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Expression analysis of rice VQ genes in response to biotic and abiotic stresses

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ABSTRACT

WRKY transcription factors are encoded by a large gene superfamily with a broad range of roles in plants. Proteins containing a short *VQ* (FxxxVQxLTG) motif have been recently shown to interact with WRKY transcription factors, implying that AtVQ proteins are important in the plant defense responses in *Arabidopsis*, either as positive or negative cofactors of WRKY transcription factors. Thirty-nine *Oryza sativa* genes containing the VQ motif (*OsVQs*) were identified and the genome structures of OsVQ proteins were characterized through genome-wide analysis in rice. Also, phylogenetic tree analysis was performed with the VQ domain of *Arabidopsis* and rice. The expression patterns of these *OsVQ* genes in plants under several stress treatments were assessed, specifically, following infection with the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), treatment with abscisic acid (ABA), or exposure to drought. The cellular localization of a few OsVQ proteins was examined using rice protoplast system. Based on our results, we suggest that OsVQ proteins function as important co-regulators during the plant defense response to biotic and abiotic stresses.

1. Introduction

Rice (*Oryza sativa* L.) is an important crop on a global scale and particularly important in East Asia, but it is susceptible to environmental stress. These stresses include pathogen infection as well as abiotic environmental stresses such as cold and drought. Specifically, the bacterial rice pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which causes a blight disease, has caused major crop losses worldwide. Clearly, it would be beneficial to management practices of this important crop if its defense mechanism were well understood. We have used the rice–*Xoo* interaction as a pathosystem for examining molecular responses in the rice plant defense mechanism (Zhang and Wang, 2013).

Generally, plants resist pathogen infection through two different immune systems: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI is an early defense response in which the plant's pattern recognition receptors (PRRs) on the plasma membrane recognize the PAMP of an invader. ETI is a late defense response induced by interaction between plant resistance genes and pathogen effectors and is associated with programmed cell death called the hypersensitive response (Coll et al., 2011).

WRKY transcription factors possess a conserved WRKYGQK amino acid sequence and represent a large gene family in many monocotyledon and dicotyledon plants, including *Arabidopsis* (74 genes) and rice (109 genes) (Eulgem and Somssich, 2007; Eulgem et al., 2000; Feng et al., 2012; Ross et al., 2007). WRKY transcription factors have one or two WRKY domains and have been classified into three groups (Groups I–III) based on the homologous sequence of the WRKY domain (Zhang and Wang, 2005). They have been implicated in plant developmental processes; specifically, *OsWRKY72* may play a role in flowering and seed germination (Yu et al., 2010) and *OsWRKY23* was implicated in leaf senescence (Jing et al., 2009). WRKY transcription factors have also been reported to be involved in the defense response to many biotic and abiotic stimuli. For examples, *OsWRKY13, -31, -53*, and *-71* were implicated in response to pathogens *Xoo* and *Magnaporthe grisea* (Chujo et al., 2007; Liu et al., 2007; Zhang et al., 2008), *OsWRKY11* in response to drought stress (Wu et al., 2009), *OsWRKY89* in response to ultraviolet B irradiation (Wang et al., 2007), *OsWRKY24, 51*, and *72* in response to exogenous abscisic acid (ABA) (Xie et al., 2005).

VQ domain proteins contain a region of 57 amino acids containing a highly conserved "$F_{XXX}VQ_X$(L/V/F)TG" motif and interact physically with WRKY transcription factors. In *Arabidopsis*, both AtWRKY25 and AtWRKY33 are Group I WRKY transcription factors and form complexes with a VQ domain protein (MKS1, called AtVQ21), which interacted

Abbreviations: OsVQ, Oryzae sativa VQ domain protein; PAMP, Pathogen-associated molecular pattern; PTI, PAMP-triggered immunity; ETI, Effector-triggered immunity; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*; PCD, Programmed cell death; NLS, Nuclear localization signal; LRR, Leucine rich repeat domain; R gene, Resistance gene.

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with MAP kinase 4 protein (MPK4; Andreasson et al., 2005; Qiu et al., 2008). Another VQ domain protein, HAIKU1 (IKU1, called AtVQ14) functions in the regulation of endosperm and seed growth through its interaction with WRKY10 (Wang et al., 2010a). A calmodulin-binding protein, AtCAMBP25, called AtVQ15, functions as a negative regulator in the plant's response to osmotic stress (Perruc et al., 2004). Recently, two VQ domain proteins, SIGMA FACTOR-BINDING PROTEIN 1 and 2 (SIB1 and SIB2), were shown to interact with WRKY33 and were renamed AtVQ23 and AtVQ16, respectively (Lai et al., 2011; Xie et al., 2010). More recently, AtVQ9 was reported to interact antagonistically with AtWRKY8 in response to salt stress (Hu et al., 2013). Based on these reports, it seems that the interaction of the WRKY transcription factor with VQ domain proteins regulates the DNA binding activity of the transcription factors. *OsVQ22* was highly upregulated during infection of rice by the rice-blast fungus (*Magnaporthe oryzae*) as shown by RNA-Seq analysis (Kawahara et al., 2012). According to genome-wide expression data derived from the hybridization of Affymetrix GeneChip Rice Genome Arrays generated with 39 tissues from two rice *indica* varieties (Minghui 63 and Zhenshan 97) by Wang et al. (2010b), two *OsVQ* genes (*OsVQ7* and *OsVQ31*) had tissue-specific expression at the late stage of leaf development. *OsVQ18* and *OsVQ36* showed variety-specific expression in these varieties. Expression of the *OsVQ12* gene gradually decreased from early to late stages of panicle development.

In this study, we identified 39 VQ domain family genes in the rice genome and analyzed the phylogenetic tree with the VQ domain of *Arabidopsis* and rice. Next, we investigated expression profiles of rice VQ domain genes in the stress response. The compatible and incompatible interactions of *Xoo* bacteria and rice were applied to a biotic stress, while ABA treatment and drought stress were studied as abiotic stresses. We also observed the cellular localization of OsVQ proteins using a rice protoplast system. From our results, we suggest that rice VQ domain proteins have important roles in plant defense responses against a variety of stresses.

2. Materials and methods

2.1. Plant materials and growth conditions

Rice seeds (*Oryza sativa* L. cultivar Ilmi) were sterilized with 70% ethanol for 1 minute, incubated in a 50% bleach solution for 30 minutes, washed in distilled water five times, and sown on wet Whatman 3MM filter paper for 5 days in a plant growth chamber at 28 °C with a 14-h light 10-h dark photoperiod. After germination, rice seedlings were transferred into soil and grown for 3 weeks in a greenhouse.

2.2. Inoculation with pathogens and ABA and drought treatment

All treatments were applied to rice young plants at the 3-week-old stage. *Xanthomona. oryzae* pv. *oryzae* strain KACC10859 was used as the compatible strain and strain KXO97K1 was used as the incompatible strain. The bacterial cultures were grown on PSA medium (10 g/L peptone, 10 g/L sucrose, 1 g/L sodium-glutamate, and 15 g/L agar) for 2 days and suspended in 10 mM $MgCl_2$ to a final 0.2 OD_{600}. Plants were inoculated by stroking a toothpick that had been dipped in the bacterial suspension across a leaf. Leaves were harvested at 0, 6, 12, 24, and 48 h after inoculation. For ABA treatment, rice plants were grown on soil for 3 weeks in green house and then we sprayed on plant leaves with 100 μM ABA solutions in Tween 20 for 0 and 24 hours and finally harvested to isolate total RNA. For drought treatment, rice plants were grown on soil for 3 weeks in green house and washed whole plants with distilled water and then placed on the Whatman filter paper without watering for 0, 3, and 12 h and then harvested. All harvested samples were immediately frozen in liquid nitrogen and stored in a deep-freezer until use.

2.3. Sequence alignment and phylogenetic analysis

39 VQ domain genes from rice were retrieved from a rice genome information website (http://rice.plantbiology.msu.edu). Amino acid sequence alignment and phylogenetic tree analysis of the VQ domain regions from *Arabidopsis* and rice were performed from the CLUSTAL W alignments. The neighbor-joining method was used to make the phylogenetic tree.

2.4. Gene expression analysis

Total RNA was isolated from prepared samples using the Plant RNA Purification Reagent (Invitrogen, USA) and treated with RNase-free DNase I to remove contaminated genomic DNA. For reverse transcription, total RNA (1 μg) was used for the synthesis of first-strand cDNAs using RocketScript Reverse Transcriptase kit (Bioneer, Korea) following the manufacturer's protocol. Quantitative real-time RT-PCR was performed using AccuPower Greenstar qPCR master mix (Bioneer, Korea) in a MyiQ Real-Time PCR (Bio-Rad, USA). The expression levels of the *OsVQ* genes tested were calibrated with the constitutive expression level of *OsActin* and then calculated using the $2^{-\Delta\Delta t}$ method (Schmittgen and Livak, 2008). The *OsVQ* gene-specific primers used in this study are listed in Supplementary Table 1. The real-time qRT-PCR experiments were biologically repeated three times.

2.5. Subcellular localization assay

For cloning yellow fluorescent protein (YFP)-OsVQ fused constructs, we used the Gateway cloning system (Invitrogen, USA). The open reading frames (ORF) of *OsVQ11*, *OsVQ14*, and *OsVQ24* were amplified by PCR. The *OsVQs* PCR primer sets were used as follows: OsVQ11-For, 5′-AAAAAGCAGGCTCGATGGACGCCGCCTCCTGCGT-3′, OsVQ11-Re, 5′-AGAAAGCTGGGTATCAAACAACCTCGTCCTTGCC-3′; OsVQ14-For, 5′-AAAAAGCAGGCTCGATGACCATGACAGTGGCCAT-3′, OsVQ14-Re, 5′-AGAAAGCTGGGTATTAGAACAAGGGGCTGAGCT-3′; OsVQ24-For, 5′-AAAAAGCAGGCTCG-ATGGGTGAGTGCAGCTACAA-3′, OsVQ24-Re, 5′-AGAAAGCTGGGTATTAAGATG-CGTACATTTCACC-3′. The first PCR products were purified and then the second PCR was performed, using attB1 forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ and attB2 reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′ for the Gateway entry cloning. These PCR products were cloned into pDONR221 to make each entry clones using BP clonase (Invitrogen, USA). These entry clones were confirmed by sequencing. The YFP-fused constructs (35S-YFP-OsVQ11, 35S-YFP-OsVQ14, and 35S-YFP-OsVQ24) were finally constructed via LR reaction into pEarleyGate104 vectors (Keith et al., 2006). The fusion constructs and control construct were transformed into rice protoplasts to determine the cellular localization of these OsVQs proteins.

The isolation of rice leaf and stem protoplasts was performed as previously described (Zhang et al., 2011). Briefly, protoplasts were isolated from leaves and stems of 2-week-old rice plants. Plasmid DNA (15–30 μg) of YFP::OsVQ-fused constructs or NLS::red fluorescent protein (RFP) as a nuclear marker were transfected into 300 μL of protoplast mixture (1×10^6 protoplasts) using a PEG-mediated DNA transfection method. To allow expression of the introduced genes, protoplasts were incubated in the dark at room temperature for 20 h. The YFP/green fluorescent protein (GFP) and RFP fluorescence were observed using an Olympus IX71 confocal laser scanning microscope.

3. Results and discussion

3.1. Identification of VQ domain proteins in rice

To identify the VQ domain proteins in rice genome, we investigated the available sequence data from a rice genome information website (http://rice.plantbiology.msu.edu). We found 39 VQ domain-containing