J Plant Biotechnol (2016) 43:422–431
DOI:https://doi.org/10.5010/JPB.2016.43.4.422

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Overexpression of rice premnaspirodiene oxygenase reduces the infection rate of *Xanthomonas oryzae* pv. *oryzae*

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Received: 1 November 2016 / Revised: 3 November 2016 / Accepted: 3 November 2016
ⓒ Korean Society for Plant Biotechnology

Abstract Plants utilize cytochrome P450, a large superfamily of heme-containing mono-oxygenases, in the synthesis of lignins, UV protectants, pigments, defense compounds, fatty acids, hormones, and signaling molecules. Despite the overwhelming assortment of rice P450 accession numbers in the database, their functional studies are lacking. So far, there is no evidence involving rice P450 in disease immunity. Most of our understanding has been based on other plant systems that are mostly dicot. In this study, we isolated the cytochrome P450 (*OsCYP71*) in rice, and screened the gene using gain-of-function technique. The full-length cDNA of *OsCYP71* was constitutively overexpressed using the 35S promoter. We then explored the functions of *OsCYP71* in the rice - *Xanthomonas oryzae* pv. *oryzae* pathosystem. Using the gene expression assays, we demonstrate the interesting correlation of PR gene activation and the magnitude of resistance in P450-mediated immunity.

Keywords Cytochrome P450, *Premnaspirodiene oxygenase*, Rice, *Xanthomonas oryzae* pv. *oryzae*

Introduction

Pathogens invading the host system more often than not perturb innate signal network which eventually leads to activation of defense. *Xanthomonas* species tried to sneak in without being compromised by employing a combination of different protein secretion systems such as T2S, T5S, and the major virulence effectors known as the T3S system proteins (*AvrXa7*, *AvrXa10*, *PthX06,* and *PthX07*) (Buttner and Bonas 2010). However, a number of *Xa* resistance genes have been reported taking part in an efficient perception of PAMPs or effectors such in the case of *Xa21* and *Xa3/Xa26*, both an LRR receptor kinase-type genes (Eulgem and Somssich 2007). In the case of WKRY-mediated immunity, invasion of pathogen often leads to modulation of endogenous signal molecule salicylic acid which eventually triggers systemic acquired resistance (Yu et al. 2001). OsWRKY45 was reported to induce the expression of pathogenesis-related (*PR*) genes resulting in enhanced resistance to *Pseudomonas syringae* (Qiu and Yu 2009).

PR proteins, which are classified into 17 families, have been frequently used as markers for systemic acquired resistance in plants after an accumulating evidence of their activation after pathogen infection (van Loon et al. 2006). *PR* gene expression in rice is correlated with developmentally controlled *Xa21*-mediatd resistance against *Xoo* (Ponciano et al. 2007). It was also reported to respond against *Magnaporthe grisea* (Jwa et al. 2001).

Cytochrome P450 (CYP) enzymes are a superfamily of heme-containing mono-oxygenases involved in the oxidative degradation of various compounds, and which show diverse reaction chemistry (Danielson 2002). It is a large gene family widely distributed in plants, animals, bacteria and fungus (Lamb et al. 2009). Generally in plants, P450s function in a multiple biosynthesis reactions that lead to fatty acid conjugates, plant hormones, secondary metabolites, lignin, a variety of defense compounds, and signaling molecules (Schuler and Werck-Reichhart 2003). Because of their roles in metabolic processes, plant P450 proteins and transcripts can act as

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downstream reports for many different biochemical pathways responding to chemical, developmental, biological, and environmental cues (Schuler and Werck-Reichhart 2003). Some members of this gene family lead to the synthesis of plant defense compounds including psoralen derivatives that inhibit replication (Berenbaum 1991), monoterpene indole alkaloids that deters microtubule formation and cell division (Kutchan 1995), and DIMBOA that inhibits protease and oxidative enzymes in fungi, bacteria, and insects (Niemeyer 1988).

Due to their functions in hydroxylation of large array of secondary compounds, P450s in plants have duplicated and diverged to an unprecedented degree (Schuler and Werck-Reichhart 2003). The completion of rice genome sequencing aids in the assortment of several hundreds of functional P450s and pseudogenes to databases. Protein sequence analysis and classification database such as Interpro keeps a record of P450 proteins especially in model organisms. In rice alone, about 980 accessions are recorded, there are 711 in Arabidopsis, 438 in human, and 258 in Zebra fish, among others. However, despite the overwhelming lists of P450 proteins particularly in rice, functional studies are lacking. Some are only named to the family level and only very few have been assigned into subfamilies due to complexities in classification. The response of different P450 loci to various stresses would provide means of classifying these subfamily members and assigning them to possible biochemical pathways and/or stress response cascades (Schuler and Werck-Reichhart 2003).

In this study, we cloned the *Oryza sativa* cytochrome P450 71 (*OsCYP71*) that we previously identified from our *Xanthomonas oryzae pv oryzae*-induced microarray data set. The full-length cDNA was overexpressed into the genome of wild type, Dongjin, which is susceptible to bacterial blight Korean strain K2. To gain additional insights on biological function of *OsCYP71* in plants, we evaluated the resistance of *OsCYP71*-overexpression rice lines against *Xanthomonas oryzae* pv. *oryzae* and determined the participation of downstream defense-related key genes such as PR proteins.

Materials and Methods

Isolation of *OsCYP71* gene

Total RNA was isolated from the young leaves of *japonica* rice *cv.* Jinbaek using the RNAiso Plus extraction reagent (Takara Bio Inc. Tokyo, Japan). The first stand cDNA was synthesized from 1 µg of high quality total RNA in a 20-µl reaction volume using Superscript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). cDNA was used to amplify the target gene, *OsCYP71*, using the primer pair, (Fw) ATGGACGAGCTCTTCTACCAGTC and (Rv) CTAATTGGCAGAGACCACAGGGA. PCR conditions employed to amplify the target gene were as follows: denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR product was electrophoresed on 1% (w/v) agarose gel stained with ethidium bromide. The expected amplicon with the right band size was eluted using the gel purification kit (Bioneer, CA, USA). The purified PCR product was sub-cloned into pGEMT-easy vector (Promega, Madison, USA) and sent to Macrogen Inc. (Macrogen, Seoul, Korea) for sequence analysis. Sequence homology was verified using multiple sequence alignment with hierarchical clustering. The full-length cDNA sequence of *OsCYP71* was submitted to NCBI which was then assigned with a GenBank accession number *KY366226*.

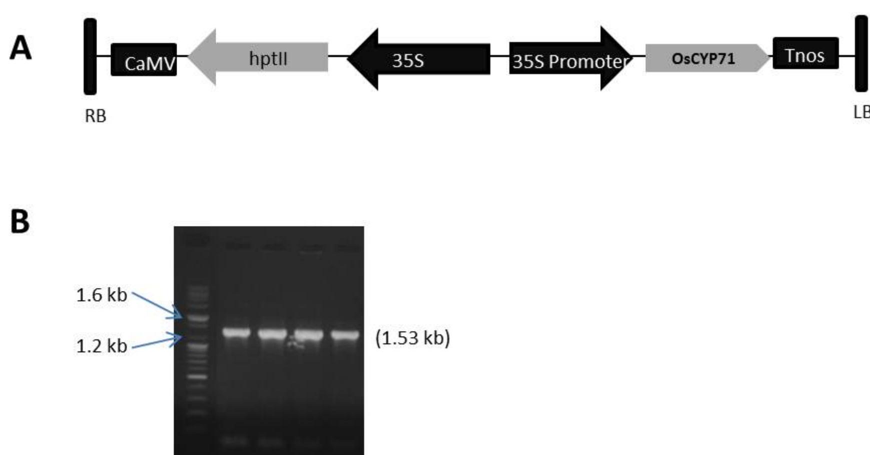

Fig. 1 A. Vector map construct of *OsCYP71* integrated into pCAMBIA1300; B. Colony PCR of recombinant *Agrobacterium* strain EHA105 containing *OsCYP71*

Vector construction and rice transformation

The full-length cDNA of *OsCYP71* was ligated to KpnI and XbaI sites of pCAMBIA1300 (Fig. 1A) using the T4 ligase kit (Promega, Madion, USA). Ligated product was transformed into *Agrobacterium tumefaciens* strain EHA105 (Fig. 1B), cultured on AB medium containing 50 mg/L kanamycin sulfate solidified with 1.5% microagar for three days at 28°C in the dark. *Agrobacterium* culture was harvested and suspended in AAM medium to yield an OD_{600} of 0.01. Ten-day-old cultured rice seed calli were immersed in the *Agrobacterium* suspension by gently inverting the tube for 3 min and blotted dry. The inoculated calli were placed on 2N6-AS medium solidified with 0.4% plant agar.

After two days of co-cultivation at 25°C in the dark, calli were washed with sterile distilled water treated with 500 mg/L carbenicillin. The calli were blotted dry and planted on 2N6 medium treated with 50 mg/L hygromycin and 400 mg/L carbenicillin under continuous light at 32°C for two weeks. Proliferating calli were transferred to MSR medium for shoot and root induction. Regenerated plants were acclimatized for two weeks and transplanted to soil (a mixture of 50% composed peat and 50% earth soil) in the greenhouse.

DNA extraction and genomic PCR

Genomic DNA was extracted as described in Cho et al. (2007) with some modifications. The relative purity and concentration of extracted DNA was estimated using NanoDrop-One (Thermo Scientific). To verify successful integration of transgenes, PCR assay was performed using the Takara ExTaq (Takara, Shiga, Japan) with the *OsCYP71*-specific primers. The target gene was amplified using the following conditions: denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min. The PCR product was electrophoresed on 1% (w/v) agarose gel stained with ethidium bromide and viewed under UV light.

Copy number assay using TaqMan qPCR

To detect and measure transgene copy number variation in transgenic rice, TaqMan® copy number assay was performed using TaqMan® reagents (Applied Biosystems, CA USA). Briefly, 10 ng of purified genomic DNA obtained from T_0 plants was combined with a FAM™ dye-labeled MGB probe to detect the genomic DNA target sequence, VIC® dye-labeled TAMRA™ probe to detect the genomic DNA reference sequence, and TaqMan Genotyping Master Mix which contains AmpliTaq Gold® DNA polymerase, ultrapure, and dNTPs. All qPCR reactions were run in two replicates on an ABI 7900HT instrument (Applied Biosystems, CA USA) using the following conditions: enzyme activation at 95°C for 10 min, denaturation at 95°C for 15 sec, and annealing at 60°C for 60 sec. After amplification, data files were analyzed using CopyCaller® Software v 2.0 (Applied Biosystems, CA USA). The number of copies of the target sequence in each test sample is determined by relative quantitation (RQ) using the comparative C_T ($\Delta\Delta C_T$) method. This method measures the C_T difference (ΔC_T) between target and reference sequences then compares the ΔC_T values of test samples to a calibrator sample known to have two copies of the target sequence. The copy number is calculated to be two times the relative quantity.

Gene expression analysis

To check the spatio-temporal expression of *OsCYP71*, total RNAs were extracted from different organs, growth stages, and different time points following bacterial inoculation in the genetic source cultivar Jinbaek and the wild type background Dongjin. Rice tissues were sampled from roots, stem, leaf sheath, leaf, and flag leaf. Tissues were also sampled from a germinating seeds, 3-leaf stage seedling, plants at maximum tillering, and panicle initiation stage. For determination of transcript levels of pathogenesis-related (*PR1a, PR1b, PR1c*) genes, leaf tissues were collected from the transgenic and wild type plants after infection with *Xoo*. Total RNAs and cDNA synthesis were performed as described above. qRT-PCR mixture was prepared using KOD SYBR® qPCR kit (Toyobo Life Science, Japan) and *OsCYP71*-specific internal primers. Ubiquitin was used as internal control to normalize the results of the quantitative RT-PCR reaction. Reactions were done in triplicates.

Bacterial blight screening

Newly generated T_0 transgenic plants were acclimatized in greenhouse and transplanted in the field after 28 days. Plants were allowed to grow until eight weeks prior to inoculation. Bacterial inoculum suspension was prepared from a 48 hr-old culture of *Xanthomonas oryzae* pv. *oryzae* (Korean isolate K2) grown on a plated peptone sucrose agar (PSA) medium and adjusted to 1 x 10^8 cfu/ml (Niño et al. 2015) The plants were inoculated by leaf-clipping method. Since the plants are in T_0 generation, replications are employed by inoculating five leaves per plant. Control plants were provided including the wild type background ‘Dongjin’ (susceptible) and resistant check ‘Jinbaek’. Lesion length was measured at 8, 12, 16 days after inoculation (DAI) in centimeter. The mean lession was interpreted using the standard evaluation system (SES IRRI,

1996) with the following scale: 0.0 ~ 5.0 cm (Resistant, R), 5.1 ~ 10.0 (Moderately resistant, MR), 10.1 ~ 15.0 (Moderately susceptible), ≥ 15.1 (Susceptible, S).

In silico analysis of deduced AA sequence of *OsCYP71*

The target gene sequence was verified using the BLAST program in NCBI database. Alignment of the deduced *OsCYP71* and other *CYP71* genes in plants was performed by ClustalX program (Thompson et al. 1997). The phylogenetic tree was constructed with MEGA program v 7.0 employing the neighbor-joining (NJ) method with 1000 bootstraps.

The presence or absence of N-terminal signal peptides was predicted by SignalP 4.0 (Petersen et al. 2011), Phobius (Käll et al. 2007), and PrediSi (Hiller et al. 2004). Degree of hydrophobicity was determined using ProtScale (Kyte and Doolittle 1982).

Results

Sequence, phylogenetic, and structural analyses of *OsCYP71*

KY366226 has an open reading frame (ORF) of 1,530 bp which encodes a polypeptide of 509 amino acid residues with a predicted molecular mass of 56.50223 kDA and a theoretical isoelectric point of 9.2397 (Fig. 2A). Phylogenetic analysis based on multiple sequence alignment and conserved

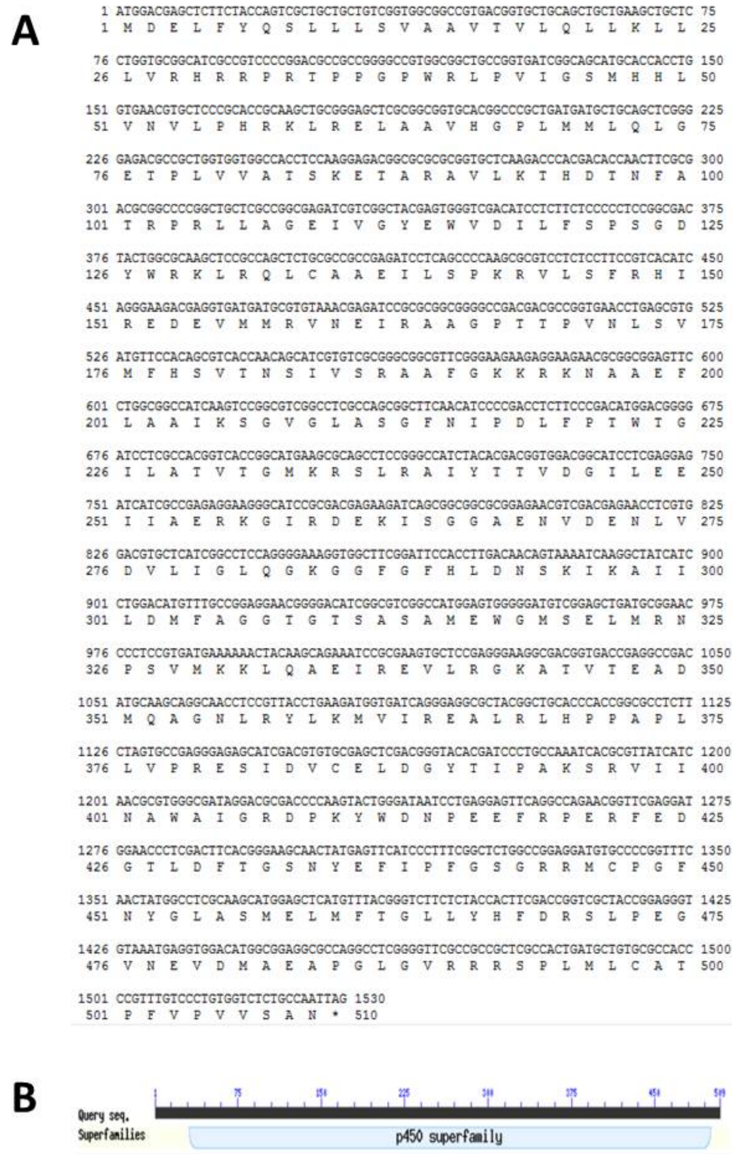

Fig. 2 Nucleotide and amino acid sequence of the full-length cDNA of KY366226 (*OsCYP71*). A. The full-length cDNA of *CYP71* is 1,530 bp in length, encoding a polypeptide of 509AA with a MW of 56.50223 kDa. B. Conserved domain of *OsCYP71*

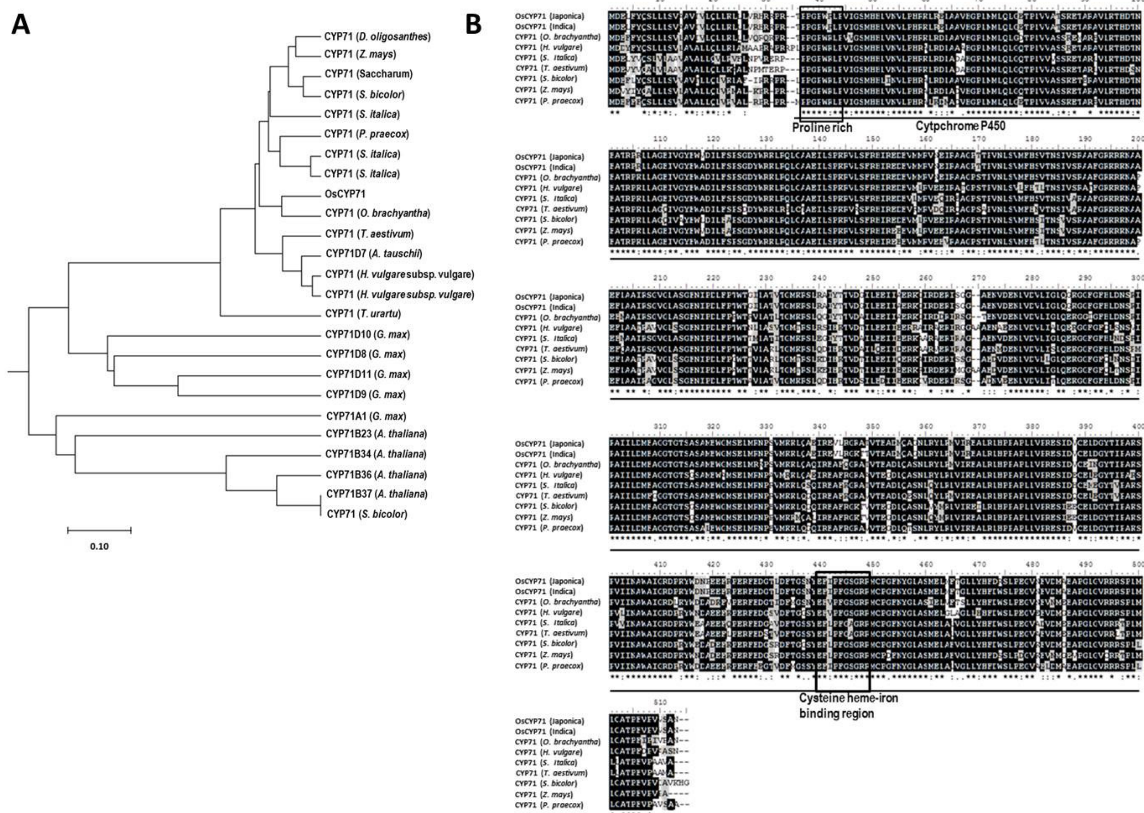

Fig. 3 A. Phylogenetic relationship of *OsCYP71* with other members of CYP71 in other plant species. The evolutionary distances were computed using the neighbor-joining method, and are in the units of the number of amino acid substitutions per site. The analysis involved 25 amino acid sequences. Evolutionary analyses were conducted in MEGA7. B. Alignment of OsCYP71 amino acid sequences with those of closely related proteins in other plant species

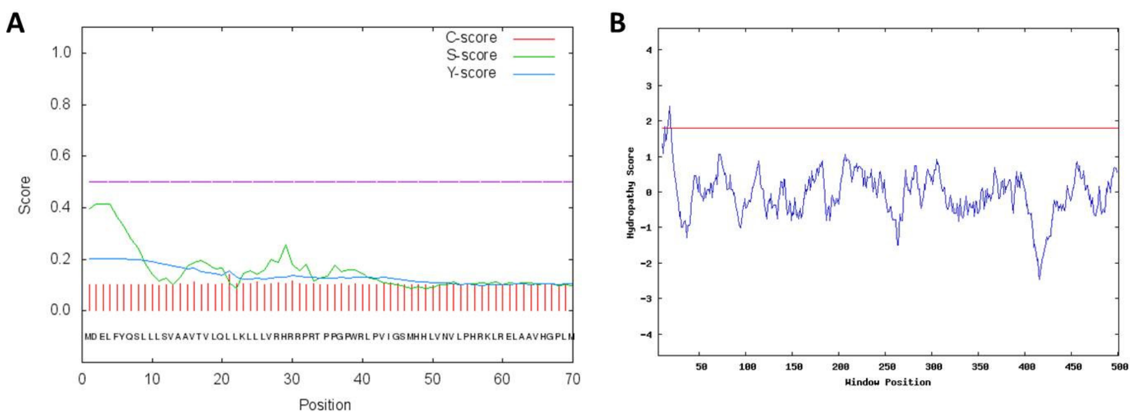

Fig. 4 A. Graphical output of SIgnalIP for predicting signal peptide in N terminal (1-70AA) of OsCYP71 protein. The neural networks produce three output scores for each position in the input sequence: C-score distinguishes signal peptide cleavage sites from everything else; S-score distinguishes positions within signal peptide from positions in the mature part from proteins and proteins without signal peptide; Y-score represents combined cleavage peptide score. B. Kyte-Doolittle hydrophobicity plot. Peaks with scores greater than 1.8 (red line) indicate possible transmembrane regions. The Window Position values shown on the x-axis of the graph reflect the average hydropathy of the entire window, with the corresponding amino acid as the middle element of that window

domain proteins predicted the gene to encode premnaspirodiene oxygenase, a cytochrome P450 enzyme (Fig. 2B). Gene ontology assigned this gene to function in oxidation-reduction process and heme-iron binding.

Smart blast search analysis of the deduced amino acid of OsCYP71 revealed high homology (88%) with XP006660483.1, a *CYP71* gene, from its distant relative species *Oryza branchyantha*. Phylogenetic tree (Fig. 3A) for subfamily

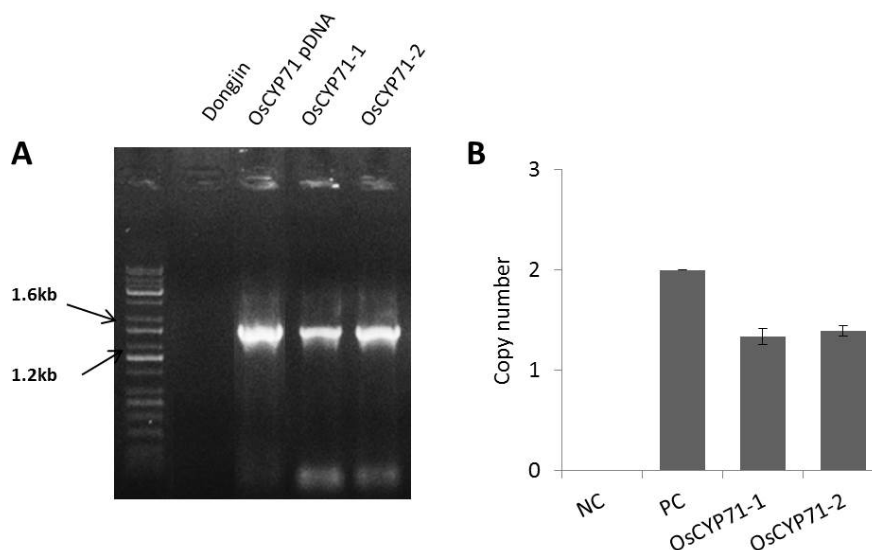

Fig. 5 A. Molecular characterization of overexpression plants. A. Genomic PCR assay for the presence of *OsCYP71*. B. Copy number of T_0 *OsCYP71* overexpression rice plants assayed using TaqMan real-time PCR; NC=negative control, PC=double copy positive control

members of CYP71 showed three clusters. OsCYP71 fell under the group of unclassified CYP71 genes from different plant species including XP004981824.1 (*S. italica*), ACM69384.1 (*P. praecox*), XP 002441847.1 (*S. bicolor*), NP 001146497.1 (*Z. mays*), OEL32185.1 (*D. oligosanthes*), CDM81270.1 (*T. aestivum*), and BAJ89686.1 (*H. vulgare* subsp. vulgare). The second cluster comprises protein members of CYP71D subfamily, while CYP71A and CYP71B subfamilies compose cluster 2. Since KY366226 (OsCYP71) is more closely related to the unclassified subfamily protein members of CYP71, we referred the gene as *Oryza sativa cytochrome P450 71* (*OsCYP71*). Clustal alignment of amino acid sequence shows homology with other CYP71 genes in other plant species. Motif scan confirmed the inclusion of cysteine heme-iron ligand signature at the C terminal of the protein which is highly conserved among cytochrome P450 enzymes (Fig. 3B).

To assess N-terminal signal peptides, three prediction methods including Phobius, PrediSi, and SignalIP were employed. All of them predicted that OsCYP71 does not contain signal peptide, while it is probable that this protein is either a transmembrane or non-secreted. Kyte-Doolittle scale for quantitative analysis of the degree of hydrophobicity of OsCYP71 indicates a strong single peak of positive value signifying alpha helix crossing the membrane. This hydrophobic amino acid chain region interacts with the phospholipid tails.

Generation of transgenic rice overexpressing *OsCYP71*

The full-length cDNA of *OsCYP71* was integrated into expression vector pCAMBIA1300 and overexpression was driven by the CaMV 35S promoter (Fig. 1A). The recombinant *Agrobacterium* (strain EHA105) was used to transfect rice calli. After *in vitro* screening and regeneration, we transplanted the plants into soil in the greenhouse. Successful integration of the gene was initially confirmed by genomic PCR (Fig. 5A). Using TaqMan qRT-PCR, we were able to obtain two independent, single copy lines (*OsCYP71-1, OsCYP71-2*) (Fig. 5B) which were established in the field for phenotypic analysis.

Spatio-temporal expression of *OsCYP71*

Real-time PCR analysis of *OsCYP71* in different tissues of rice revealed that the gene is tissue-specific (Fig. 6A). It is overexpressed in the leaf, flag leaf, and roots while almost no expression was noted in the stem and leaf sheath. Expression pattern in different growth stages of rice indicates high level of expression during late stages including maximum tillering and panicle initiation, while no trace of transcript was observed during germination and 3-leaf stage (Fig. 6B).

Expression of *OsCYP71* was induced in Dongjin (susceptible wildtype) and Jinbaek (resistant) following infection of *Xoo* strain K2. Almost the same pattern was observed in both cultivars although higher levels were evident in the resistant cultivar Jinbaek. In particular, elevation of transcript level peaked at 4 and 8 hours post inoculation (hpi) (Fig. 6C).

OsCYP71 confers resistance to *Xoo*

When the lesion length was plotted vs. observation time (Fig.

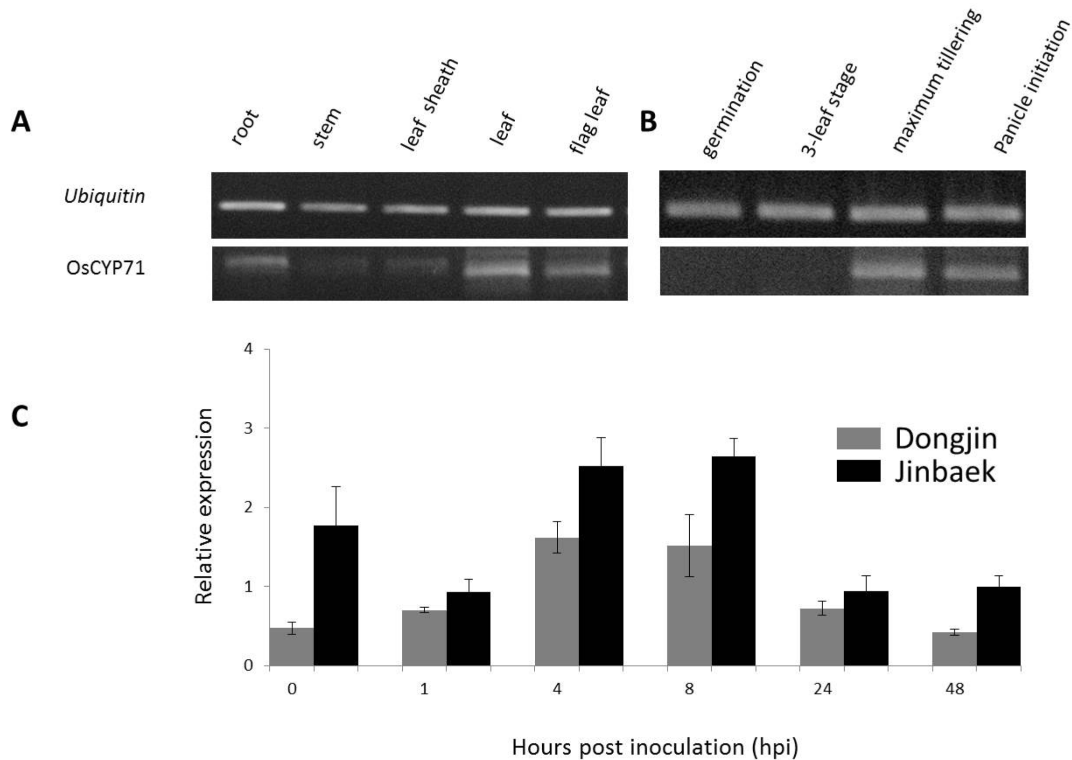

Fig. 6 mRNA expression of *OsCYP71*: A. in different organs, and B. in different growth stages of wild type Dongjin (susceptible). C. Transcript levels of *OsCYP71* in Dongjin and Jinbaek after infection of *Xoo* strain K2. Observation time: 0, 1, 4, 8, 24, 48 hpi

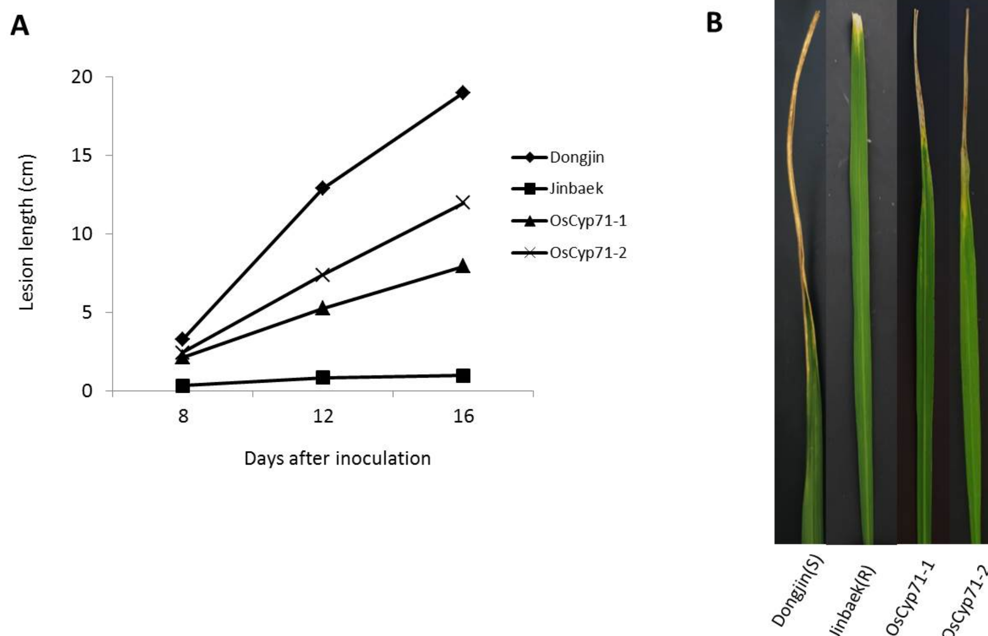

Fig. 7 Screening of overexpression plants against *Xoo*. A. Disease progression curve in transgenic and control plants plotted at 8, 12, and 16 days after infection (DAI). B. Phenotypic reaction of transgenic and control plants at 16 DAI

7A), a distinct trend is portrayed by different treatment plants. Resistant cultivar Jinbaek has the lowest area under the disease progress curve (AUDPC) signifying slowest disease progress, followed by *OsCYP71-1* and *OsCYP71-2*. These transgenic lines showed smaller disease progress curves than the susceptible wild type Dongjin.

After 16 days of infection, the two transgenic plants significantly reduced the lesion length compared with Dongjin

Table 1 Mean lesion length of bacterial blight in the leaves of transgenic (*OsCYP71-1*,-2) and control (Dongjin-S, Jinbaek-R). R=Resistant, MR-Moderate resistant, MS= Moderate susceptible, S=Susceptible

Lines	Mean±SD	SES (Index)
Dongjin	19.0±1.66	S
Jinbaek	1.0±0.04	R
OsCYP71-1	8.0±1.27	MR
OsCYP71-2	12.0±0.86	MS

(Table 1, Fig. 7B). Specifically, the mean lesion length in Dongjin (19 cm) is reduced almost by half in *OsCYP71-1* (8 cm). Using the disease index scale (SES 1996), *OsCYP71-1* is moderately resistant to *Xoo* strain K2.

Involvement of *PR* genes in *OsCYP71*-mediated immunity

Transcriptional regulation of pathogenesis related (*PR*) genes were likely induced by *Xoo* in *OsCYP71*-mediated resistance as shown in Figure 8. Transcript levels of the three PR genes were elevated in the *OsCYP71-1* while only PR1c was induced in *OsCYP71-2*. Looking at the overall pattern, differences in expression levels of PR genes among the transgenic plants correlated with the magnitude of resistance conferred by *OsCYP71*. It was very clear that these PR genes are not activated in the wild type.

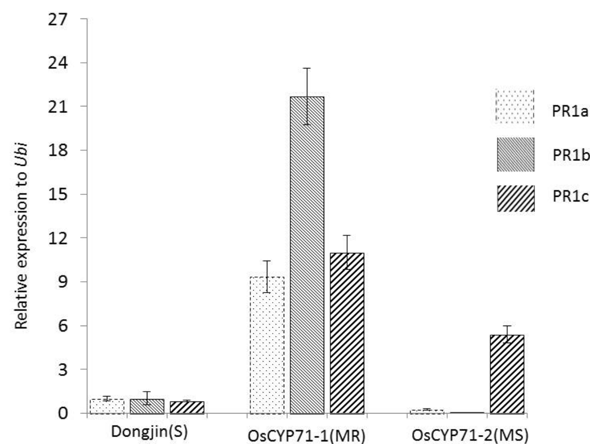

Fig. 8 Transcript level of *OsPR* genes (*PR1a*, *b*,*c*) in the transgenic and wild type plants inoculated with *Xoo* strain K2

Discussion

Prior to this study, we have identified *OsCYP71* from our cDNA microarray data set to be differentially induced by *Xanthomonas oryzae* pv. *oryzae* strain K2 (unpublished data). OsCYP71 (GenBank accession number KY366226) is predicted to encode premnaspirodiene oxygenase, a cytochrome P450 enzyme, bound to function in heme-iron binding and oxidoreductase activity. Phylogenetic analysis of its deduced amino acid sequence revealed close relationship with the unclassified subfamily members of P450 proteins under CYP71 thus the name *OsCYP71*. P450s are encoded by a highly divergent gene superfamily that maintains secondary and tertiary structural conservations but relatively few primary sequence conservations (Schuler and Werck-Reichhart 2003). Using three signal peptide prediction methods, the gene was consistently predicted to be devoid of signal peptide sequence in its N-terminus, and that it is likely a transmembrane or non-secretory protein. This corroborates the positive value peak in hydrophobicity plot which signifies the presence of non-polar amino acid side chain integrated in the phospholipid bilayer, a characteristic of a membrane protein. Most classical plant P450s, according to Schuler (1996), are localized with a single N-terminal transmembrane helix anchoring the protein in the endoplasmic reticulum (ER) membrane and the remainder of the protein in the cytosol.

We cloned the gene from the rice cultivar Jinbaek which is highly resistant to *Xoo* and overexpressed in the genome of wild type Dongjin which is susceptible to bacterial blight. Clustal alignment of the nucleotide base sequence of the gene in Jinbaek with the reference Nipponbare revealed a single nucleotide polymorphism which caused an amino acid change TGG(Trp) → CGG(Arg).

Functional screening of genes using gain-of-function is an effective technique for unbiased functional exploration of the genome. We generated independent, single copy transgenic rice plants overexpressing *OsCYP71* and the plant selection marker *hygromycin phosphotransferase* (hpt) gene using the strong constitutive 35S promoter. Spatio-temporal expression profiling of the gene in the wild type rice revealed that *OsCYP71* is tissue-specific. It is expressed in the roots, leaves, and flag leaf. Moreover, high levels of transcripts are noted only during maximum tillering and panicle initiation stages implying that it is likely developmentally controlled. The same transcriptional characteristic has been reported for the model *Xa21*- *Xoo* pathosystem wherein *Xa21*-mediated resistance to *Xoo* is not fully expressed in early stages of development but showed increasing magnitude of resistance as the plant matures (Ponciano et al. 2007). Expression of *OsCYP71* peaked at 4 to 8 hours after infection of *Xoo* which indicates crucial involvement during early response.

Reduction in infection rate as depicted by shorter lesion is evident in the transgenic rice overexpressing *OsCYP71*. The rate may not as low as the resistant control but it is relatively lower than the wild type Dongjin implying that *OsCYP71* confers a partial resistance to *Xoo*. This, in part, indicates the

inhibiting role of premnaspirodiene oxygenase against *Xoo*. This enzyme has been reported to catalyze regio- and stereo-specific hydroxylations of diverse sesquiterpene substrate resulting in solavetivone, a potent antifungal phytoalexin (Takahashi et al. 2007). Members of this family of compounds are known to inhibit mycelial growth of *Phytophthora infestans* (Engstorm et al. 1999) and *Rhizoctonia solani* (Yao et al. 2003), spore germination of *Fusarium oxysporum* (Yokose et al. 2004), and the growth of *Staphylococcus aureus* and *Bacillus substilis* (Kuroyanagi et al. 1999).

PR genes have been frequently used as marker genes of systemic acquired resistance in many plants (Mitsuhara et al. 2007). In our study, we inferred the involvement of PR genes in *OsCYP71*-mediated immunity at transcription level by real time-PCR. Three members of *PR1* gene family namely *OsPR1a*, *OsPR1b*, and *OsPR1c* were selected. The transcripts of the three were differentially expressed in two transgenic lines where in, all are highly induced in CYP71-1 plant while only *OsPR1c* elevated in CYP71-2. Considering the phenotypic reaction of the two overexpression plants against *Xoo* infection, differences in expression levels of PR genes among the transgenic plants seemed to correlate with the magnitude of resistance conferred by *OsCYP71*. Alexander et al. (1993) indicated that *PR1a* encodes putative acidic protein which inhibits oomycete infection in tobacco. Induction of *OsPR1a* and *OsPR1b* in rice was also noted against blast fungus infection (Jwa et al. 2001). Because of the roles of P450 in wide diversity of metabolic processes, plant P450 proteins and transcripts can serve as downstream reporters for many different biochemical pathways (Schuler and Werck-Reichhart 2003), and in our study we can speculate that *OsCYP71* may act as regulator of downstream defense-related genes such as OsPR proteins, although this requires further comprehensive genetic analysis for validation.

Acknowledgments

This work was supported by a grant (National Center for GM Crops No. PJ011245: Development of GM Event Line Enhanced Multiple Disease Resistance by Antimicrobial Peptide in Rice) of the Next-Generation BioGreen 21 Program funded by the Rural Development Administration, Republic of Korea. We are also grateful for the support from Chungbuk National University in 2014.

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