACGGCGTCACC-3' Reverse: 5'-CTCGTTACCTTCTTGGCGTG-3'
<i>OsCHS</i>	Forward: 5'-CATCACCCACCTCGTCTTCTGC-3' Reverse: 5'-GGAGGATGGTCTGGCTCGCCG-3'
<i>OsPOX</i>	Forward: 5'-CTGCTTCGTCCAAGGGTGCGAC-3' Reverse: 5'-GTCCCTGAAGTTCTGGCACTGC-3'
<i>OsPRI0</i>	Forward: 5'-CAGTTCAACTTCACCTCAGCCA-3' Reverse: 5'-GCAAAACCAACAGGTAGATGCT-3'
<i>Actin</i>	Forward: 5'-TGTCATGGTTGGAATGGGCCA-3' Reverse: 5'-AGGCAGTCAGTCAGATCACGA-3'

from total RNA of the transgenic or non-transgenic rice leaves that were sampled at 9 hr after inoculation with *X. oryzae* pv. *oryzae* strain KXO18, respectively. Briefly, 40 µg total RNA was labeled using a 3DNA Array 50 Expression Array Detection Kit (Genisphere) according to the manufacturer's protocols. Total RNA was reverse-transcribed using SuperScript III reverse transcriptase (Gibco) with RT primer containing a specially designed 3DNA capture sequence for Cy3 and Cy5 detection. Following incubation at 42°C for 2 hours, the reaction was stopped by adding 7 µl of 0.5M NaOH/50 mM EDTA, and the template RNA was degraded by incubation at 65°C for 10 min. The reaction solution was neutralized with 10 µl of 1M Tris-HCl, pH 7.5. Two cDNA samples were combined into one tube then precipitated in ethanol. The dried sample was resuspended in water and then used for hybridization. The glass slide was placed in a sealed hybridization cassette and submerged in a 60°C water bath for 16–20 h. After hybridization, the slide was washed in 2× SSC, 0.2% SDS for 15 min at 55°C, then in 2× SSC for 15 min, and finally in 0.2× SSC for 15 min at room temperature with agitation using stirring bar. The dried slide was then hybridized with the fluorescent 3DNA reagent in a 60°C water bath for 4 hours. After finishing 3DNA hybridization, the slide was washed as described above. Microarray slide was scanned using Axon scanner with GenePix Pro software and further analysis was performed with the GeneSpring software (Silicon Genetics, SanCarlos, CA).

Northern blotting. Total RNA (10 µg) was denatured by heating at 65°C for 15 min in RNA loading dye (Ambion, USA) with 5.5 µg ethidium bromide, followed by electrophoresis on 18% formaldehyde gels. The fractionated RNA was transferred to the nylon membranes (Hybond N+, Amersham, USA) and cross-linked by irradiation with UV light. The *EcoRI* fragment of *hpa1* gene and the full length of the *Oryza sativa* 1-aminocyclopropane-1-carboxylate

synthase (*OsACS1*) gene were ³²P-labeled with a random priming kit (Amersham, USA) to be used as a probe. Pre-hybridization was performed at 65°C for 30 min in the hybridization solution (1% BSA, 1 mM EDTA, 0.5 M NaPO₄ and 7% SDS). Hybridization was performed overnight at 65°C in the hybridization solution added with 50 µl of ³²P-labeled probes. The membranes were washed with 2× SSC, 0.1% SDS for 15 min and then 1× SSC, 0.1% SSC for 15 min at 65°C. The hybridized blots were exposed to X-ray films.

Results and Discussion

Generation of transgenic rice over-expressing the *hpa1* gene. Previously, it was reported that expression of *hpa1* gene of *X. oryzae* pv. *oryzae* in tobacco enhances defense responses to diverse pathogens (Peng et al., 2004). Based on this report, we tried to determine the effect of Hpa1 expression on disease resistance in rice and generated *hpa1* construct for rice transformation. In this construct, *hpa1* expression is controlled by rice *actin1* (*act1*) promoter (McElroy et al., 1990), as shown in Fig. 1A. This *hpa1* construct was transformed into rice cultivar Nakdong, which belongs to a japonica type and is very susceptible to both bacterial leaf blight and rice blast diseases (Ahn et al., 2005a; Ahn et al., 2005b; Koh et al., 1987). Total 56 of T0 transgenic lines resistant to Basta® treatment were generated. First, regardless of *hpa1* expression, most of these regenerated plants (53 out of 56 lines) displayed normal phenotypes (data not shown), indicating that overexpression of *hpa1* does not affect rice growth and development, as did not in tobacco (Peng et al., 2004). Then, we checked expression of *hpa1* gene in these transgenic lines. 51 of 53 T0 transgenic lines showed certain level of *hpa1* expression, but two lines did not (Fig. 1B). Fig. 1B shows *hpa1* expression determined by northern blotting with *hpa1* gene as a probe in 20 representatives of T0 transgenic lines.

Fig. 1. *hpa1* expression in the transgenic rice. (A) Genetic structure of T-DNA region carrying *hpa1* gene under actin promoter (*pact1*). Arrows indicate direction of transcription. *p35S*, CaMV 35S promoter; *Pin2* and *nos*, transcriptional terminators, MAR, matrix attachment region. (B) *hpa1* expression in 20 representatives of T0 transgenic rice by northern blot analysis with *hpa1* gene as a probe. Ribosomal RNAs (rRNA) were shown by ethidium bromide staining as loading controls. NT, non-transformed.

Table 2. Grouping of total 53 T0 transgenic rice based on response to *X. oryzae* pv. *oryzae* (BLB) and *M. grisea* (RB)

RB \ BLB	R	S	Total
R	30	3	33 (62%)
S	15	5	20 (38%)
Total	45 (85%)	8 (15%)	53 (100%)

R, resistant; S, susceptible

Disease response in transgenic rice plants harboring *pact1::hpa1* to both bacterial and fungal plant pathogens. We investigated whether the transgenic rice lines displayed disease resistance to bacterial and fungal pathogens. For the disease response to bacterial pathogen, 53 of T0 transgenic rice showing normal growth were inoculated with *X. oryzae* pv. *oryzae* KXO18 using the leaf clipping method. Lesion length was measured on three leaves at 14 days after inoculation, and the grouping was done as R (resistant) or S (susceptible) based on more than 50% reduction of lesion length relative to that of untransformed rice plants as the control. Among 53 T0 lines, 45 lines (85%) showed resistance to *X. oryzae* pv. *oryzae* (Table 2). For the response to the fungal pathogen, *M. grisea*, transgenic plants were inoculated with spore solution by spraying, and disease were observed at third to fourth leaf stage of T0 plants. Disease severity was scaled from 0 to 9, as described (IRRI, 1996). Lesion scales, 0 through 3, were considered as R, and scales, 5 through 9, were considered as S. Among 53 of T0 lines, 33 (62%) showed resistance to *M. grisea* (Table 2).

Based on responses to both pathogens, 53 T0 lines can be categorized into four types, R/R, R/S, S/R, and S/S types. As shown in Table 2 and Fig. 2A, 30 of T0 lines including AP42 line showed resistance to both pathogens (R/R type). 15 of T0 lines only showed disease resistance to *X. oryzae* pv. *oryzae* (R/S type), while only 3 lines showed disease resistance to *M. grisea* (S/R type). Finally, 5 lines were susceptible to both pathogens (S/S type). Disease severity in the representative lines for each type was shown in Fig. 2A, and disease symptom in AP42 line belonging to R/R type, compared to the untransformed rice plants, was shown in Fig. 2B. These results indicate that expression of *hpa1* gene in rice enhances disease resistance to both bacterial and fungal pathogens.

Harpins including Hpa1 protein in bacterial pathogens are secreted out from bacterial cells and targeted to inter-cellular space of plant tissues (Alfano and Collmer, 1997; Perino et al., 1999). In addition, when infiltrated into inter-cellular space of plant tissues, harpins induce the HR, which is a strong resistance response (Kim et al., 2003). This means that harpins normally function outside plant cells.

Fig. 2. Effect of *hpa1* expression in T0 transgenic rice on disease resistance against plant pathogens. (A) Disease severity in four different groups of T0 transgenic rice by two pathogens shown in (B) and (C). NT, non-transformed; S, susceptible; R, resistant. (B) Response to *X. oryzae* pv. *oryzae* strain KXO18, causing bacterial leaf blight. The top of rice leaves were cut by scissors after dipping in bacterial solution (5×10^8 cfu/ml). Photos were taken 14 days after inoculation. AP42, T0 transgenic rice; NT, non-transformed. (C) Response to *M. grisea*, causing rice blast. Rice plants were sprayed with spore solution (1×10^5 spores/ml), and photos were taken 6 days after inoculation. AP42, T0 transgenic rice; NT, non-transformed.

When *hpa1* was expressed in plant cells, this protein is very likely located inside plant cells. Based on our study and the previous study (Peng et al., 2004), the presence of Hpa1 proteins inside plant cells may induce a weak disease resistance, compared to HR induction by the presence of Hpa1 proteins outside plant cells.

Disease response in transgenic *Arabidopsis* harboring *p35S::hpa1* to both bacterial and fungal plant pathogens. To examine the effect of *hpa1* expression in *Arabidopsis* on defense responses to plant pathogens, the full length of this gene was cloned into pCambia2301 vector

Fig. 3. Effect of *hpa1* expression in *Arabidopsis* on disease resistance against two plant pathogens. (A) Genetic structure of T-DNA region carrying *hpa1* gene under CaMV 35S promoter (p35S). Arrows indicate direction of transcription. Tnos, nopaline synthase (nos) transcriptional terminator. (B) Response to *B. cineria* in T2 plants of transgenic *Arabidopsis* (line 7) carrying *p35S::hpa1*. Photos were taken 6 days after spray inoculation with 1×10^5 spore/ml of the fungus. NT, non-transformed. (C) Response to *X. campestris* pv. *campestris* in T2 plants of transgenic *Arabidopsis* (lines 1 and 7) carrying *p35S::hpa1*. Photos were taken 14 days after inoculation with the bacterium (OD=0.03) by the leaf clipping method. NT, non-transformed. (D) Expression of *hpa1* gene in transgenic *Arabidopsis* by RT-PCR. *EF1α* gene was used as an internal control. NT, non-transformed.

to generate pCAM-*p35S::hpa1* construct (Fig. 3A). This construct was used for transformation in *Arabidopsis* ecotype Col-0 by floral dipping method. Two different plant pathogens, *B. cineria* and *X. campestris* pv. *campestris*, were used to determine the effect of *hpa1* expression on defense responses in transgenic *Arabidopsis*. First, *B. cineria* fungus was inoculated by spray in 95 of T1 plants at growth stage 3.7 (Boyes et al., 2001). The survived plants were considered as resistant (R) and the dead plants were considered as susceptible (S). 36 out of 95 T1 plants (37.9%) showed resistance to the *B. cineria* and 59 transformants (62.1%) were susceptible. Next, 36 transformants that are resistant to *B. cineria* were inoculated with *X. campestris* pv. *campestris* strain 1366R using the leaf clipping method. Plants with lesion length under 50% to the non-transformant control were considered as R and plants with lesion length over 50% to the non-transformant control were considered as S. Only 4 out of 36 transformants (11%) showed resistance to the *X. campestris* pv. *campestris* strain 1366R. To exclude interference by the prior inoculation with *B. cineria*, 171 more T1 plants were inoculated with *X. campestris* pv. *campestris* strain 1366R. 28 out of 171

plants (16%) showed resistance to this bacterium. These results indicate that Hpa1 expression in *Arabidopsis* enhances resistance to both pathogens, but resistance was more efficient to the fungal pathogen. Similarly, in tobacco carrying *hpa1* gene, resistance to *R. solanacearum* was more efficient than that to *Alternaria alternata* (Peng et al., 2004). These indicate that difference in efficiency may be not originated from identity or types of pathogens. What causes these differences still remains to be determined.

To determine if Hpa1-mediated resistance to plant pathogens is stably inherited to the next generation, 5 different T2 transgenic *Arabidopsis* lines were generated from T1 plants. Two T2 lines were generated from T1 plants that showed resistance to both *B. cineria* and *X. campestris* pv. *campestris* (R/R type), and three lines were generated from T1 plants that showed resistance to *B. cineria*, but susceptible to *X. campestris* pv. *campestris* (R/S type). 5 plants per each line were inoculated with either *B. cineria* or *X. campestris* pv. *campestris*. In the case of T2 lines originated from R/R type T1 plants, all of 5 plants showed resistance to *B. cineria* (Fig. 3B). However, only 2 plants showed resistance to *X. campestris* pv. *campestris* (Fig. 3C). *hpa1* gene was well expressed in these two T2 plants (Fig. 3D). In the case of T2 lines originated from R/S type T1 plants, 3 to 5 out of 5 plants showed resistance to *B. cineria*, but all of them showed susceptible to *X. campestris* pv. *campestris*. These results indicate that Hpa1-mediated resistance is stably inherited to the next generation, particularly in the R/R type for *B. cineria*.

Expression pattern of defense-related genes in transgenic rice plants. We showed that expression of Hpa1 protein enhanced resistance to both bacterial and fungal pathogens in rice. To examine which defense pathway(s) might be

Fig. 4. Expression of defense-related genes in transgenic rice (AP42), compared to that in the non-transformed rice (NT) at the indicated time points after inoculation of *X. oryzae* pv. *oryzae* strain KXO18 by RT-PCR. Actin gene was used as an internal control.

Table 3. Locus numbers of rice genes that are up-regulated by treatment of *X. oryzae* pv. *oryzae* strain KXO18 in AP42 T0 transgenic rice, compared to the wild type rice

Locus #	Homologous genes or proteins	Fold change
NIAB5210	<i>Oryza sativa</i> 1-aminocyclopropane-1-carboxylate synthase (<i>OsACS1</i>)	5.19
NIAB3303	<i>Zea mays</i> thiamine biosynthetic enzyme (<i>thi1-1</i>)	4.95
NIAB2023	<i>Arabidopsis thaliana</i> At5g40630	4.43
NIAB2343	<i>Nicotiana sylvestris</i> chloroplast 29 kD ribonucleoprotein	4.25
NIAB2262	<i>Arabidopsis thaliana</i> putative sorbitol dehydrogenase (At5g51970)	4.12
NIAB2208	<i>Arabidopsis thaliana</i> At1g54320	4.06
NIAB0534	<i>Zea mays</i> tonoplast membrane integral protein ZmTIP4-2	3.65
NIAB4320	<i>Arabidopsis thaliana</i> At2g16590	3.49
NIAB3529	<i>Arabidopsis thaliana</i> putative arginine/serine-rich protein (At1g16610)	3.43
NIAB5211	<i>Oryza sativa</i> Ran	3.33
NIAB2097	<i>Arabidopsis thaliana</i> At5g25754	3.24
NIAB0219	<i>Oryza sativa</i> glycine-rich protein (<i>OsGRP1</i>)	3.11

responsible for Hpa1-mediated disease resistance in rice, five of previously known defense-related genes, *OsPR1*, *OsPAL*, *OsCHS*, *OsPOX*, and *OsPRI0* were checked by RT-PCR in transgenic rice before and after inoculation with *X. oryzae* pv. *oryzae* strain KXO18. Although *PR1* and *OsCHS* genes looked more expressed in transgenic rice than in the non-transformed plant at 0 hr, none of them showed any significant difference in gene expression (Fig. 4).

In order to examine any genes that are up- or down-regulated after inoculation with *X. oryzae* pv. *oryzae* strain KXO18, we performed microarray experiment with a DNA chip carrying 5,000 random rice genes. From this analysis, at least 12 up-regulated genes, which were shown over 3-fold difference, were found, compared to those in the wild type rice plants (Table 3). All of them except *OsACS1* gene, which showed the highest induction level, did not show strong connection to disease resistance. Therefore, expression pattern of *OsACS1* gene after inoculation with *X. oryzae* pv. *oryzae* strain KXO18 was determined. Consistent with microarray data, its expression was induced about 3 hrs after inoculation in the transgenic rice plant, compared to about 24 hrs in the non-transformed rice plant (Fig. 5). *ACS1* gene encodes 1-aminocyclopropane-1-carboxylate synthase (ACC synthase), which is known as a critical enzyme for ethylene biosynthesis in plants, and five *ACS* genes are present as a multigene family in rice (Zarembinski and Theologis, 1997). These results indicate that ethylene-mediated pathway might be involved in Hpa1-mediated disease resistance.

The relationship between harpin function and ethylene-mediated pathway has been reported in diverse plant species, including tomato and *Arabidopsis*. By microarray analysis in tomato, expression of *SIERF1* gene encoding ethylene-response factor 5 was shown to be induced to the highest level, when HrpN of *E. amylovora* was treated (Chuang et

Fig. 5. Expression of rice *OsACS1* gene, encoding 1-aminocyclopropane-1 carboxylate synthase in transgenic rice. Rice transgenic plants (AP42) were inoculated with *X. oryzae* pv. *oryzae* strain KXO18, and leaves were collected at the designated time points after pathogen inoculation, and northern blot analysis was carried out with the indicated genes as probes. Ribosomal RNAs (rRNAs) were shown by ethidium bromide staining as loading controls. NT, non-transformed.

al., 2010). In addition, treatment with the same harpin in *Arabidopsis* increases ethylene level, and the ethylene receptor gene *ETR1* was required for harpin-induced plant growth enhancement and insect resistance because these effects were impaired in the *etr1-1* mutant (Dong et al., 2004). Recently, transcriptome profiling was carried out in cotton carrying *hpa1* gene, and it showed that expression of genes involved in multiple defense signaling pathways, including and ethylene-mediated defense pathways, was induced (Miao et al., 2010).

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