The Rice Dwarf Virus P2 Protein Interacts with *ent*-Kaurene Oxidases in Vivo, Leading to Reduced Biosynthesis of Gibberellins and Rice Dwarf Symptoms[1]

Shifeng Zhu, Feng Gao, Xuesong Cao, Mao Chen[2], Gongyin Ye[2], Chunhong Wei, and Yi Li*

Peking-Yale Joint Center for Plant Molecular Genetics and Agrobiotechnology, National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Science, Peking University, Beijing 100871, China

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_3 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 Metzlaff, 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 Metzlaff, 2005) and altered metabolism (Roger, 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; Zhong 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

[1] This work was supported by grants of the National Outstanding Youth Grant (contract no. 30125004) and the National Science Foundation of China (to Y.L.), and by the National Key Basic Research Program (973; contract no. G6200016201 to C.H.W.).

[2] Present address: Plant Protection Department, Zhejiang University, Hua Jia Chi, Hangzhou 310029, China.

* Corresponding author; e-mail liyi@pku.edu.cn; fax 86–10–6275–4427.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Yi Li (liyi@pku.edu.cn).

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.072306.

```
OsKOS1   ---------MESMLVAGAGAAAVAAVGGLVAAAALADKLVAAPPPRKNRANPPPAVPGLPKLPKAMSVLTRE-SMVAISDYGDYQKMAKRNIMIGMLGFNAQKQFRGTRERMISNVLSTL
OsKOL4   ---------MESMLVAGAGAAAVAAVGGLVAAAALADKLVAAPPPRKNRANPPPAVPGLPKLPKAMSVLTRK-SMVAISDYGDYQKMAKRNIMIGMLGFNAQKQFRGTRERMISNVLSTL
OsKOS2   ---------MESLLAAGAGGIGVA------AAAAVVAATLAVVPPKDRGNNPPPADPGLPKLPKALSVISRK-NMVSISDYGDFYKMAKRNIMLAILGFNAQKRFCDTRERMVSNVLSSL
OsKOL5   ---------MESLLAAGAGGIGVA------AAAAVVAATLAVVPPKDRGNNPPPAVPGLPKLPKALSVISRK-NMVSISDYGDFYKMAKRNIMLAILGFNAQKHFCDTRERMVSNVLSSL
OsKOS3   ---------MEAFVPGGAGVAA-AAVGGFVAAAALAERAGVIA-PRKRPNA-PPAVPGLPKLSKALTVLTRDKSMVATSDYCDFHKMVKRYVMSSMLGTSAQKQFRDIRDMMIHNMLSTF
OsKO2    ---------MEAFVPGGAGAAA-AAVGGFVAAAALAERAGVIA-PRKRPNA-PPAVPGLPKLSKALTVLTRDKSMVATSDYCDFHKMVKRYVMSSMLGTSAQKQFRDIRDMMIHNMLSTF
OsKOS4   ------MESLLAAGAGGIGVAA-AAVGGFIAAATLAVAPPKYR-RNP-----PPAVPGLPKLSKALTVLSHDKSMVATSDSGDFHKMGKRYIMLSMLGTSAQKQFRDTRDMIINNMLSTF
OsKO1    ------MESLLAAGAGGIGVAA-AAVGGFIAAATLAVAPPKNR-RNP-----PPAVPGLPKLSKALTVLSHDKSMVATSDSGDFHKMGKRYIMLSMLGTSAQKQFRDTRDMIINNMLSTF
AtKO1    ---------MAFFSMISILLGFVISSFIFIFFFKKLLSFSRKNMSEVSTLPSVPVVPGFPKLSNALTVLTCDKSMVATSDYDDFHKLVKRCLLNGLLGANAQKRKRHYRDALIENVSSKL
CmKO1    MAVATDPLGCMQKLVQMLQAPPYVAAAVQSSALLLTFFIGDWRKRRRSPLPLLPAIPGIPKLSKALTILTADKCMVAMSDYNDFHKLVKRYILANVLGANAQKRLRQRRDTMIDNISREL
PsKO1    -------------------MDTLTLSLGFLSLFLFLFLLKRSTHKHSKLSHVPVVPGLPKLSTALTILTSDKCMVAMSDYNDFHKMVKKHILASVLGANAQKRLRFHREVMMENMSSKF
                                                                  *  ** *** *.....   **. **  *. *. *. ..  .** ***.   *. .. *.

OsKOS1   HKLVSLDPHSPLNFRDVYINELFSLSLIQSLGEDVSSVYVEEFGREIPKDEIFDVLVHEMMMCAVEADWRDYFPYLSWLPNKSFDTIVSTTEFRRDAIMNALIKKQKERIARGEARASYI
OsKOL4   HKLVSLDPHSPLNFRDVYINELFSLSLIQSLGEDVSSVYVEEFGREISKDEIFDVLVHEMMMCAVEADWRDYFPYLSWLPNKSFDTIVSTTEFRRDAIMNALIKKQKERIARGEARASYI
OsKOS2   HKLVAVDPHSPLNFREVYTTELFGLSLIQNLGEDVCSVYVEEFGREISKEEIFHVLVHEILSCVVEPDWRDYFPYLSWLPNKSFETIVSSTEFRRDAVMNALIKRQKERIARGEARISYI
OsKOL5   HKLVAVDPHSPLNFREVYTTELFGLSLIQNLGEDVCSVYVEEFGREISKEEIFHVLVHEILSCVVEPDWRDYFPYLSWLPNKSFETIVSSTEFRRDAVMNALIKRQKERIARGEARISYI
OsKOS3   HKLVKDDPHAPLIFRDVFKDELFRLSMIQSLGEDVSSVYVDEFGRDISREEIYNATVTDMMMCAIEVDWRDFFPYLSWVPNKSFETRVFTTETRRTAVMRALIKQQKERIVRGEAKTCYL
OsKO2    HKLVKDDPHAPLIFRDVFKDELFRLSMIQSLGEDVSSVYVDEFGRDISKEEIYNATVTDMMMCAIEVDWRDFFPYLSWVPNKSFETRVFTTETRRTAVMRALIKQQKERIVRGEAKTCYL
OsKOS4   HQLVKDDPHAPLIFRDVFKNELFRLSMIQSLGEDVSSVYVDEFGRDISKEEIYNATVTDMMMCAIEVDWRDFFPYLSWVPNKSFETRVFTTESRRTAVMRALIKQQKERIVRGEARTCYL
OsKO1    HQLVKDDPHAPLIFRDVFKDELFRLSMIQSLGEDVSSVYVDEFGRDISKEEIYNATVTDMMMCAIEVDWRDFFPYLSWVPNKSFETRVFTTESRRTAVMRALIKQQKERIVRGEARTCYL
AtKO1    HAHARDHPQEPVNFRAIFEHELFGVALKQAFGKDVESIYVKELGVTLSKDEIFKVLVHDMMEGAIDVDWRDFFPYLKWIPNKSFEARIQQKHKRRLAVMNALIQDRLKQNGSESDDDCYL
CmKO1    FACVKDSSSESVNFRKIFESELFGLALKETFGRDMESLYVDGLGTTLLREDLFRTLVIDPMEGAIEVDWRDFFPYLRWIPNKGVEDRIRKMDFRRRVTMKSLMEEKKKQIAAGEDLNCYS
PsKO1    NEHVKTLSDSAVDFRKIFVSELFGLALKQALGSDIESIYVEGLTATLSREDLYNTLVVDFMEGAIEVDWRDFFPYLKWIPNKSFEKKIRRVDRQRKIIMKALINEQKKRLTSGKELDCYY
                   . ** ..  *** ... .  * *. *.**       . .....  * . .   .. ****.**** *.***  .  .     .*  * .*.  .  .       .*

OsKOS1   DFLLEAE-RSAQLTDDQLMLLLSESILAAADTVLVTTEWTMYEIAKNPDKQELLYQEIREACGGEAVTEDDLPRLPYLNAVFHETLRLHSPVPVLPPRFVHDDTTLAGYDIAAGTQMMIN
OsKOL4   DFLLEAE-RSAQLTDDQLMLLLSESILAAADTVL-----------------ELLYQEIREACGGEAVTEDDLPRLPYLNAVFHETLRLHSPVPVLPPRFVHDDTTLAGYDIAAGTQMMIN
OsKOS2   DFLLEAK-NSTQLTDHQLMLLLAESIAAAVDTVLVTTEWAMYELAKNPDKQEWLYREIREVCGGKAVTEEDLPRLPYLDAVLHETLRLHSPVPVLPTRFVHDDTTLAGYDVPAGTQVMIN
OsKOL5   DFLLEAK-NSTQLTDHQLMLLLAESIAAAVDTVLVTTEWAMYELAKNPDKQEWLYREIREVCGGKAVTEEDLPRLPYLDAVLHETLRLHSPVPVLPTRFVHDDTTLAGYDVPAGTQVMIN
OsKOS3   DFLLAEN-T---LTDEQLMMLVWEALIEAADTTLVTTEWAMYELAKNPDKQERLYQEIREVCGDETVTEEHLPRLPYLNAVFHETLRRHSPVPLIPPRFVHEDTKLAGYDVPAGTEMVIN
OsKO2    DFLLAEN-T---LTDEQLMMLVWEALIEAADTTLVTTEWAMYELAKNPDKQERLYQEIREVCGDETVTEEHLPRLPYLNAVFHETLRRHSPVPLIPPRFVHEDTKLAGYDVPAGTEMVIN
OsKOS4   DFLLAEN-T---LTDEQLMMLVWEALIEAADTTLVTTEWAMYELAKNPDKQERLYQEIREVCGDEAVTEEHLPWLPYLNAVFQETLRRHSPVPLIPPRFVNEDTMLAGYDVPAGTEMVIN
OsKO1    DFLLAEN-T---LTDEQLMMLVWEALIEAADTTLVTTEWAMYELAKNPDKQERLYQEIREVCGDEAVTEEHLPWLPYLNAVFQETLRRHSPVPLIPPRFVNEDTMLAGYDVPAGTEMVIN
AtKO1    NFLMSEAKT---LTKEQIAILVWETIIETADTTLVTTEWAIYELAKHPSVQDRLCKEIQNVCGGEKFKEEQLSQVPYLNGVFHETLRKYSPAPLVPIRYAHEDTQIGGYHVPAGSEIAIN
CmKO1    EFLLSEAKS---LTEEQISMLLWEIIIETSDTTLVVTEWAMYELAQNPKRQERLYQHIQSVCGSAKITEENLSQLPYLTAVFHETLRKYSPVSIVPLRYAHEDTQLGGYFIPAGSEVAVN
PsKO1    DYLVSEAKE---VTEEQMIMLLWEPIIETSDTTLVTTEWAMYELAKDKNRQDRLYEELLNVCGHEKVTDEELSKLPYLGAVFHETLRKHSPVPIVPLRYVDEDTELGGYHIPAGSEIAIN
          .*.       .*  *. .*. * .  .** *                 . *  .   **    .. *  .***  * .****  **  ..* *.  .** . ** . **... .*

OsKOS1   VYACHMDEKVWESPGEWSPERFLGEGFEVADRY-KTMAFGAGRRTCAGSLQAMNIACVAVARLVQELEWRLREGDGDKEDTMQFTALKLDPLHVHLKPRGRM-
OsKOL4   VYACHMDEKVWESPGEWSPERFLGEGFEVADRY-KTMAFGAGRRTCAGSLQAMNIACVAVARLVQELEWRLREGDGDKEDTMQFTALKLDPLHVHLKPRGRM-
OsKOS2   VFGCHMDEEAWESPGEWSPERFLGEGFKLADRY-KTLAFGAGRRTCAGSQQAVSIACVAIARFVQELQWTLREGDGDKEDTTQYTALKLHPLHVHLKPRGS--
OsKOL5   VFGCHMDEEAWESPGEWSPERFLGEGFKLADRY-KTLAFGAGRRTCAGSQQAVSIACVAIARFVQELQWTLREGDGDKEDTMQYTALKLHPLHVHLKPRGS--
OsKOS3   LYGCNMNRKEWESPEEWVPERFAGGRLEVADMY-KTMAFGAGRRACAGSLQATHIACAAVARFVQEFGWRLREGDEEKVDTVQLTAYKLHPLHVHLTRRGRM-
OsKO2    LYGCNMNRKEWESPEEWVPERFAGGRLEVADMY-KTMAFGAGRRACAGSLQATHIACAAVARFVQEFGWRLREGDEEKVDTVQLTAYKLHPLHVHLTRRGRM-
OsKOS4   LYGCNMNKKEWESPEEWAPERFAGGRFKVADMY-KTMAFGAGRRVCAGSLQATHIACAAIARFVREFGWRLREGDEEKVDTVQLTAYKLHPLHVHLTRRGRM-
OsKO1    LYGCNMNKKEWESPEEWAPERFAGGRFKVADMY-KTMAFGAGRRVCAGSLQATHIACAAIARFVQEFGWRLREGDEEKVDTVQLTAYKLHPLHVHLTPRGRM-
AtKO1    IYGCNMDKKRWERPEDWWPERFLDDGKYETSDLHKTMAFGAGKRVCAGALQASLMAGIAIGRLVQEFEWKLRDGEEENVDTYGLTSQKLYPLMAIINPRRS--
CmKO1    IYACNMDKKQWESPEEWKPERFLDE-SYDPMDLYKTMAFGGGKRVCAGAPKAMLIACTTLGRLVQGFTWKLREGEEDKVDTLGLTARKLQPLHIVAKPRIN--
PsKO1    IYGCNMDSNLWENPDQWIPERFLDE-KYAQADLYKTMAFGGGKRVCAGSLQAMLIACTAIGRLVQEFEWELGHGEEENVDTMGLTTHRLHPLQVKLKPRNRIY
         .. *.*   ** *  * ****            **.*** *.* ***. .*  .* .. * *.   * *  *. .  **   *. .* **      *
```

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

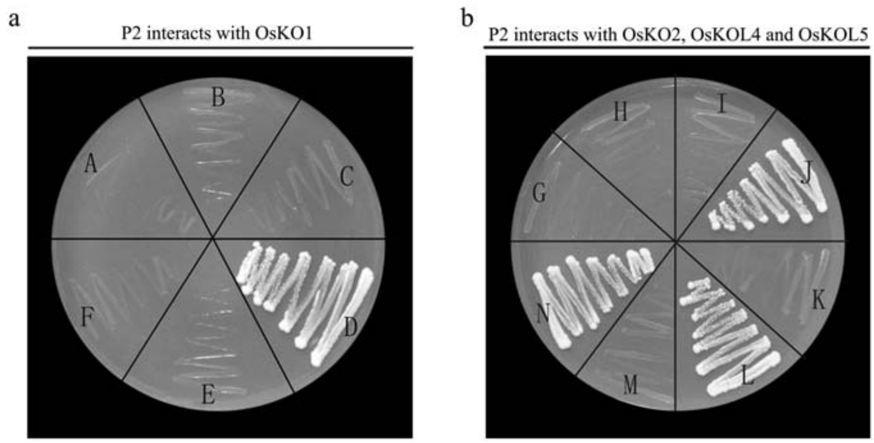

Figure 2. Interaction of P2 with rice *ent*-kaurene oxidases or *ent*-kaurene oxidase-like proteins in yeast. a, P2 interacts with OsKO1. b, P2 interacts with OsKO2, OsKOL4, and OsKOL5. The transformants were plated on a SD/−Leu/−Trp/−His/−Ade medium. A and G, pGBKT7 and pGADT7; B and H, pGBKS2 and pGADT7; C, pGBKT7 and pGAD-*OsKO1*; D, pGBKS2 and pGAD-*OsKO1*; E, pGBKS8 and pGADT7; F, pGBKS8 and pGAD-*OsKO1*; I, pGBKT7 and pGAD-*OsKO2*; J, pGBKS2 and pGAD-*OsKO2*; K, pGBKT7 and pGAD-*OsKOL4*; L, pGBKS2 and pGAD-*OsKOL4*; M, pGBKT7 and pGAD-*OsKOL5*; N, pGBKS2 and pGAD-*OsKOL5*.

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 GA_1, 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 GA_3 (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

Figure 1. (*Continued.*)
numbers as follows: AAT46567 (OsKOS1), BAD54592 (OsKOL4), AAT81229 (OsKOS2), BAD54586 (OsKOL5), AAT81230 (OsKOS3), BAD54598 (OsKO2), AAT91065 (OsKOS4), BAD54595 (OsKO1), AAC39507 (AtKO1), AAG41776 (CmKO1), and AAP69988 (PsKO1), respectively.

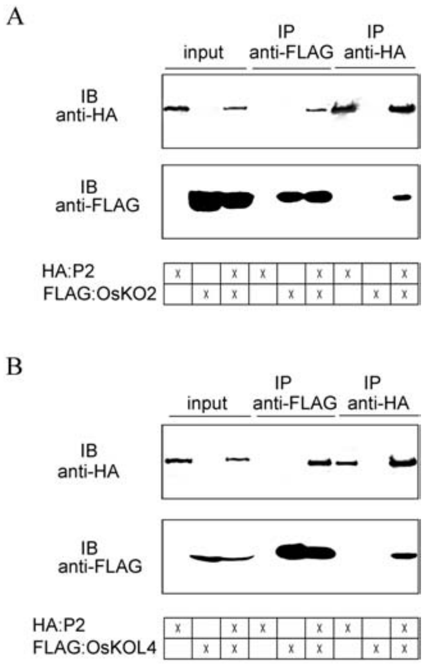

Figure 3. P2 associates with OsKO2 (OsKOL4) in plant cells. A, Immunoblot showing P2 coimmunoprecipitated with OsKO2. The total proteins were isolated from Agrobacterium-infiltrated *N. benthamiana* leaves expressing HA:P2 and the FLAG:OsKO2, and immunoprecipitated with anti-HA (top) and anti-FLAG (bottom) antibodies. B, P2 (HA:P2) coimmunoprecipitates with FLAG:OsKOL4.

representing a rice cDNA library. Seven positive colonies were identified among the approximately 2×10^6 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://btn.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,

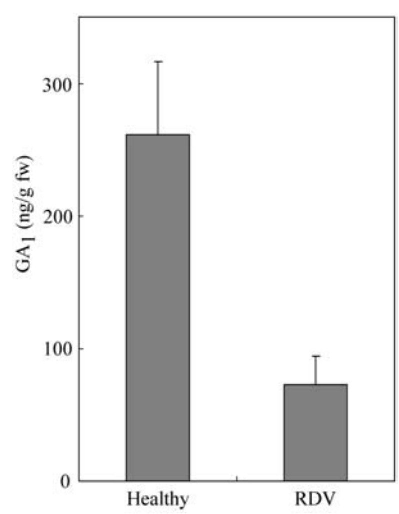

Figure 4. GA_1 content in RDV-infected rice plants. Each column represents the mean ± SE of measurement from four plants. g fw, Gram fresh weight. Extract residues were dissolved in phosphate-buffered saline (0.01 M, pH 7.4) to determine GA content. The content of GA_1 was measured by an indirect ELISA technique with anti-GA_3 antibodies. Healthy means uninfected healthy rice seedlings. RDV means RDV-infected rice seedlings.

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 pGBKT7 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 pGBKS2 into yeast. Yeast cells cotransformed with constructs pGBKT7/pGADT7, pGBKS2/pGADT7, pGBKT7/pGAD-*OsKO1*, pGBKS8/pGADT7, pGBKS8/pGAD-*OsKO1*, pGBKT7/pGAD-*OsKO2*, pGBKT7/pGAD-*OsKOL4*, and pGBKT7/pGAD-*OsKOL5* served as negative controls. The pGBKS8 construct encodes one of the RDV outer capsid proteins. Only the yeast cells cotransformed with pGBKS2/pGAD-*OsKO1*, pGBKS2/pGAD-*OsKO2*, pGBKS2/pGAD-*OsKOL4*, and pGBKS2/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 coimmunoprecipitated 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_1 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

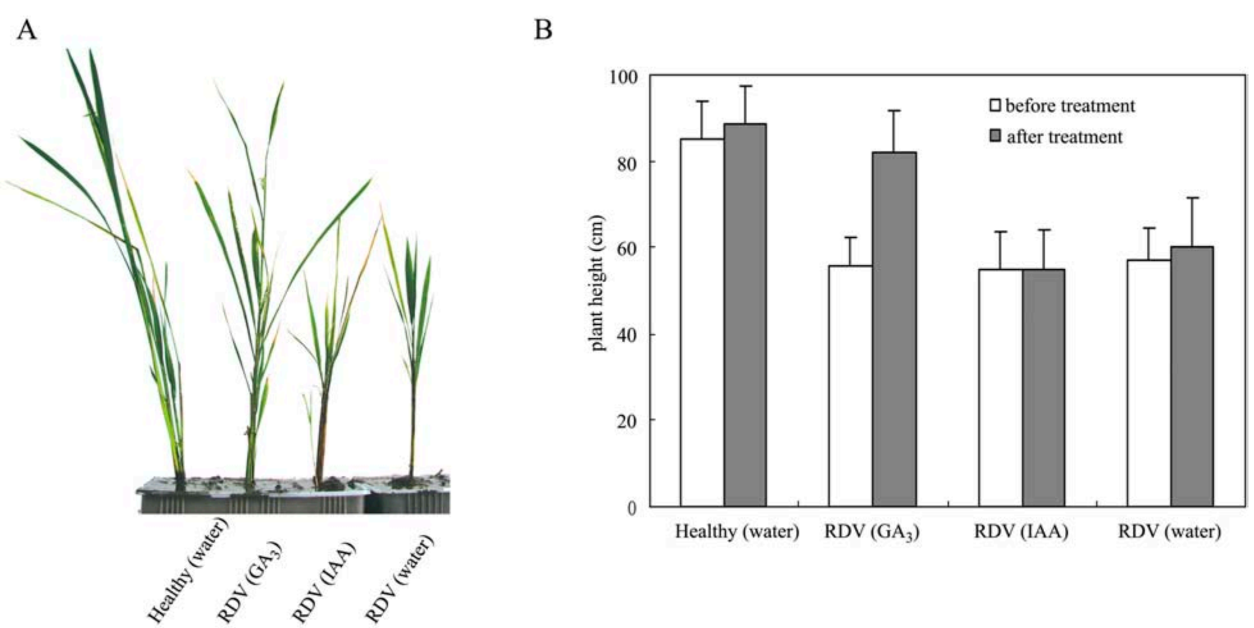

Figure 5. Restoration of the dwarf phenotype of RDV-infected rice plants by application of GA_3. A, GA_3 restored the phenotype of RDV-infected rice plants. Rice plants at 30 d postinfection, inoculation at the stage of five leaves, were treated with GA_3 (50 mg/L), IAA (30 mg/L), or water. The height of RDV-infected plants was measured before and after treatment. The RDV-infected plants were sprayed with water, GA_3, or IAA once every 5 d. The spray volume was about 10 mL. Ten days after the second spraying, the height of the treated plants was measured. In contrast to RDV-infected rice plants, wild-type plants were only treated with water and were measured correspondingly. Each group had more than 10 plants and the experiments were repeated three times. B, Statistical analysis of plant height treated with GA_3. Each column represents the mean ± SE of 30 wild-type (white bars) or RDV-infected plants (gray bars). The SDs are also indicated. Healthy means uninfected healthy rice seedlings. RDV means RDV-infected rice seedlings.

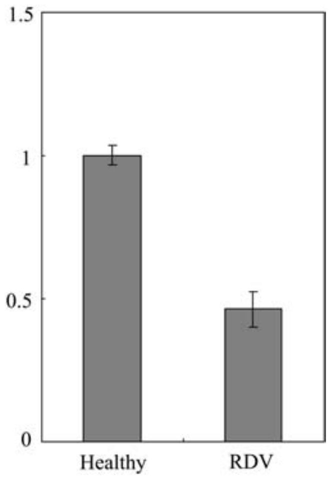

Figure 6. The relative accumulation levels of *OsKO* (*OsKOL*) mRNAs in leaves of healthy and RDV-infected rice plants by quantitative real-time RT-PCR. Data represent means plus SDs based on results from five replicate experiments.

phenotypes of RDV-infected plants. To address this question, we analyzed the content of GA_1, a major active GA component in rice vegetative tissues, in RDV-infected and healthy rice plants. The results indicated that the amount of endogenous GA_1 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 GA_3 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 *ent*-kaurene oxidases or *ent*-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 *ent*-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

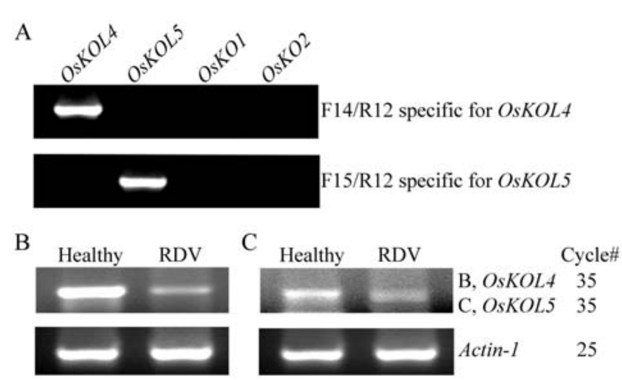

Figure 7. The expression levels of *OsKOL4* and *OsKOL5* in healthy and RDV-infected rice plants. RT-PCR was used to detect the transcriptional levels of *OsKOL4* and *OsKOL5*. A, PCR analysis of primer specificity for *OsKOL4* and *OsKOL5*; B, *OsKOL4*; C, *OsKOL5*; Healthy, RNA was extracted from uninfected healthy rice plants; RDV, RNA was extracted from RDV-infected rice plants. As an internal standard, the rice *Actin 1* gene was amplified using *Actin 1*-specific primers.

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 GA_1 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 *d35*$^{Tan\text{-}Ginbozu}$, which also showed GA deficiency (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 interactions, 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; Olszewski 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 I. *Primers used in this study*

RE, Restriction enzyme; EN, pENTR/D-TOPO vector.

Primers	Primer Sequences	RE Site	Constructs
F1	5′-TCACGCCAGATGTGGTTAGA-3′		pGBKS8
R1	5′-GAAGATCTGCGCTCGAGATTCAGGACCG-3′	*Bgl*II	
F2	5′-CACCCCATGGAGTCGATGCTCGTAGC-3′	*Nco*I	EN-*OsKOL4*
R2	5′-AGTCGACTCACATCCTTCCTCTGGGCTTG-3′	*Sal*I	
F3	5′-CACCATGGAGTCGCTGCTCGCAGC-3′	*Nco*I	EN-*OsKOL5*
R3	5′-AGTCGACCTAGCTTCCTCTGGGCTTGAGGTG-3′	*Sal*I	
F4	5′-CACCAGATCTTGTCCATGGAGGCGTTCGTGCCG-3′	*Bgl*II	EN-*OsKO2*
R4	5′-GCGAATTCACATCCTTCCTCTGCGCGTGAG-3′	*Eco*RI	
F5	5′-CACCAGATCTGGTCGGCCATGGAGTCGCTGCTCG-3′	*Bgl*II	EN-*OsKO1*
R5	5′-GCGAATTCTAATGGTTCACATCCTTCCTCTG-3′	*Eco*RI	
F6	5′-ATTCCATGGACTACCCATACGATGTTCCTGACTATGCGGCTTATCCTAATGAC-GTCAGAAACG-3′	*Nco*I	pRTL2-HAS2
R6	5′-ACTGGATCCTCACAATGCATCATAGATAGATTG-3′	*Bam*HI	
F7	5′-GACTACAAGGACGACGATGACAAGGAGTCGCTGCTCGCAGCCGGTG-3′		pRTL2-FLAG-OsKO1
R7	5′-AGTGCAAGATCTTCACATCCTTCCTCTGCGCGTGA-3′	*Bgl*II	
F8	5′-GACTACAAGGACGACGATGACAAGGAGGCGTTCGTGCCGGGCGGCG-3′		pRTL2-FLAG-OsKO2
R7	5′-AGTGCAAGATCTTCACATCCTTCCTCTGCGCGTGA-3′	*Bgl*II	
F9	5′-TTACCATGGACTACAAGGACGACGATGACAAGGAGTCGATGCTCGTAGC-CGGAGC-3′	*Nco*I	pRTL2-FLAG-OsKOL4
R8	5′-AGTGCAGGATCCTCACATCCTTCCTCTGGGCTTGA-3′	*Bam*HI	
F10	5′-TTACCATGGACTACAAGGACGACGATGACAAGGAGTCGCTGCTCG-CAGCCGGTGC-3′	*Nco*I	pRTL2-FLAG-OsKOL5
R9	5′-AGTGCAGGATCCCTAGCTTCCTCTGGGCTTGAGGT-3′	*Bam*HI	
F11	5′-GTACAAGACGATGGCGTTCG-3′	For *OsKO(L)s*	
R10	5′-CTTCCTCTGCGCTTGAGGTG-3′	For *EF1α*	
F12	5′-ACATTGCCGTCAAGTTTGCTG-3′		
R11	5′-AACAGCCACCGTTTGCCTC-3′	For *OsKO1* and *OsKO2*	
F13	5′-TTTCATAAACTGGTGAAAGA-3′		
F14	5′-AGCAATGTCTGTGCTAACTC-3′	For *OsKOL4*	
F15	5′-AAGCATTGTCTGTGATAAGC-3′	For *OsKOL5*	
R12	5′-CCTGTTTGTCAGGGTTCTTG-3′	For *OsKO1*, *OsKO2*, *OsKOL4*, and *OsKOL5*	
F16	5′-GGAACTGGTATGGTCAAGGC-3′	For *Actin 1*	
R13	5′-CCGTCAGGATCTTCATGAGG-3′		

of *OsKO* homologs in the *d35*$^{Tan\text{-}Ginbozu}$ 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 diphosphate 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 citron. 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 momilactones 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 *Nco*I/*Eco*RI site (Promega) within the GAL4 DNA binding domain vector pGBKT7 (CLONTECH). RDV gene segment S8 (GenBank accession no. U36565) was also cloned and placed into the pGBKT7 vector. Briefly, the pGBKT7 vector was digested with *Nco*I, the ends made blunt with T4 DNA polymerase (Promega), and then digested with *Bgl*II. PCR products of S8 amplified with primer pairs F1 and R1 were made blunt and digested with *Bgl*II, followed by ligation into the pGBKT7 vector. The recombinant plasmid containing the RDV S2 or S8 segment was designated as pGBKS2 or pGBKS8. The cDNA fragment identified during the yeast two-hybrid screening had sequence identity with the C terminus of CYP701A8 (*japonica* cultivar group), a putative gene encoding a rice (*Oryza sativa*) *ent*-kaurene oxidase-like protein (http://drnelson.utmem.edu/rice.color.dec29.html), designated as *OsKOL4* presently. The full-length ORF of the gene was cloned using RNA extracted from rice seedlings (cv Xiu shui 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. 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 *Bgl*II and *Eco*RI, and fragments were ligated into *Bgl*II/*Eco*RI-linearized pGADT7 vector. Constructs containing *OsKOL4* or *OsKOL5* ORF were digested with *Nco*I and *Sal*I, and fragment was ligated into *Nco*I- and *Sal*I-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 *Nco*I and *Bam*HI (Restrepo et al., 1990). The FLAG-OsKO1 or FLAG-OsKO2 fragments were first made blunt and then digested with *Bgl*II. The pRTL2 vector was digested with *Nco*I, made blunt, and then digested with *Bam*HI. The FLAG-OsKO1 or FLAG-OsKO2 fragments were then ligated into the *Bam*HI 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 *Pst*I and inserted into the *Pst*I 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 *Hin*dIII and inserted into the *Hin*dIII site of the vector pCAMBIA1301. The restriction fragments representing FLAG-OsKOL4 were cut from pRTL2-FLAG-*OsKOL4*, made blunt, and then ligated into the *Sma*I 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 6×10^8 cfu/mL. The cDNA encoding full-length P2 protein was inserted in frame into the GAL4 DNA binding domain vector pGBKT7 (CLONTECH). The rice cDNA library in GAL4 activation domain vector pGADT7 was 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 a binary vector was infiltrated into leaves of *Nicotiana benthamiana* essentially as described previously (Tai et al., 1999). *Agrobacterium tumefaciens* was grown overnight at 28°C on Luria-Bertani agar containing 50 mg/mL of rifampicin and 50 mg/mL of kanamycin. Cells were resuspended in induction media (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 mM acetosyringone) and incubated at room temperature for 3 h before inoculation.

Immunoprecipitation

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 1.5× Complete Protease Inhibitor [Roche]; Leister et al., 2005). The crude lysates were then spun at 20,000*g* 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× 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 GA_1 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 10,000*g* 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 N_2, and measured for GA_1 content with ELISA using anti-GA_3 antibody (He, 1993; Yang et al., 2001).

The responses of RDV-infected rice plants to gibberellin (GA_3) 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 GA_3 (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 TriReagent (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. *EF1α* 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* (*OsKOLs*) and *EF1α* 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 LightCycler system). Due to the high nucleotide sequence similarity among *OsKO* (*OsKOL*) genes, the primers used for PCR were as follows: F11 and R10 designed as universal primers for four *OsKO* (*OsKOL*) genes, and F12 and R11 for *EF1α*.

Semiquantitative RT-PCR

Semiquantitative RT-PCR was used to analyze the transcription level of *OsKO1*, *OsKO2*, *OsKOL4*, and *OsKOL5* with specific primers in RDV-infected and healthy rice plants, respectively. Due to the high nucleotide sequence similarity among these four genes, the upstream primers used were F13 for *OsKO1* and *OsKO2*, F14 for *OsKOL4*, and F15 for *OsKOL5*, and the downstream primer used for all reactions was R12. Total RNA was extracted from frozen rice leaves using TriReagent (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 35 cycles for *OsKO1*, *OsKO2*, *OsKOL4*, or *OsKOL5* at 94°C, 5 min; 94°C for 50 s; 55°C for 1 min; 72°C for 90 s; and 72°C for 10 min. 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, AY660664, AY660665, and AY660666.

ACKNOWLEDGMENTS

We thank James C. Carrington for providing pRTL2; Hongbing Shu for assistance with the coimmunoprecipitation experiments; and Biao Ding, Richard S. Nelson, Xingshun Ding, Yuxian Zhu, and Sara Berg for critical comments on the manuscript.

Received October 6, 2005; revised October 6, 2005; accepted October 19, 2005; published November 18, 2005.

LITERATURE CITED

Bailey JA, Vincent GG, Burden RS (1976) The antifungal activity of glutinosone and capsidiol and their accumulation in virus-infected tobacco species. Physiol Plant Pathol **8:** 35–41

Boccardo G, Milne RG (1984) Plant reovirus group. *In* AF Morant, BD Harrison, eds, CM/AAB Descriptions of Plant Viruses. Commonwealth Mycological Institute and Association of Applied Biologists, Unwin Brothers, Gresham Press, Old Woking, England, pp 294

Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana.* EMBO J **17:** 6739–6746

Callaway A, Giesman-Cookmeyer D, Gillock ET, Sit TL, Lommel SA (2001) The multifunctional capsid proteins of plant RNA viruses. Annu Rev Phytopathol **39:** 419–460

Cao XS, Zhou P, Zhang XM, Zhu SF, Zhong XH, Xiao Q, Ding B, Li Y (2005) Identification of an RNA silencing suppressor from a plant double-stranded RNA virus. J Virol **79:** 13018–13027

Causier B, Davies B (2002) Analysing protein-protein interactions with the yeast two-hybrid system. Plant Mol Biol **50:** 855–870

Chellappan P, Vanitharani R, Fauquet CM (2005) MicroRNA-binding viral protein interferes with Arabidopsis development. Proc Natl Acad Sci USA **102:** 10381–10386

Chen J, Li WX, Xie D, Peng JR, Ding SW (2004) Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microRNA in host gene expression. Plant Cell **16:** 1302–1313

Chien CT, Bartel PL, Sternglanz R, Fields S (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci USA **88:** 9578–9582

Dai S, Zhang Z, Chen S, Beachy RN (2004) RF2b, a rice bZIP transcription activator, interacts with RF2a and is involved in symptom development of rice tungro disease. Proc Natl Acad Sci USA **101:** 687–692

Davidson SE, Smith JJ, Helliwell CA, Poole AT, Reid JB (2004) The pea gene *LH* encodes *ent*-kaurene oxidase. Plant Physiol **134:** 1123–1134

Fernandez I, Candresse T, Le Gall O, Dunez J (1999) The 5′ noncoding region of grapevine chrome mosaic nepovirus RNA-2 triggers a necrotic response on three Nicotiana spp. Mol Plant Microbe Interact **12:** 337–344

Fukazawa J, Sakai T, Ishida S, Yamaguchi I, Kamiya Y, Takahashi Y (2000) Repression of shoot growth, a bZIP transcriptional activator, regulates cell elongation by controlling the level of gibberellins. Plant Cell **12:** 901–915

Hagiwara K, Higashi T, Namba K, Uehara-Ichiki T, Omura T (2003) Assembly of single-shelled cores and double-shelled virus-like particles after baculovirus expression of major structural proteins P3, P7 and P8 of rice dwarf virus. J Gen Virol **84:** 981–984

Hammerschmidt R (1999) Phytoalexins: What have we learned after 60 years? Annu Rev Phytopathol **37:** 285–306

He Z (1993) A Laboratory Guide to Chemical Control Technology on Field Crop. Beijing Agricultural University Press, Beijing, pp 60–68

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol **48:** 431–460

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci **5:** 523–530

Helliwell CA, Poole A, Peacock WJ, Dennis ES (1999) Arabidopsis *ent*-kaurene oxidase catalyzes three steps of gibberellin biosynthesis. Plant Physiol **119:** 507–510

Helliwell CA, Sheldon CC, Olive MR, Walker AR, Zeevaart JA, Peacock WJ, Dennis ES (1998) Cloning of the Arabidopsis *ent*-kaurene oxidase gene *GA3.* Proc Natl Acad Sci USA **95:** 9019–9024

Hooley R (1994) Gibberellin: perception, transduction and responses. Plant Mol Biol **26:** 1529–1555

Itoh H, Tatsumi T, Sakamoto T, Otomo K, Toyomasu T, Kitano H, Ashikari M, Ichihara S, Matsuoka M (2004) A rice semi-dwarf gene, *Tan-Ginbozu (D35)*, encodes the gibberellin biosynthesis enzyme, *ent*-kaurene oxidase. Plant Mol Biol **54:** 533–547

Jameson PE, Clarke SF (2002) Hormone-virus interactions in plants. CRC Crit Rev Plant Sci **21:** 205–228

Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. Dev Cell **4:** 205–217

Kimura I, Minobe Y, Omura T (1987) Changes in a nucleic acid and a protein component of rice dwarf virus particles associated with an increase in symptom severity. J Gen Virol **68:** 3211–3215

Kobayashi M, Sakurai A, Saka H, Takahashi N (1989) Quantitative analysis of endogenous gibberellins in normal and dwarf cultivars of rice. Plant Cell Physiol **30:** 963–969

Kong LJ, Orozco BM, Roe JL, Nagar S, Ou S, Feiler HS, Durfee T, Miller AB, Gruissem W, Robertson D, et al (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. EMBO J **19:** 3485–3495

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Leister RT, Dahlbeck D, Day B, Li Y, Chesnokova O, Staskawicz BJ (2005) Molecular genetic evidence for the role of SGT1 in the intramolecular complementation of Bs2 protein activity in *Nicotiana benthamiana.* Plant Cell **17:** 1268–1278

Li Y, Bao YM, Wei CH, Kang ZS, Zhong YW, Mao P, Wu G, Chen ZL, Schiemann J, Nelson RS (2004) Rice dwarf phytoreovirus segment S6-encoded nonstructural protein has a cell-to-cell movement function. J Virol **78:** 5382–5389

Mao Z, Li Y, Xu H, Chen ZL (1998) The 42K protein of rice dwarf virus is a post-translational cleavage product of the 46K outer capsid protein. Arch Virol **143:** 1831–1838

Miyazaki N, Hagiwara K, Naitow H, Higashi T, Cheng RH, Tsukihara T, Nakagawa A, Omura T (2005) Transcapsidation and the conserved interactions of two major structural proteins of a pair of phytoreoviruses confirm the mechanism of assembly of the outer capsid layer. J Mol Biol **345:** 229–337

Nakagawa A, Miyazaki N, Taka J, Naitow H, Ogawa A, Fujimoto Z, Mizuno H, Higashi T, Watanabe Y, Omura T, et al (2003) The atomic structure of rice dwarf virus reveals the self-assembly mechanism of component proteins. Structure **11:** 1227–1238

Ogawa S, Toyomasu T, Yamane H, Murofushi N, Ikeda R, Morimoto Y, Nishimura Y, Omori T (1996) A step in the biosynthesis of gibberellins that is controlled by the mutation in the semi-dwarf rice cultivar Tan-ginbozu. Plant Cell Physiol **37:** 363–368

Olszewski N, Sun TP, Gubler F (2002) Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell (Suppl) **14:** S61–S80

Omura T, Yan J (1999) Role of outer capsid proteins in transmission of *Phytoreovirus* by insect vectors. Adv Virus Res **54:** 15–43

Omura T, Yan J, Zhong B, Wada M, Zhu Y, Tomaru M, Maruyama W, Kikuchi A, Watanabe Y, Kimura I, et al (1998) The P2 protein of rice dwarf phytoreovirus is required for adsorption of the virus to cells of the insect vector. J Virol **72:** 9370–9373

Otomo K, Kenmoku H, Oikawa H, Konig WA, Toshima H, Mitsuhashi W, Yamane H, Sassa T, Toyomasu T (2004) Biological functions of *ent*- and *syn*-copalyl diphosphate synthases in rice: key enzymes for the branch point of gibberellin and phytoalexin biosynthesis. Plant J **39:** 886–893

Padmanabhan MS, Goregaoker SP, Golem S, Shiferaw H, Culver JN (2005) Interaction of the tobacco mosaic virus replicase protein with the Aux/IAA protein PAP1/IAA26 is associated with disease development. J Virol **79:** 2549–2558

Prisic S, Xu M, Wilderman PR, Peters RJ (2004) Rice contains two disparate *ent*-copalyl diphosphate synthases with distinct metabolic functions. Plant Physiol **136:** 4228–4236

Restrepo MA, Freed DD, Carrington JC (1990) Nuclear transport of plant potyviral proteins. Plant Cell **2:** 987–998

Rodriguez-Cerezo E, Klein PG, Shaw JG (1991) A determinant of disease symptom severity is located in the 3′-terminal noncoding region of the RNA of a plant virus. Proc Natl Acad Sci USA **88:** 9863–9867

Roger H (2002) Matthew's Plant Virology, Ed 4. Academic Press, San Diego, pp 373–436

Ross JJ, Murfet IC, Reid JB (1997) Gibberellin mutants. Physiol Plant **100:** 550–560

Sakamoto T, Miura K, Itoh H, Tatsumi T, Ueguchi-Tanaka M, Ishiyama K, Kobayashi M, Agrawal GK, Takeda S, Abe K, et al (2004) An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol **134:** 1642–1653

Shepherd DN, Martin DP, McGivern DR, Boulton MI, Thomson JA, Rybicki EP (2005) A three-nucleotide mutation altering the Maize streak virus Rep pRBR-interaction motif reduces symptom severity in maize and partially reverts at high frequency without restoring pRBR-Rep binding. J Gen Virol **86:** 803–813

Sun TJ, Melcher U, Essenberg M (1988) Inactivation of cauliflower mosaic virus by photoactivatable cotton phytoalexin. Physiol Mol Plant Pathol **33:** 115–126

Suzuki N, Sugawara M, Kusano T, Mori H, Matsuura Y (1994) Immunodetection of rice dwarf phytoreoviral proteins in both insect and plant hosts. Virology **202:** 41–48

Suzuki N, Sugawara M, Nuss DL, Matsuura Y (1996) Polycistronic (tri- or bicistronic) phytoreoviral segments translatable in both plant and insect cells. J Virol **70:** 8155–8159

Swain SM, Singh DP (2005) Tall tales from sly dwarves: novel functions of gibberellins in plant development. Trends Plant Sci **10:** 123–129

Tai TH, Dahlbeck D, Clark ET, Gajiwala P, Pasion R, Whalen MC, Stall RE, Staskawicz BJ (1999) Expression of the Bs2 pepper gene confers resistance to bacterial spot disease in tomato. Proc Natl Acad Sci USA **96:** 14153–14158

Tomaru M, Maruyama W, Kikuchi A, Yan J, Zhu Y, Suzuki N, Isogai M, Oguma Y, Kimura I, Omura T (1997) The loss of outer capsid protein P2 results in nontransmissibility by the insect vector of rice dwarf phytoreovirus. J Virol **71:** 8019–8023

VanEtten H, Temporini E, Wasmann C (2001) Phytoalexin (and phytoanticipin) tolerance as a virulence trait: Why is it not required by all pathogens? Physiol Mol Plant Pathol **59:** 83–93

Vidal AM, Ben-Cheikh W, Talón M, García-Martínez JL (2003) Regulation of gibberellin 20-oxidase gene expression and gibberellin content in citrus by temperature and citrus exocortis viroid. Planta **217:** 442–448

Wang MB, Metzlaff M (2005) RNA silencing and antiviral defense in plants. Curr Opin Plant Biol **8:** 216–222

Weiler EW (1986) Plant hormone immunoassay based on monoclonal and polyclonal antibodies. *In* HF Linskens, JF Jackson, eds, Immunology in Plant Sciences. Springer-Verlag, New York, pp 1–17

Whitham SA, Wang Y (2004) Roles for host factors in plant viral pathogenicity. Curr Opin Plant Biol **7:** 365–371

Xu H, Li Y, Mao ZJ, Chen ZL (1998) Rice dwarf phytoreovirus segment S11 encodes a nucleic acid binding protein. Virology **240:** 267–272

Yan J, Tomaru M, Takahashi A, Kimura I, Hibino H, Omura T (1996) P2 protein encoded by genome segment S2 of rice dwarf phytoreovirus is essential for virus infection. Virology **224:** 539–541

Yang YM, Xu CN, Wang BM, Jia JZ (2001) Effects of plant growth regulators on secondary wall thickening of cotton fibres. Plant Growth Regul **35:** 233–237

Zheng H, Yu L, Wei C, Hu D, Shen Y, Chen Z, Li Y (2000) Assembly of double-shelled, virus-like particles in transgenic rice plants expressing two major structural proteins of rice dwarf virus. J Virol **74:** 9808–9810

Zhong B, Kikuchi A, Moriyasu Y, Higashi T, Hagiwara K, Omura T (2003) A minor outer capsid protein, P9, of rice dwarf virus. Arch Virol **148:** 2275–2280