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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(Received on September 4, 2012; Revised on September 23, 2012; Accepted on September 23, 2012)

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 OsACS1 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 monocalot and dicot, can enhance disease resistance to both fungal and bacterial plant pathogens.

Keywords: bacterial leaf blight pathogen, defense response, harpin, OsACS1 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 homologs 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 *PRI* 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 *hpn* expression under Cauliflower mosaic virus (CaMV) 35S promoter in *Arabidopsis* induces expression of *PRI* gene by activating SAR (Dong et al., 1999), and also that *hpal* 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 *hpal* expression in cotton showed that genes responsible for multiple defense signaling pathways are constitutively expressed (Miao et al., 2010).

In this study, *hpal* gene of *X. oryzae* pv. *oryzae* strain KXO85 was transformed in rice and *Arabidopsis* and was expressed under a constitutive promoter (*act1* promoter in rice or CaMV 35S promoter in *Arabidopsis*). In these transgenic rice and *Arabidopsis*, the effect of *hpal* expression on disease resistance to both bacterial and fungal pathogens was examined. Here, we show that expression of *hpal* gene increases disease resistance to both bacterial and fungal pathogens in both rice and *Arabidopsis*, and expression of rice *OsACS1* gene was induced in the *hpal*-transformed rice plant.

**Materials and Methods**

**Bacterial and fungal strains and growth conditions.** *Escherichia coli* strains, DH5α and Top10 (Invitrogen, USA) and *Agrobacterium tumefaciens* strains GV3015 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 µg/ml; kanamycin (Km), 50 µg/ml; ampicillin (Ap), 50 µg/ml. The fungal pathogens, *Magnaporthe grisea* and *Botrytis cinerea* 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 *hpal* gene constructs for plant transformation, 0.42-kb DNA fragment carrying the full length *hpal* 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'-GGATCCGGATCCTCTTGGTACACAC-3' with BamHI site) and the reverse primer (5’-TCTAGATGGCGGTAGGGCGG-3’ with XbaI site). The PCR product was inserted into pCR2.1 (Invitrogen, USA) to produce pMS-*hpa1*TOPO. The *hpal* gene from pMS-*hpa1*TOPO was transferred into the *BamHI*-digested pBluescriptII KS(+) vector to produce pMS-*hpal1*.

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 *EcoRV*-XbaI DNA fragment, including the *act1* promoter and *hpal* 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 HindIII-BamHI DNA fragment of pBI221 (Clontech, USA), including the 35S promoter, was ligated into HindIII-BamHI digested pMS-*hpal* vector to produce pMS-p35S::*hpa1*. Then, p35S::*hpal* 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. tum-