The Rice Dwarf Virus P2 Protein Interacts with ent-Kaurene Oxidases in Vivo, Leading to Reduced Biosynthesis of Gibberellins and Rice Dwarf Symptoms

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The mechanisms of viral diseases are a major focus of biology. Despite intensive investigations, how a plant virus interacts with host factors to cause diseases remains poorly understood. The *Rice dwarf virus* (RDV), a member of the genus Phytoreovirus, causes dwarfed growth phenotypes in infected rice (*Oryza sativa*) plants. The outer capsid protein P2 is essential during RDV infection of insects and thus influences transmission of RDV by the insect vector. However, its role during RDV infection within the rice host is unknown. By yeast two-hybrid and coimmunoprecipitation assays, we report that P2 of RDV interacts with *ent*-kaurene oxidases, which play a key role in the biosynthesis of plant growth hormones gibberellins, in infected plants. Furthermore, the expression of *ent*-kaurene oxidases was reduced in the infected plants. The level of endogenous GA$_1$ (a major active gibberellin in rice vegetative tissues) in the RDV-infected plants was lower than that in healthy plants. Exogenous application of GA$_1$ to RDV-infected rice plants restored the normal growth phenotypes. These results provide evidence that the P2 protein of RDV interferes with the function of a cellular factor, through direct physical interactions, that is important for the biosynthesis of a growth hormone leading to symptom expression. In addition, the interaction between P2 and rice *ent*-kaurene oxidase-like proteins may decrease phytoalexin biosynthesis and make plants more competent for virus replication. Moreover, P2 may provide a novel tool to investigate the regulation of GA metabolism for plant growth and development.

Knowledge of viral disease mechanisms has fundamental importance in understanding the evolution of virus-host interactions, basic cellular functions, and engineering of host resistance. For plant viruses, viral symptom determinants have been mapped to specific viral proteins (Brigneti et al., 1998; Kong et al., 2000; Chellappan et al., 2005; Padmanabhan et al., 2005; Shepherd et al., 2005; Wang and Metzloff, 2005) and DNA/RNA sequences (Rodriguez-Cerezo et al., 1991; Fernandez et al., 1999; Dai et al., 2004). Changes in gene expression of infected plants (Whitham and Wang, 2004; Wang and Metzloff, 2005) and altered metabolism (Rogers, 2002) have also been reported. Recent studies suggest that viral suppressors of RNA silencing may also alter host microRNA metabolism that contributes to symptom development (Kasschau et al., 2003; Chen et al., 2004). In general, however, there is a lack of mechanistic insights about how a viral protein interacts directly with a specific host factor(s), thereby altering the function of a cellular pathway leading to disease development.

We use Rice dwarf virus (RDV) infection as a model system to address molecular mechanisms of viral diseases that have vital economic importance. RDV is a member of the genus Phytoreovirus, family Reoviridae (Boccardo and Milne, 1984). The genome of RDV is composed of 12 segmented double-stranded RNAs encapsidated within an icosahedral double-shelled particle having a diameter of approximately 700 Å (Zheng et al., 2000; Nakagawa et al., 2003). RDV can propagate in cells of host plants and insect vectors and is transmitted in nature by leafhoppers (*Nephotettix cincticeps* or *Recilia dorsalis*). RDV infects rice (*Oryza sativa*) plants systemically and is known to be one of the major viral diseases in rice in south Asia, including China and Japan (Boccardo and Milne, 1984).

RDV encodes at least seven structural proteins and five nonstructural proteins. The seven structural proteins, namely, P1, P2, P3, P5, P7, P8, and P9, are products of segments S1, S2, S3, S5, S7, S8, and S9, respectively (Mao et al., 1998; Hagiwara et al., 2003; Zheng et al., 2003; Miyazaki et al., 2005). The P2 and P8 proteins are components of the virion outer shell. Coexpression of the P3 and P8 proteins in transgenic plants provide a novel tool to investigate the regulation of GA metabolism for plant growth and development.

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Figure 1. Alignment of ent-kaurene oxidases or ent-kaurene oxidase-like proteins from rice, Arabidopsis, pumpkin, and pea. The alignment was made using DNAMAN (4.0). Identical (*) and conservatively substituted (.) amino acid residues are shown. The 66 amino acid residues identified in the yeast two-hybrid screening are underlined. The additional 17 amino acid residues in OsKOS1 but absent from OsKOL4 are indicated in the open box. The proteins can also be identified by their GenBank accession numbers.
rice plants or insect cells results in the formation of double-shelled virus-like particles (Zheng et al., 2000; Hagiwara et al., 2003). The five nonstructural proteins, namely, P4, P6, P10, P11, and P12, are encoded by the S4, S6, S10, S11, and S12 segments, respectively (Suzuki et al., 1996; Xu et al., 1998; Li et al., 2004; Cao et al., 2005). Functions of these six nonstructural proteins are not well understood, except that the Pns11 was reported to be a nucleic acid-binding protein, the Pns6 was identified as a viral movement protein, and Pns10 as a RNA silencing suppressor of RDV (Xu et al., 1998; Li et al., 2004; Cao et al., 2005).

The P2 protein was previously determined to be essential for RDV infection in its insect vectors and subsequent transmission to its host plants from these vectors (Yan et al., 1996; Tomaru et al., 1997; Omura et al., 1998). It was proposed that the P2 protein interacts with receptors encoded by the insect vector cells and this interaction was necessary for the recognition of virus particles by these insect cells (Omura and Yan, 1999). However, the viral proteins of plant viruses often have multiple functions (Callaway et al., 2001). After analysis of P2 protein in extracts from RDV-infected rice leaves and leafhoppers, Suzuki et al. (1994) concluded that the P2 protein was more abundant in rice leaves than leafhoppers. In addition, virus concentration in rice plants infected by a RDV transmission-defective (TD) isolate, in which the S2 segment contained an early termination codon in the 5' end of the open reading frame (ORF) resulting in no P2 protein, was much lower than that in rice plants infected by the RDV transmission-competent (TC) isolate (Tomaru et al., 1997). RDV TD isolate-infected rice plants showed a semidwarf phenotype compared with the dwarf phenotype caused by infection with the RDV TC isolate containing the wild-type S2 segment (T. Omura, personal communication). These results indicate that the P2 protein may have a function in the host plant to induce dwarfing. However, the mechanism by which the P2 protein induces the dwarf phenotype remains unknown.

The yeast two-hybrid technology is a proven tool for identifying protein-protein interactions that lead to an understanding of the function of the protein of interest (Chien et al., 1991; Causier and Davies, 2002). In our study, we used P2 protein as bait in yeast two-hybrid experiments to screen a rice cDNA library. These experiments led to the identification of an interaction between an ent-kaurene oxidase-like protein and P2. Our further investigation indicates that P2 can interact with three other ent-kaurene oxidases or ent-kaurene oxidase-like proteins from rice in yeast (Saccharomyces cerevisiae) cells, and, more importantly in plant cells, ent-kaurene oxidase is required during the biosynthesis of GA. We have determined that the endogenous GA1, the major active GA in rice vegetative tissues, was significantly reduced in rice plants infected with RDV. The dwarf phenotype in infected rice plants could be partially restored by supplying infected plants with GA3 (gibberellic acid). These findings suggest that the P2 protein interacts in vivo with an enzyme in the GA biosynthesis pathway, leading to diminished accumulation of GA and to the dwarf phenotype exhibited by RDV-infected rice plants.

RESULTS

Identification of an ent-Kaurene Oxidase-Like Protein That Interacts with RDV P2

To identify rice proteins that interact with the RDV P2 protein, we used a yeast two-hybrid system with RDV P2 as the bait to screen prey plasmids
representing a rice cDNA library. Seven positive colonies were identified among the approximately $2 \times 10^6$ cDNA clones that were screened. The cDNA fragments from all the seven colonies encode an identical polypeptide containing 66 amino acid residues (Fig. 1). Sequence analysis showed that the polypeptide shares a high degree of identity with rice ent-kaurene oxidases (OsKO1, BAD54595, 75% identity; and OsKO2, BAD54598, 75% identity), rice ent-kaurene oxidase-like proteins (OsKOL4, BAD54592, 100% identity; and OsKOL5, BAD54586, 84% identity), Arabidopsis (Arabidopsis thaliana) ent-kaurene oxidase (AtKO1, AAC39507, 53% identity), pumpkin (Cucurbita pepo) ent-kaurene oxidase (CmKO1, AAG41776, 58% identity), and pea (Pisum sativum) ent-kaurene oxidase (PsKO1, AAP69988, 60% identity).

Cloning of the Full-Length ORF Genes Encoding Rice ent-Kaurene Oxidases or ent-Kaurene Oxidase-Like Proteins

The full-length ORF of the rice ent-kaurene oxidase-like gene was cloned from rice through reverse transcription (RT)-PCR and primers designed based on sequence information available in the rice genome database (http://btjn.genomics.org.cn/rice). Sequence analysis of the coding region indicated that the ORF of the rice ent-kaurene oxidase-like gene contains 1,530 nucleotides and encodes a protein of 510 amino acids (Fig. 1). The cloned gene was subsequently named OsKOS1 (GenBank accession no. AY579214).

The deduced amino acid sequence of OsKOS1 was used to search the protein database for similar sequences using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The BLAST results show that there are at least four genes encoding ent-kaurene oxidases or ent-kaurene oxidase-like proteins in the rice genome. To determine whether P2 could interact with other ent-kaurene oxidases or ent-kaurene oxidase-like proteins, the cDNA fragments containing the full-length ORFs of rice cDNAs (GenBank accession nos. AK071743, AK066285, and AK100964) were cloned from rice by RT-PCR and named OsKOS2 (GenBank accession no. AY660664), OsKOS3 (GenBank accession no. AY660665), and OsKOS4 (GenBank accession no. AY660666). We compared the sequences of our cDNA fragments with the genes encoding ent-kaurene oxidases or ent-kaurene oxidase-like proteins cloned and analyzed previously (Itoh et al., 2004). The results of the amino acid sequences and gene structure comparison showed that OsKOS1 contained an additional 17 amino acid residues compared with OsKOL4, and these 17 additional amino acid residues also existed in OsKOL5, OsKO1, and OsKO2 (Fig. 1). Apart from 17 additional amino acid residues, OsKOS1 has 99.6% identity to OsKOL4 (BAD54592). The OsKOS2 sequence corresponds to OsKOL5 (BAD54586, 99.4% identity), OsKOS3 corresponds to OsKO2 (BAD54598, 99.6% identity), and OsKOS4 corresponds to OsKO1 (BAD54595, 99.0% identity; Fig. 1). Based on these analyses, we renamed OsKOS1, OsKOS2, OsKOS3,
and OsKOS4 as OsKOL4, OsKOL5, OsKO2, and OsKO1, respectively.

**P2 Interacts with Full-length Rice *ent*-Kaurene Oxidases and *ent*-Kaurene Oxidase-Like Proteins in Yeast**

To test whether P2 can interact with the four full-length rice *ent*-kaurene oxidases and *ent*-kaurene oxidase-like proteins, the cDNAs encoding the protein of interest were inserted in frame into the GAL4 DNA binding domain vector pGBK7 or GAL4 activation domain vector pGADT7 (CLONTECH), respectively. pGAD-OsKO1, pGAD-OsKO2, pGAD-OsKOL4, and pGAD-OsKOL5 encoding the fusions between the GAL4 activation domain and the respective *ent*-kaurene oxidases or *ent*-kaurene oxidase-like proteins were cotransformed with pGBK2 into yeast. Yeast cells cotransformed with constructs pGBK7/pGADT7, pGBK2/pGADT7, pGBK7/pGAD-OsKO1, pGBK8/pGADT7, pGBK8/pGAD-OsKO1, pGBK7/pGAD-OsKO2, pGBK7/pGAD-OsKOL4, and pGBK7/pGAD-OsKOL5 served as negative controls. The pGBK8 construct encodes one of the RDV outer capsid proteins. Only the yeast cells cotransformed with pGBK2/pGAD-OsKO1, pGBK2/pGAD-OsKO2, pGBK2/pGAD-OsKOL4, and pGBK2/pGAD-OsKOL5 were able to grow on the selective media (Fig. 2). These results established that P2 interacts specifically with OsKO1, OsKO2, OsKOL4, and OsKOL5 in yeast cells.

**P2 Interacts with Rice *ent*-Kaurene Oxidases and *ent*-Kaurene Oxidase-Like Proteins in Plant Cells**

Specific interaction of P2 with rice *ent*-kaurene oxidase and *ent*-kaurene oxidase-like proteins in yeast suggests functional significance. To test this further, we used coimmunoprecipitation to determine whether such interaction occurs in plant cells. As shown in Figure 3, the hemagglutinin (HA)-epitope-tagged P2 coimmunoprecipitated with the FLAG (synthetic octapeptide)-OsKO2 or FLAG-OsKOL4 after Agrobacterium-mediated transient expression in *Nicotiana benthamiana*. This interaction was confirmed with the reciprocal experiments, in which FLAG-OsKO2 and FLAG-OsKOL4 communoprecipitated with the HA-P2, respectively (Fig. 3). These results provided evidence that P2 interacts with OsKO2 or OsKOL4 in plant cells. Furthermore, similar assays demonstrated that P2 interacts with OsKO1 or OsKOL5 (data not shown).

**Decrease of GA$_3$ in RDV-Infected Rice Plants**

*ent*-Kaurene oxidases play an essential role in GA biosynthesis (Helliwell et al., 1998, 1999). The interaction between P2 and the rice *ent*-kaurene oxidases and *ent*-kaurene oxidase-like proteins raised the important question of whether such interaction would interfere with GA biosynthesis that contributes to the dwarfed...
phenotypes of RDV-infected plants. To address this question, we analyzed the content of GA1, a major active GA component in rice vegetative tissues, in RDV-infected and healthy rice plants. The results indicated that the amount of endogenous GA1 in RDV-infected plants was only 27.9% of that in the healthy plants (Fig. 4). Furthermore, the stunting and leaf darkening symptoms of the RDV-infected plants are reminiscent of those developed in the GA-deficient mutant rice plants (Ross et al., 1997).

To further test whether the reduced accumulation of GAs in RDV-infected plants contributed to the disease symptoms, we asked whether symptoms caused by RDV infection could be rescued by exogenously supplied GA. As shown in Figure 5, RDV-infected rice plants sprayed with GA3 grew almost as tall as the uninfected rice plants. Significantly, application of indole-3-acetic acid (IAA) to the RDV-infected plants failed to restore the height of infected plants (Fig. 5). These results provided compelling evidence that the reduced endogenous GA level in RDV-infected plants is specifically responsible for the development of growth stunting symptoms.

RDV Infection Resulted in Down-Regulation of OsKO1, OsKO2, OsKOL4, and OsKOL5 Expression Levels

The reduced GA levels in RDV-infected plants could be attributed to inhibited activity and/or expression of enl-kaurene oxidases or enl-kaurene oxidase-like proteins as a result of P2 interactions. While testing the activity of these enzymes awaits development of biochemical assay systems, their expression levels could be investigated by standard molecular methods. To this end, we determined the accumulation levels of OsKO and OsKOL mRNAs by quantitative real-time RT-PCR. Such analysis showed that the overall accumulation levels of OsKO or OsKOL transcripts in leaves of RDV-infected rice were 50% of that in healthy rice plants (Fig. 6).

Semiquantitative RT-PCR was used to analyze the accumulation levels of OsKO1, OsKO2, OsKOL4, and OsKOL5 mRNAs in RDV-infected and healthy rice plants using specific primers of longer lengths (>500 bp), respectively. The results showed that the accumulation levels of OsKOL4 and OsKOL5 were reduced in RDV-infected rice plants compared with that from healthy plants (Fig. 7). The transcription of OsKO1 and OsKO2 was also down-regulated in RDV-infected rice plants (data not shown).

DISCUSSION

Plant virus infections often lead to alterations in physiological, biochemical, and metabolic processes, resulting in symptoms such as plant stunting and leaf mottling and/or wrinkling (Jameson and Clarke, 2002). For many virus diseases, alterations in plant growth were thought to be the result of cytopathic effects leading to changes in plant hormone metabolism upon virus infection (Jameson and Clarke, 2002). Despite extensive research efforts, the host factors that are primary targets for viral proteins in disease formation remain largely elusive. RDV infection causes growth stunting and leaf darkening in rice, which are also typical of GA-deficiency symptoms. P2 has been implicated as a pathogenicity determinant of RDV (Tomaru et al., 1997; T. Omura, personal communication). In this study, we have identified rice enl-kaurene oxidases as the primary targets of the RDV P2 protein for symptom expression. First, P2 interacts with these enzymes in yeast (Fig. 2). More importantly, this interaction occurs in plant cells (Fig. 3). Second, the growth stunting and leaf darkening symptoms of RDV-infected plants are correlated with reduced levels of GAs (Fig. 4). Such symptoms were alleviated with
exogenously supplied GAs but not IAA (Fig. 5). This observation indicates that GA perception and signal transduction pathways appeared to be functional in RDV-infected rice plants and there was a correlation between GA1 content and dwarf phenotype. Third, RDV-infected plants exhibit down-regulated expression of ent-kaurene oxidases (Fig. 6). These observations are fully consistent with the established role of ent-kaurene oxidases in GA biosynthesis and with the established role of GAs in plant growth and development. It is significant to note that the phenotypes of RDV-infected plants are strikingly similar to those exhibited by the ent-kaurene oxidase mutant rice plant d35Tan-Ginbozu (Kobayashi et al., 1989; Ogawa et al., 1996; Itoh et al., 2004). Taking all data together, we propose that in an RDV-infected plant, the RDV P2 interacts with ent-kaurene oxidases. The reduced expressions and/or activities of these enzymes, as a result of P2 interaction, compromise the biosynthesis of GAs. The reduced accumulation of GAs contributes directly to the abnormal functions of the GA-regulated cellular processes that lead to symptom expression.

Our findings have broad significance in studying the mechanisms of GA metabolism in plant growth and development and in plant-viral interactions. The GAs are a large family of tetracyclic diterpenoid plant regulators that are involved in a number of plant growth and developmental processes, including seed germination, stem elongation, flowering, fruit development, apical dominance, and regulation of gene expression in the cereal aleurone layer (Hooley, 1994; Ross et al., 1997; Swain and Singh, 2005). The ent-kaurene oxidase is an enzyme in the GA biosynthesis pathway. It is involved in the first microsomal step of GA biosynthesis and catalyzes the three-step oxidation of ent-kaurene to ent-kaurenoic acid (Hedden and Kamiya, 1997; Helliwell et al., 1998, 1999; Hedden and Phillips, 2000; Olzewski et al., 2002). Plants with ent-kaurene oxidase mutated display characteristic phenotypes, including plant stunting at internodes and leaf darkening (Helliwell et al., 1998; Davidson et al., 2004; Itoh et al., 2004). In addition, a Nicotiana tabacum plant with decreased expression of ent-kaurene oxidase exhibits a prominent decrease in plant height (Fukazawa et al., 2000). Sequence analysis

Table 1. Primers used in this study

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<th>Primers</th>
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Plant Physiol. Vol. 139, 2005 1941
of OsKO homologs in the d35-Tan-Ginbozu genome revealed a Ser-to-Arg substitution located in exon 5 of OsKO2 (Itoh et al., 2004). The OsKO2 rice mutants showed severe dwarf symptoms without flowering or seed development similar to the null mutants of ent-copalyl dipiphosphate synthase or ent-kaurenoic acid oxidase (Sakamoto et al., 2004). All of these results suggest that OsKO2 plays an important role in GA-mediated plant growth and development. As shown in this study, the RDV P2 may serve as a novel tool to investigate the regulation of GA biosynthesis in plant growth and development.

Decreases in GA_{1} level were reported in two uninfected dwarf rice cultivars (Tan-Ginbozu and Waito-C; Kobayashi et al., 1989; Ogawa et al., 1996) and in virus-infected plants (Jameson and Clarke, 2002). Citrus exocortis viroid infection likely reduces the content of the active GA_{1} and leads to a dwarf phenotype in Etrog citrus. Here, the transcription level of CcGA20ox1 is down-regulated (Vidal et al., 2003). In general, the mechanisms by which the viral or viroid infection causes a decrease in GA levels are not understood. Our study reported here showed that a viral pathogenicity determinant can target directly a key component of the GA biosynthetic pathway, pointing out an important direction for future research on the molecular interactions between viral and host factors underlying disease formation.

Although the P2 protein plays a role in inhibiting plant growth, other RDV factors may also be involved in this process. Kimura et al. (1987) isolated a severe (S) strain of RDV from rice plants inoculated with the ordinary (O) strain of RDV. Of the 12 segments of the RDV genome, the S4 segment from the S strain was larger than that from the O strain. In addition, there was a single amino acid substitution (Ile235Thr) in S8-encoded P8 protein of the S strain compared with the O strain. These results suggested that the S4 segment and P8 protein may also modulate the dwarf phenotypes.

Our yeast two-hybrid and coimmunoprecipitation assays showed that P2 also interacted with rice ent-kaurene oxidase-like proteins (OsKOL4 and OsKOL5; Figs. 2 and 3). The interaction of P2 with the two ent-kaurene oxidase-like proteins (OsKOL4 and OsKOL5) suggests other possibilities of functional significance. The expression of OsKOL4 and OsKOL5 was also reduced upon RDV infection (Figs. 6 and 7). Itoh et al. (2004) reported that OsKOL4 and OsKOL5 may participate in phytoalexin biosynthesis. Rice plants not only produce ubiquitous GAs but also labdane-related diterpenoids, such as demilactones A and B, oryzalexins A to F, oryzalexin S, and phytocassanes A to E (also known as phytoalexins; Otomo et al., 2004; Prisic et al., 2004). Phytoalexins play important roles in plant defense (Hammerschmidt, 1999). Successful pathogens must have evolved strategies for circumventing or counteracting the effects of these defense compounds. Most studies on phytoalexin tolerance mechanisms have been conducted in the context of plant interactions with fungal or bacterial pathogens (VanEtten et al., 2001). In plants infected with viruses, accumulation of phytoalexin was previously thought to be a response rather than a resistance to virus infection because these compounds had no obvious effects on virus replication (Bailey et al., 1976). In a later study, Sun et al. (1988) reported that a phytoalexin in cotton may inhibit the replication of Cauliflower mosaic virus. Thus, whether phytoalexins serve as plant defense factors against the infection of viruses in general and of RDV in particular remains an open issue. Our finding that RDV P2 interacts with OsKOL4 and OsKOL5 raises the intriguing question of whether this interaction can lead to a decrease in phytoalexin biosynthesis to compromise host defense reactions. In this regard, it is interesting to note that the TD isolate of RDV with no P2 protein expressed can infect rice (Tomaru et al., 1997). However, the titers for major structural proteins are lower for the TD than for the TC isolate. These results suggest that P2, although not required for RDV replication in rice plants, is important for the efficient replication. The role of phytoalexins in RDV infection and the role of P2 in this process are clearly important issues to be addressed in future studies.

In summary, our study suggests that, during RDV infection, the P2 interacts with ent-kaurene oxidases, resulting in inhibition of their activities and/or transcription. This further leads to reduced GA levels that contribute to growth stunting and the associated symptoms. The interaction of P2 with ent-kaurene oxidase-like proteins may have important roles that remain to be understood. Our findings establish a foundation to further investigate the molecular mechanisms of how a viral protein interacts with critical cellular factors that lead to altered cellular functions to cause disease symptoms.

MATERIALS AND METHODS

PCR Primer Sequences

The oligonucleotides used in this study are listed in Table I.

Plasmid Construction

RDV gene segment S2 (GenBank accession no. AY847464) containing the S2 ORF was ligated into the Ncol/EcoRI site (Promega) within the GAL4 DNA binding domain vector pGBK7 (CLONTECH). RDV gene segment S8 (GenBank accession no. U86656) was also cloned and placed into the pGBK7 vector. Briefly, the pGBK7 vector was digested with Ncol, the ends made blunt with T4 DNA polymerase (Promega), and then digested with BglII. PCR products of S8 amplified with primer pairs F1 and R1 were made blunt and digested with BglII, followed by ligation into the pGBK7 vector. The recombinant plasmid containing the RDV S2 or S8 segment was designated as pGBK52 or pGBK58. The cDNA fragment identified during the yeast two-hybrid screening had sequence identity with the C terminus of CyP701A8 (Japanese cultivar group), a putative gene encoding a rice (Oryza sativa) ent-kaurene oxidase-like protein (http://drnelson.utmem.edu/rice.colordec29.html), designated as OsKOL4 presently. The full-length ORF of the gene was cloned using RNA extracted from rice seedlings (cv Xiushu 11 japonica). First-strand cDNA was prepared from the total RNA using SuperScript II RT (Invitrogen) and primer R2 specific for the OsKOL4. The cDNA was then subjected to PCR using primers R2 and F2 and F1. A 1.5-kb PCR product containing the ORF of OsKOL4 was cloned directly into the pENTR/D-TOPO vector as described (Invitrogen). The three other genes encoding rice ent-kaurene oxidases or ent-kaurene oxidase-like proteins were also cloned from rice seedlings with primer pairs F3/R3, F4/R4, and F5/R5, which were
designed according to rice cDNA sequences (GenBank accession nos. AK071743, AK066285, and AK100964). The specific fragments were cloned into pENTR/D-TOPO vector, respectively. Constructs containing OsKO1 or OsKO2 ORF were digested with RglII and EcoRI, and fragments were ligated into RglII/EcoRI-linearized pGADI7 vector. Constructs containing OsKOL4 or OsKOL5 ORF were digested with Ncol and Sfil, and fragment was ligated into Ncol- and Sfil-digested pGADT7 vector. The recombinant plasmids were designated as pGAD-OsKO1, pGAD-OsKO2, pGAD-OsKOL4, and pGAD-OsKOL5, respectively. HA- and FLAG-epitope tags were added to S2 or OsKO (OsKOL) constructs by PCR with gene-specific primers (F6 and R6, F7 and R7, F8 and R7, F9 and R8, and F10 and R9). Restriction fragments containing ORFs of HA-S2, FLAG-OsKOL4, or FLAG-OsKOL5 were ligated into a cauliflower mosaic virus 35S-based pRTL2 transient-expression vector digested with Ncol and BamHI (Restrepo et al., 1990). The FLAG-OsKO1 or FLAG-OsKO2 fragments were first made blunt and then digested with RglII. The pRTL2 vector was digested with Ncol, made blunt, and then digested with BamHI. The FLAG-OsKO1 or FLAG-OsKO2 fragments were then ligated into the BamHI site in the pRTL2 vector. The above recombinant plasmids containing S2 or OsKOs (OsKOLs) were designated pRTL2-HAS2, pRTL2-FLAG-OsKO1, pRTL2-FLAG-OsKO2, pRTL2-FLAG-OsKOL4, and pRTL2-FLAG-OsKOL5, respectively. Restriction fragments representing HAS2 or FLAG-OsKOL5 were cut from pRTL2-HAS2 or pRTL2-FLAG-OsKOL5 using PstI and inserted into the PstI site of the binary vector pCAMBIA1301 (http://www.cambia.org). Restriction fragments representing FLAG-OsKO1 or FLAG-OsKO2 were cut from pRTL2-FLAG-OsKO1 or pRTL2-FLAG-OsKO2 using HindIII and inserted into the HindIII site of the vector pCAMBIA1301. The restriction fragments representing FLAG-OsKOL4 were cut from pRTL2-FLAG-OsKOL4, made blunt, and then ligated into the Xhol site in the pCAMBIA1301 vector. All the constructs were sequenced from 5’ and 3’ ends to confirm the sequences and frames (Shanghai BioAsia Biotechnology).

**Yeast Two-Hybrid Assay**

A rice seedling two-hybrid cDNA library from rice cv Xiu shui 11 was constructed with CLONTECH protocols. The titer of the library was determined after amplification and was approximately 6 x 10^7 cfu/mL. The cDNA encoding full-length P2 protein was inserted in frame into the GAL4 DNA binding domain vector pBKT7 (CLONTECH). The rice cDNA libraries in GAL4 activation domain vector pGAD7 were screened and the isolation of positive clones was performed using MATCHMAKER GAL4 Two-Hybrid System 3 and Libraries (CLONTECH).

**Agrobacterium-Mediated Transient Expression**

*Agrobacterium* strain EHA105 carrying the gene of interest expressed from System 3 and Libraries (CLONTECH). A rice seedling two-hybrid cDNA library from rice cv Xiu shui 11 was constructed with CLONTECH protocols. The titer of the library was determined after amplification and was approximately 6 x 10^7 cfu/mL. The cDNA encoding full-length P2 protein was inserted in frame into the GAL4 DNA binding domain vector pBKT7 (CLONTECH). The rice cDNA libraries in GAL4 activation domain vector pGAD7 were screened and the isolation of positive clones was performed using MATCHMAKER GAL4 Two-Hybrid System 3 and Libraries (CLONTECH).

**Immuno precipitation**

After Agrobacterium-mediated transient expression for 24 h, N. benthamiana leaves (approximately 0.3 g) were harvested and ground to a powder in liquid nitrogen. Ground tissues were resuspended in 3.0 mL of IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 5 mM dithiothreitol, and 0.1% Complate Proteinase Inhibitor [Roche]; Leister et al., 2005). The crude lysates were spun at 20,000g for 15 min at 4°C. After centrifugation, 1 mL of supernatant was incubated with 0.5 μg of the indicated monoclonal antibody for each immunoprecipitation. After a 1-h incubation at 4°C, immunocomplexes were collected by the addition of 50 μL of protein G Sepharose-4 fast flow beads (Amersham) and incubated end over end for 4 h at 4°C. After incubation, the immunocomplexes were washed four times with 1 mL of IP buffer and the pellet was resuspended in 3 x SDS-PAGE loading buffer (Laemmli, 1970).

**Protein Separation and Immunoblotting**

Protein samples were separated by SDS-PAGE on 8% polyacrylamide gels and transferred by electroblotting to nitrocellulose membranes. Membranes were probed with anti-HA horseradish peroxidase (Roche) or anti-FLAG peroxidase (Sigma-Aldrich) to detect HA- and FLAG-epitope-tagged proteins, respectively. All immunoprecipitation experiments were repeated at least three times, and the identical results were obtained.

**Measurement of GA1 Content and Application of Plant Growth Regulators**

Procedure used to purify GAs from RDV-infected or healthy rice plant was as described previously (Weiler, 1986; He, 1993). Leaf tissue from each plant was homogenized in liquid nitrogen and then extracted in 4 mL of 80% (v/v) ice-cold aqueous methanol containing butylated hydroxytoluene (1 mmol/liter) and polyvinylpyrrolidone (60 mg/g fresh weight). The samples were incubated overnight at 4°C and centrifuged at 10,000g for 10 min. The resulting supernatants were collected individually and filtered through C18 Sep-Pak cartridges (Waters). Efflux of each sample was collected, dried by evaporation with N2, and measured for GA1 content with ELISA using anti-GA1 antibody (He, 1993).

The responses of RDV-infected rice plants to gibberellin (GA1) and IAA were examined with RDV-infected rice plants of 30 d post-RDV infection, which were inoculated at the stage of five leaves. RDV-infected rice seedlings of similar size were chosen and divided into four groups. The seedlings were sprayed once every 5 d with 10 mL of GA1 (50 mg/L), IAA (30 mg/L), or water (Fukazawa et al., 2000). All treated plants were measured for their height at 10 d after the second spray. Healthy rice seedlings at the same stage sprayed with water were used as controls during the experiment. Each group had 10 plants and the experiments were repeated three times.

**Real-Time PCR Analysis**

Total RNA was extracted from rice leaves using TRI Reagent (Sigma) and was treated with RQ1 RNase-free DNase (Promega). Five micrograms of total RNA was reverse transcribed by SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexamers according to the manufacturer’s instructions. The real-time PCR reaction was performed using DyNAmo SYBR Green qPCR kits (FINNZYMES) following the manufacturer’s instructions. EF1a was used as an internal control. Quantifications of each cDNA sample were made in triplicate, and the consistent results from at least five separately prepared RNA samples were used. Purified plasmids or cDNAs representing OsKOs (OsKOL) and EF1a were serially diluted for standard curve preparation. The Ct, defined as the PCR cycle number at reporter fluorescence detection threshold, is used as a measure for the starting copy numbers of the target gene. Relative quantitation of the target OsKO (OsKOL) expression level was performed using the comparative Ct method (Roche Light Cycler system). Due to the high nucleotide sequence similarity among these four genes, the upstream primers used were F13 for OsKO1 and OsKO2, F14 for OsKO4, and F15 for OsKO5, and the downstream primer used for all reactions was R12. Total RNA was extracted from frozen rice leaves using TRI Reagent (Sigma) as described above. The RT condition was 42°C for 50 min. Each PCR reaction (25 μL) contained 2 μL of cDNA template, and PCR was conducted for 25 cycles for Actin 1 and 30 cycles for OsKO1, OsKO2, OsKO4, or OsKO5 at 94°C, 5 min; 94°C for 50 s, 95°C for 1 min; 72°C for 40 s; 72°C for 10 min. The transcription level of Actin 1 gene in these rice samples was analyzed using primers F16 and R13 as a control. The PCR products were visualized in 1.0% agarose gel after staining with ethidium bromide.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY579214, AY660064, AY660065, and AY660066.

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