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Rice Gene Network Inferred from Expression Profiling of Plants Overexpressing OsWRKY13, a Positive Regulator of Disease Resistance

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ABSTRACT Accumulating information indicates that plant disease resistance signaling pathways frequently interact with other pathways regulating developmental processes or abiotic stress responses. However, the molecular mechanisms of these types of crosstalk remain poorly understood in most cases. Here we report that OsWRKY13, an activator of rice resistance to both bacterial and fungal pathogens, appears to function as a convergent point for crosstalk among the pathogen-induced salicylate-dependent defense pathway and five other physiologic pathways. Genome-wide analysis of the expression profiles of *OsWRKY13*-overexpressing lines suggests that OsWRKY13 directly or indirectly regulates the expression of more than 500 genes that are potentially involved in different physiologic processes according to the classification of the Gene Ontology database. By comparing the expression patterns of genes functioning in known pathways or cellular processes of pathogen infection and the phenotypes between *OsWRKY13*-overexpressing and wild-type plants, our data suggest that OsWRKY13 is also a regulator of other physiologic processes during pathogen infection. The OsWRKY13-associated disease resistance pathway synergistically interacts via OsWRKY13 with the glutathione/glutaredoxin system and flavonoid biosynthesis pathway to monitor redox homeostasis and to putatively enhance the biosynthesis of antimicrobial flavonoid phytoalexins, respectively, in *OsWRKY13*-overexpressing lines. Meanwhile, the OsWRKY13-associated disease resistance pathway appears to interact antagonistically with the SNAC1-mediated abiotic stress defense pathway, jasmonic acid signaling pathway, and terpenoid metabolism pathway via OsWRKY13 to suppress salt and cold defense responses as well as to putatively retard rice growth and development.

Key words: abiotic stress; bacterial blight; microarray; *Oryza sativa*; signal transduction.

INTRODUCTION

Plants are constantly exposed to diverse biotic and abiotic stresses and must cope with and adapt to environmental challenges. To survive under stress conditions, plants have evolved intricate mechanisms to rapidly reallocate metabolic resources among different physiologic pathways and to maximize metabolic resources for adaptation to changed environments. Such adaptive responses, if exceeding the regular modulation of plants, may lead to fitness cost of plants that frequently show growth retardation and reduced metabolism (Heil and Baldwin, 2002; Burdon and Thrall, 2003; van Hulten et al., 2006). Thus, elucidation of the complicated mechanisms of adaptive responses may facilitate engineering or breeding a decrease in this fitness cost in crop production or allow development of stress-tolerant plants.

One of the major environmental stresses is pathogens. Plants have evolved an efficient defense transduction network against pathogenic attack. Although many components of this network are still unknown, different resistance (*R*) gene-mediated gene-for-gene resistance pathways, salicylic acid (SA)-dependent systemic acquired resistance pathway, and jasmonic acid (JA)/ethylene-induced resistance pathway are well-recognized branches of this network (Glazebrook, 2001; Hammond-Kosack and Parker, 2003). Some of the components functioning in the pathogen-induced defense network also play roles in other physiologic or developmental pathways. A few genes, which positively regulate both disease resistance and development, have been identified. For example, the Arabidopsis *ERECTA*, encoding a leucine-rich repeat receptor-like

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kinase, quantitatively enhances plant resistance to bacterial wilt and regulates development of all aerial organs (Godiard et al., 2003). More genes, which have opposite roles, either positive or negative, in disease resistance and other physiologic and developmental regulation, have been reported. For example, Arabidopsis *AtTIP49a*, encoding a resistance protein–interacting protein, acts as a negative regulator of some resistance function but is essential for development of sexual organs (Holt et al., 2002). Rice *Xa13* gene, encoding a plasma membrane protein, is a negative regulator of rice resistance to bacterial blight disease but a positive regulator of pollen development (Chu et al., 2006). Rice *OsMAPK5*, encoding a mitogen-activated protein kinase, negatively regulates resistance to diseases caused by bacteria and fungi and positively regulates tolerance to drought, salt and cold (Xiong and Yang, 2003). Arabidopsis *WES1* and rice *GH3-8*, encoding GH3-type proteins, act in stress resistance and suppress growth (Park et al., 2007; Ding et al., 2008).

In addition, hormones and reactive oxygen species (ROS), as signaling molecules, also play important roles in the crosstalk between disease resistance and other physiologic activities. For example, brassinolide, a plant steroid hormone required for growth and development, can induce innate immunity of tobacco and rice (Nakashita et al., 2003). JA is essential for defense responses to some types of pathogens and developmental processes (Lorenzo and Solano, 2005). ROS control various physiologic processes at the points of convergence between biotic and abiotic stress response pathways (for reviews, see Apel and Hirt, 2004, and Fujita et al., 2006). The overlapping genes and signaling molecules in different pathways are the interaction points between different physiologic processes. They are also important players in adaptive responses. Although the points of convergence in different stress responses and developmental processes have been shedding light on the crosstalk between different signaling pathways, the underlying molecular mechanisms of adaptive responses remain poorly understood in most cases.

Our previous studies have revealed that constitutive overexpression of OsWRKY13, a WRKY-type transcription factor, enhances rice resistance to both *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causing bacterial blight and *Magnaporthe grisea* causing fungal blast, two of the most devastating diseases of rice worldwide (Qiu et al., 2007). OsWRKY13 appears to function in the convergent point between SA- and JA-signaling pathways in disease resistance. It functions as an activator of the SA-dependent pathway by directly or indirectly regulating the expression of a subset of genes acting both upstream and downstream of SA. At the same time, it is a suppressor of the JA-dependent pathway by repressing the expression of genes involved in synthesis of JA. The expression of *OsWRKY13* was largely induced in rice lines carrying different *R* genes after avirulent pathogen infection, suggesting that activation of an *R* gene is the key to the initiation of OsWRKY13's function in disease resistance (Wen et al., 2003; Qiu et al., 2007). The WRKY proteins are a class of regulators that control diverse developmental and physiologic processes in addition to disease resistance responses (Eulgem et al., 2000). Although 109 WRKY genes have been identified in the rice genome (Qu and Zhu, 2006), only two of these genes have been functionally characterized to be involved in pathogen-induced defenses (Liu et al., 2006; Qiu et al., 2007). Furthermore, the signaling pathways involved in rice WRKY proteins are poorly understood. To determine whether OsWRKY13 is also involved in the regulation of genes functioning in other signal transduction pathways in addition to disease resistance signaling, we analyzed the genome-wide gene expression profiling of *OsWRKY13*-overexpressing lines. Comparing the expression patterns of a subset of differentially expressed genes in *OsWRKY13*-overexpressing and wild-type plants after pathogen infection, the results suggest that several known signaling pathways for abiotic responses and developmental processes may crosstalk with the disease resistance network with OsWRKY13 as a convergent point.

RESULTS AND DISCUSSION

Different Types of Genes Are Regulated by OsWRKY13 Transcription Factor

To determine the genes directly or indirectly regulated by OsWRKY13 in the entire genome, we performed the microarray analysis using the Affymetrix GeneChip Rice Genome Array containing a total of 57,381 probe sets. Two independent and uninfected *OsWRKY13*-overexpressing rice lines, D11UM1-1 and D11UM7-2, and wild-type Mudanjiang 8, which was susceptible to *Xoo* and *M. grisea*, were used for the analyses (Qiu et al., 2007). A total of 460 and 445 up-regulated genes and 478 and 605 down-regulated genes were identified in D11UM1-1 and D11UM7-2, respectively, as compared with wild type (the microarray data are under the accession number GSE8380 at http://www.ncbi.nlm.nih.gov/geo). Among these differentially expressed genes, 236 up-regulated (including 211 transcription units [TUs] annotated by TIGR [The Institute for Genomic Research, http://rice.tigr.org], 22 redundant probe sets, and 25 with no TU annotation) and 273 down-regulated (including 257 TUs, 30 redundant probe sets, and 16 with no TU annotation) genes were detected in both *OsWRKY13*-overexpressing lines (Supplemental Tables 1 and 2) and these genes were considered to be significantly changed. For the redundant probe sets corresponding to the same gene of the TIGR annotation, the average value was used as the expression ratio of this gene.

A total of 22,295 genes annotated with TIGR TUs were detected in the chip, of which 12,597 have annotation in the Gene Ontology (GO) database. Among the differentially expressed genes, 297 genes, including 140 up-regulated and 157 down-regulated genes, have GO information. The 12,597 genes with GO annotation were used as a baseline to analyze the differentially expressed genes in GO categories and subcategories included in "biological process," "molecular function," and "cellular component" ontologies.

Among the up-regulated genes, there are three significantly changed subcategories in the GO term "biological process," which includes "nitrogen compound biosynthesis," "amino acid and derivative metabolism," and "secondary metabolism" (Table 1 and Supplemental Table 3). Twenty-one up-regulated genes are included in the three subcategories. The up-regulated genes in the former two subcategories are both related to amino acid metabolism, mainly in the aspartate family amino acid metabolism or biosynthesis, and have a large overlap (Supplemental Table 3). The subcategory of "secondary metabolism" includes 13 up-regulated genes, which are putatively involved in phenylpropanoid and flavonoid metabolism. Four subcategories, "oxidoreductase activity," "thiol-disulfide exchange intermediate activity," "amino acid kinase activity," and "phosphotransferase activity, carboxyl group as acceptor," including total 23 up-related genes, are significantly changed in the GO term "molecular function" (Table 1 and Supplemental Table 3). Only one subcategory, "anchored to plasma membrane," is significantly changed in the GO term "cellular component," which includes two up-regulated genes encoding proteins putatively involved in abiotic metal stress responses (Roudier et al, 2002) (Supplemental Table 3).

Among the down-regulated genes, two subcategories in the GO term "biological process," "induced systemic resistance" and "jasmonic acid mediated signaling pathway," are significantly changed (Table 2). Four of the five down-regulated genes included in the two subcategories are the same (Supplemental Table 4). Another two subcategories in the "biological process," "organic acid transport" and "amine transport," including in total five non-redundant down-regulated genes and involved mainly in amino acid transport, are also significantly changed. Further another two significantly changed subcategories in the "biological process," "alkene metabolism" and "isoprenoid metabolism," share the same seven down-regulated genes (Table 2). Among the seven genes, three, *Os02g04690*, *Os02g12890*, and *Os03g22634* encoding cycloartenol synthase, thromboxane-A synthase, and terpene synthase, respectively, are involved in plant terpenoid metabolism, which has close relationships to plant developmental processes as well as biotic and abiotic stress responses (Blee, 2002; Tholl, 2006). Another two genes in the subcategory related to alkene and isoprenoid metabolism, *Os09g35010* and *Os09g35030* encoding DREB-type transcription factors, are predicted to be involved in plant stress

Table 1. Analysis of overpresented GO subcategories in up-regulated genes of *OsWRKY13*-overexpressing plants[a]

GO ID	Term of subcategory	Total[b]	Changed[c]	*P* value[d]
Biological process (GO: 0008150)				
0044271	Nitrogen compound biosynthesis	162	7	0.0022
0009309	Amine biosynthesis	152	6	0.0071
0008652	Amino acid biosynthesis	135	6	0.0040
0009067	Aspartate family amino acid biosynthesis	23	5	0.0000
0009088	Threonine biosynthesis	3	2	0.0004
0009086	Methionine biosynthesis	8	2	0.0033
0006519	Amino acid and derivative metabolism	475	12	0.0069
0009066	Aspartate family amino acid metabolism	38	5	0.0001
0006566	Threonine metabolism	5	2	0.0012
0019748	Secondary metabolism	431	13	0.0011
Molecular function (GO: 0003674)				
0016491	Oxidoreductase activity	846	20	0.0011
0030611	Arsenate reductase activity	11	2	0.0064
0008794	Arsenate reductase (glutaredoxin) activity	11	2	0.0064
0030613	Oxidoreductase activity, acting on phosphorus or arsenic in donors	11	2	0.0064
0030614	Oxidoreductase activity, acting on phosphorus or arsenic in donors, with disulfide as acceptor	11	2	0.0064
0030508	Thiol-disulfide exchange intermediate activity	61	4	0.0047
0019202	Amino acid kinase activity	5	2	0.0012
0016774	Phosphotransferase activity, carboxyl group as acceptor	12	2	0.0076
Cellular component (GO: 0005575)				
0046658	Anchored to plasma membrane	7	2	0.0025

a The detailed information of genes involved in this table is shown as Supplemental Table 3.
b The total number of genes expressed in the chip, counting (without duplication) all the genes of all of its descendant categories.
c The number of up-regulated genes within this category, counting (without duplication) all the genes of all of its descendant categories.
d The categories with P value < 0.01 using Fisher's exact test and false discovery ratio (FDR) < 0.1 correction are indicated.

Table 2. Analysis of overpresented GO subcategories in down-regulated genes of *OsWRKY13*-overexpressing plants[a]

GO ID	Term of subcategory[b]	Total[c]	Changed[d]	*P* value[e]
Biological process (GO: 0008150)				
0009682	Induced systemic resistance	12	4	0.0000
0009864	Induced systemic resistance, jasmonic acid mediated signaling pathway	12	4	0.0000
0009867	Jasmonic acid mediated signaling pathway (induced systemic resistance, jasmonic acid mediated signaling pathway)	51	5	0.0004
0015849	Organic acid transport	60	5	0.0009
0046942	Carboxylic acid transport	60	5	0.0009
0006865	Amino acid transport	55	5	0.0006
0015801	Aromatic amino acid transport	10	3	0.0002
0015827	L-tryptophan transport	10	3	0.0002
0015800	Acidic amino acid transport	13	3	0.0005
0015810	L-aspartate transport	10	3	0.0002
0015807	L-amino acid transport	14	3	0.0006
0015804	Neutral amino acid transport	17	3	0.0012
0015837	Amine transport (amino acid transport)	55	5	0.0006
0043449	Alkene metabolism	126	7	0.0011
0042214	Terpene metabolism	110	7	0.0005
0006721	Terpenoid metabolism	110	7	0.0005
0006720	Isoprenoid metabolism	130	7	0.0013
0016096	Polyisoprenoid metabolism (terpenoid metabolism)	110	7	0.0005
0009631	Cold acclimation	17	3	0.0012
Molecular function (GO: 0003674)				
0005342	Organic acid transporter activity	79	6	0.0005
0046943	Carboxylic acid transporter activity	79	6	0.0005
0015171	Amino acid transporter activity	72	6	0.0003
0015172	Acidic amino acid transporter activity	15	3	0.0008
0005275	Amine transporter activity	83	6	0.0006
0015203	Polyamine transporter activity	72	6	0.0003
0005279	Amino acid-polyamine transporter activity	72	6	0.0003
0015359	Amino acid permease activity (amino acid transporter activity)	57	5	0.0007
0016846	Carbon-sulfur lyase activity	25	4	0.0002
0043167	Ion binding	710	20	0.0006
0046872	Metal ion binding	710	20	0.0006
0005507	Copper ion binding	65	6	0.0002

a The detailed information of genes involved in this table is shown as Supplemental Table 4.
b The subcategory name presented in the parentheses indicates that the same genes as shown in the previous subcategory with the same name are involved.
c The total number of genes expressed in the chip, counting (without duplication) all the genes of all of its descendant categories.
d The number of up-regulated genes within this category, counting (without duplication) all the genes of all of its descendant categories.
e The categories with P value < 0.01 using Fisher's exact test and false discovery ratio (FDR) < 0.1 correction were indicated.

responses (e.g., drought or cold acclimation) (Dubouzet et al., 2003). A significant change in the subcategory "cold acclimation" of "biological process" is also detected (Table 2). There are four significantly changed subcategories in the GO term "molecular function," which includes "organic acid transport activity," "amine transport activity," "carbon-sulfur lyase activity," and "ion binding" (Table 2). No significantly changed subcategory was detected in the GO term "cellular component."

In addition to *OsWRKY13*, among the differentially expressed genes in *OsWRKY13*-overexpressing plants, a total of 33 genes encode putative transcription factors, including

9 up-regulated and 24 down-regulated (Supplemental Tables 3 and 4). This result suggests that the differential expression of large numbers of genes in the transgenic plants may be through the action of other transcription factors regulated by OsWRKY13.

Large numbers and different types of genes are transcriptionally regulated by OsWRKY13, suggesting that it may be an important regulator during adaptive responses. The expression profiling of *OsWRKY13*-overexpressing lines provides hints for characterizing the crosstalk between the disease resistance pathway and other known pathways and cellular processes via OsWRKY13.

OsWRKY13 Overexpression Affects the Glutathione/ Glutaredoxin System to Monitor Redox Homeostasis

Instantaneous generation of ROS, such as H_2O_2, the superoxide anion, and the hydroxyl radical, is a characteristic of both biotic and abiotic stress responses (for a review, see Fujita et al., 2006). ROS exert various effects on plant defense responses, such as antimicrobial effects and strengthening cell walls against pathogen invasion; however, ROS are also highly toxic to cells (Gozzo, 2003; Zhao et al., 2005). Based on GO classification, 20 genes in the "oxidoreductase activity" subcategory of "molecular function," which are putatively involved in redox homeostasis, were induced by OsWRKY13 (Table 1 and Supplemental Table 3). One of the cellular defense systems in response to oxidative stress is the glutathione/glutaredoxin (Grx) system. Grxs have been suggested to be protective against oxidative stress by catalyzing the reduction of protein mixed disulfides formed with tripeptide glutathione (GSH) (Wheeler and Grant, 2004). Two (*Os12g35330* and *Os11g43520*) of the 20 induced genes in the "oxidoreductase activity" subcategory encode putative glutaredoxin family proteins Grx_C12 and Grx_C17, which may function in the inhibition of ROS formation in rice (Figure 1A). Glutathione *S*-transferase (GST) is known to protect cells from a wide range of biotic and abiotic stresses (Dixon et al., 2002). GST functions by detoxifying many xenobiotic compounds, including oxidative stress metabolites, by conjugating them to GSH (Figure 1A). Three genes, *Os01g27390*, *Os11g14040*, and *Os01g72170,* putatively encoding GSTF2, GSTzeta, and GST24, were induced by OsWRKY13. The induced expression of genes involved in oxidoreductase activity suggests that *OsWRKY13*-overexpressing plants may be more resistant to oxidative stress.

To examine the above hypothesis, we treated rice leaves with different concentrations of paraquat. Paraquat, also known as methyl viologen, inhibits ferredoxin reduction in photosystem I by autooxidizing to a radical, resulting in the production of superoxide and H_2O_2 (Babbs et al., 1989). The size of the lesions was enlarged with increasing concentrations of paraquat treatment in both *OsWRKY13*-overexpressing and wild-type plants (Figure 1B). However, compared with wild-type plants, *OsWRKY13*-overexpressing lines showed smaller lesions caused by paraquat, indicating that these transgenic plants are more tolerant to ROS generated by paraquat treatment than wild-type plants.

Expression of four of the above-listed five genes, *Os12g35330*, *Os11g43520*, *Os01g27390*, and *Os11g14040* putatively encoding Grxs or GSTs, in response to *Xoo* infection, was analyzed. *OsWRKY13* expression was obviously induced at 1 to 4 d after *Xoo* infection in different rice lines (Wen et al., 2003; Qiu et al., 2007). Thus the samples collected from plants at 2 and 4 d after infection were used to analyze the expression of the genes putatively under the regulation of OsWRKY13. *Xoo* infection induced maximally 2.6- to 39.4-fold more transcripts of the four genes in susceptible wild-type plants, but induced *Os11g14040* and *Os11g43520* and suppressed *Os01g27390* and *Os12g35330* in *OsWRKY13*-overexpressing plants (Figure 1C). After PXO61 infection, the transcript levels of *Os01g27390*, *Os11g14040*, and *Os12g35330* were also higher in *OsWRKY13*-overexpressing than in wild-type plants, but the transcript level of *Os11g43520* was significantly lower in *OsWRKY13*-overexpressing than in wild-type plants, indicating that *Os11g43520* expression may also be regulated by other factors. These results suggest OsWRKY13 may monitor redox homeostasis via transcriptional activation of glutathione/glutaredoxin system in pathogen-induced defense responses.

OsWRKY13 Overexpression Activates Flavonoid Biosynthesis Pathway Genes

Our previous study has shown that OsWRKY13 induces the expression of *CHS* encoding chalcone synthase (Qiu et al., 2007), which functions on a branch of the phenylpropanoid pathway involved in flavonoid biosynthesis (Figure 2A), including defensive secondary metabolites, such as phytoalexins (Lee et al., 1995). The genome-wide gene expression profiling showed that two other genes *Os01g24980* and *Os08g44830*, putatively functioning downstream of CHS, also induced by OsWRKY13 (Figure 2A). *Os01g24980* encodes flavonone-3-hydroxylase (F3H), which catalyzes the 3-hydroxylation of flavanones to dihydroflavonols leading to the formation of anthocyanins, condensed tannins, and flavonols (Bogs et al., 2006). These flavonoid compounds are involved in coloration of plant tissues and abiotic stress; some of these compounds can function as phytoalexins in plant disease resistance when the constitutive concentrations are sufficiently high to be antimicrobial (Dixon and Paiva, 1995; Dixon, 2001; Winkel-shirley, 2001). *Os08g44830* encodes a zinc-finger protein that is highly homologous to Arabidopsis TT1 (transparent testa 1) (score = 107, E-value = 9e-22), a putative transcriptional regulator involved in flavonoid metabolism (Sagassar et al., 2002). In addition to being induced by OsWRKY13, *Xoo* infection also significantly induced the expression of *F3H* and *TT1* in both *OsWRKY13*-overexpressing and susceptible wild-type plants (Figure 2B). The two genes showed the similar induced expression patterns in the two types of plants, except that the expression levels of the two genes was significantly higher in *OsWRKY13*-overexpressing plants than in wild-type plants.

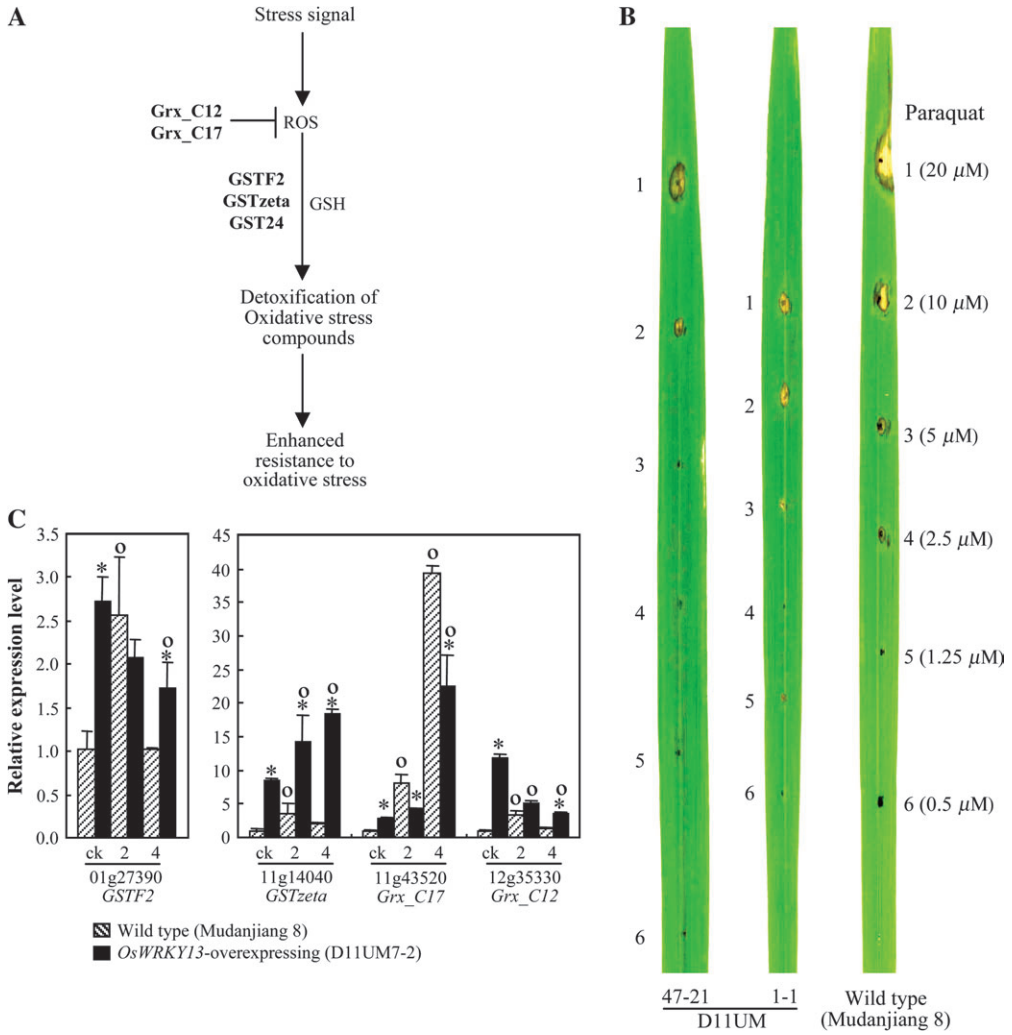

Figure 1. OsWRKY13 and pathogen infection influence the expression of genes putatively functioning in the oxidative stress response pathway.

D11UM47-21, D11UM1-1, and D11UM7-2 are *OsWRKY13*-overexpressing lines. Grx_C12, glutaredoxin_C12; Grx_C17, glutaredoxin_C17; GST, glutathione *S*-transferase.

(A) Schematic overview of the oxidative stress pathway.

(B) Rice leaves at 2 d after paraquat treatment. Totally, at least ten fully-expanded leaves from five transgenic lines and wild-type Mudanjiang 8 were examined, respectively, in three repeated experiments. Similar results were obtained in repeated experiments and typical examples are shown here.

(C) Expression of OsWRKY13-induced genes in *OsWRKY13*-overexpressing line and wild-type plants after infection with *Xoo* strain PXO61 analyzed by qRT-PCR. Bars represent mean (three replicates) ± standard deviation. Samples were obtained before PXO61 inoculation (ck) and at 2 and 4 d after inoculation. The expression level of each gene in D11UM7-2 was calculated relative to that in noninoculated wild-type plants. Circles indicate a significant difference ($P < 0.05$, T-test) between noninoculated and inoculated plants and asterisks indicate a significant difference ($P < 0.05$) between the D11UM7-2 and wild-type plants within the same treatment. Similar results were obtained in two independent biological experiments with independent transgenic lines.

The development of lesions in lesion mimic rice mutants is frequently associated with accumulation of the flavonoid phytoalexin, sakuranetin (Jung et al., 2005; Mori et al., 2007). Accumulation of sakuranetin enhances rice resistance to fungal pathogens (Dillon et al., 1997). Our results showed that accumulation of sakuranetin was associated with induction of *TT1* expression and not associated with *F3H* expression in a rice T-DNA insertion mutant that developed lesions spontaneously; sakuranetin could not be detected in rice plants without lesions (X. Ding and S. Wang, unpublished data). This result further suggests that TT1 may be involved in sakuranetin synthesis. In addition, TT1 and F3H may function in different subbranches of the flavonoid biosynthesis pathway, and F3H may be involved in the synthesis of flavonoids other than sakuranetin (Figure 2A). However, no sakuranetin was detected in *OsWRKY13*-overexpressing and wild-type plants. This may

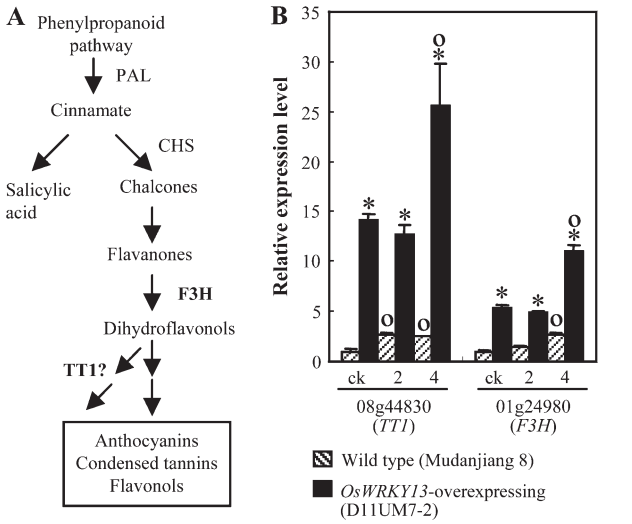

Figure 2. OsWRKY13 and pathogen infection activate genes putatively functioning in flavonoid biosynthesis pathway.

(A) Schematic overview of the branch of phenylpropanoid pathway involved in flavonoid biosynthesis. Question mark indicates that the functional position has yet to be confirmed. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; F3H, flavonone-3-hydroxylase; TT1, transparent testa 1 (a putative zinc-finger family transcription factor).

(B) Expression of OsWRKY13-induced genes in *OsWRKY13*-overexpressing line and wild-type Mudanjiang 8 after infection with *Xoo* strain PXO61 analyzed by qRT-PCR. Bars represent mean (three replicates) ± standard deviation. Samples were obtained before PXO61 inoculation (ck) and at 2 and 4 d after inoculation. The expression level of each gene in D11UM7-2 was calculated relative to that in noninoculated wild-type plants. Circles indicate a significant difference ($P < 0.05$, T-test) between noninoculated and inoculated plants and asterisks indicate a significant difference ($P < 0.05$, T-test) between the D11UM7-2 and wild-type plants within the same treatment. Similar results were obtained in two independent biological experiments with independent transgenic lines.

be because TT1-induced sakuranetin synthesis in *OsWRKY13*-overexpressing plants was under the quantitatively detectable level. This hypothesis can be supported by the fact that no lesion mimic phenotype has been observed in *OsWRKY13*-overexpressing plants. These results suggest that OsWRKY13 may be an important activator of the flavonoid biosynthesis pathway, although further quantitative analysis of different flavonoid metabolites in *OsWRKY13*-overexpressing plants is needed.

Activation of OsWRKY13 Suppresses SNAC1-Involved Abiotic Stress Resistance Pathway

A recent study reported that rice transcription factor SNAC1 was involved in abiotic stress responses (Hu et al., 2006). Overexpressing *SNAC1* enhanced rice resistance to drought, salt and cold stresses; the *SNAC1*-overexpressing plants showed increased expression of another gene *ERD1*, suggesting that ERD1 functions downstream of SNAC1 in defense responses of these abiotic stresses (Figure 3A). The genome-wide gene expression profiling showed that two genes *Os03g60080* and *Os02g32520* encoding SNAC1 and ERD1, respectively, were suppressed in *OsWRKY13*-overexpressing plants (Supplemental Table 2), indicating that *OsWRKY13*-overexpressing plants may be more susceptible to abiotic stresses. To test this hypothesis, independent *OsWRKY13*-overexpressing lines (D11UM1-1, D11UM7-2, and D11UM47-21) were treated with NaCl or cold stress, respectively. After treatment with 150 mM NaCl for 15 days, the growth of transgenic seedlings was retarded. The average height of transgenic plants was 5.2 ± 0.9 cm shorter than that of wild-type plants (Figure 3B). The average root length and fresh weight of transgenic plants were 0.65 ± 0.18 cm and 0.02 ± 0.01 g/plant less than those of wild-type plants, respectively (Figure 3C). After treatment at lower temperature (4°C) for 2 d followed by room temperature (25°C) for 6 d, approximately 57% to 64% of the transgenic plants had died, but only 28% of the wild-type plants had died (Figure 3D).

To determine whether *SNAC1* and *ERD1* were also suppressed in response to pathogen infection in wild-type plants, their expression in Mudanjiang 8 was examined. The expression level of *SNAC1* and *ERD1* was significantly suppressed at 2 d after infection and then recovered to the original level at 4 d after infection of *Xoo* in the susceptible wild-type plants (Figure 3E). The pathogen-induced suppression of *SNAC1* and *ERD1* was not observed in *OsWRKY13*-overexpressing plants, but overexpressing *OsWRKY13* resulted in a significantly lower level of *SNAC1* and *ERD1* transcripts as compared to wild-type plants. *SNAC1* was grouped into the "jasmonic acid-mediated signaling pathway" subcategory of GO term "biological process" among the down-regulated genes (Supplemental Table 4). Another gene, *02g43790* encoding the BIERF3 transcription factor in this subcategory, showed the similar expression pattern as *SNAC1* and *ERD1* on bacterial infection (Figure 3E). Overexpressing *SNAC1* significantly induced *BIERF3* expression, suggesting that BIERF3 functions downstream of SNAC1 (Figures 3A, F). However, overexpressing *SNAC1* did not significantly influence the expression of another two genes, *01g58420* and *05g41780* encoding ERF3- and AP2-containing transcription factors, respectively (data not shown), which were also grouped into the "jasmonic acid-mediated signaling pathway" subcategory (Supplemental Table 4). These results suggest that OsWRKY13, a positive regulator of disease resistance, appears to be a negative regulator of defenses against abiotic stresses through the SNAC1-involved pathway.

Activation of OsWRKY13 Suppresses the JA-Signaling Pathway and Terpenoid Metabolism Pathway

JA is an essential plant hormone that regulates certain types of disease resistance, such as resistance against necrotrophic pathogens, insect resistance, abiotic defense responses, and developmental processes (for a review, see Lorenzo and Solano, 2005). JA is synthesized following a route starting with unsaturated fatty acids, and lipoxygenase (LOX) and allene oxide synthase (AOS) are two important enzymes in this route

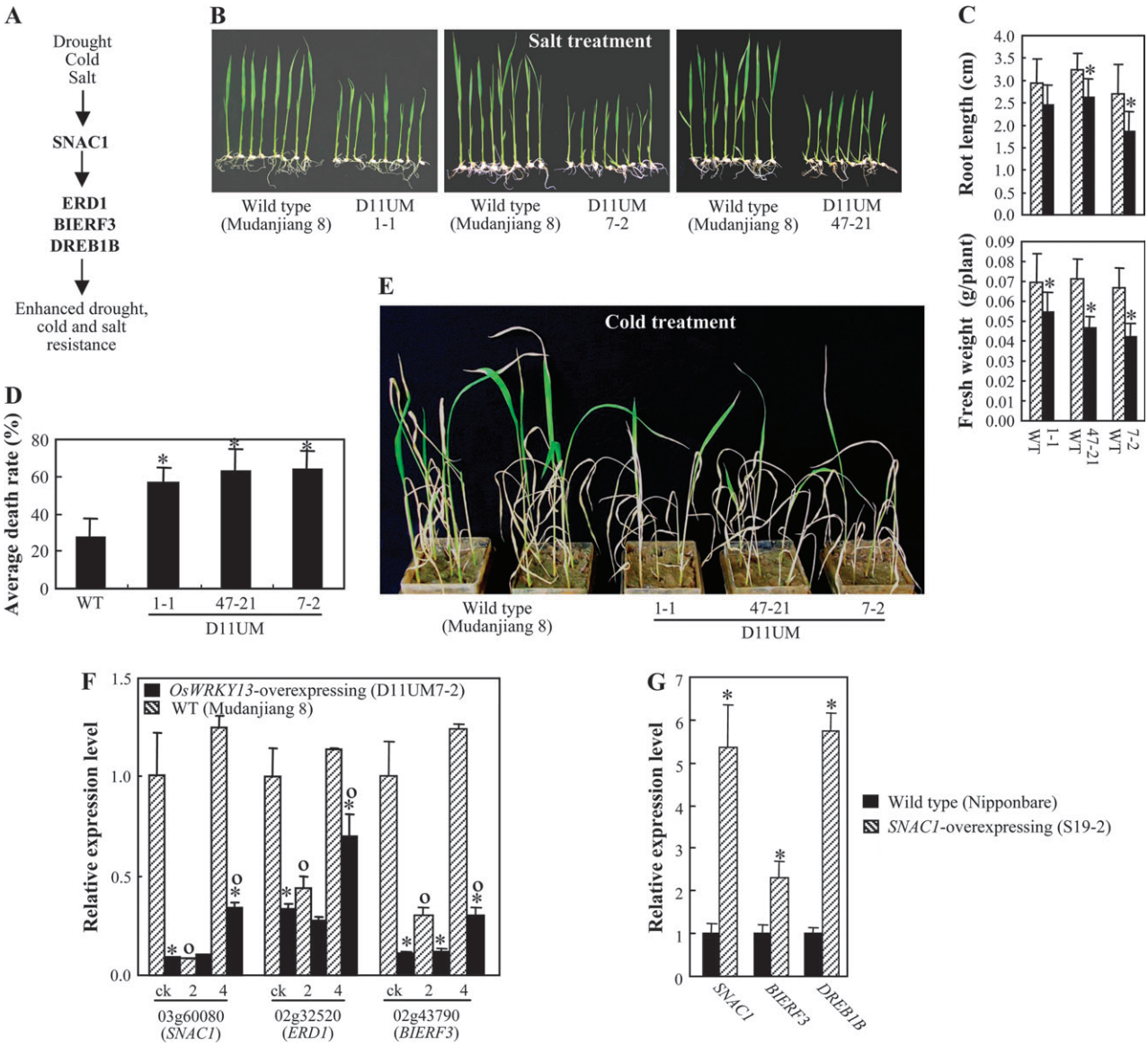

Figure 3. OsWRKY13 and pathogen infection influence the expression of genes functioning in an abiotic stress resistance pathway.
D11UM1-1, D11UM7-2, and D11UM47-21 are *OsWRKY13*-overexpressing lines. Mudanjiang 8 is wild-type (WT).
(A) Schematic overview of a known abiotic stress pathway of rice (Hu et al., 2006).
(B and C) Increased salt susceptibility of *OsWRKY13*-overexpressing plants. Transgenic and wild-type plants were treated with 150 mM NaCl for 15 d. Asterisks indicate a significant difference ($P < 0.05$, T-test) between transgenic and wild-type plants within the same treatment. Bars represent mean (8 replicates) ± standard deviation. Similar results were obtained in two independent biological experiments.
(D and E) Increased cold susceptibility of *OsWRKY13*-overexpressing plants. Transgenic and wild-type plants were treated at 4°C for 2 d followed by 6 d at room temperature (25°C). Asterisks indicate a significant difference ($P < 0.05$) between transgenic and wild-type plants. Bars represent mean (8 replicates) ± standard deviation. Similar results were obtained in two independent biological experiments.
(F) Expression of OsWRKY13-suppressed genes in *OsWRKY13*-overexpressing and wild-type plants upon infection with *Xoo* strain PXO61 analyzed by qRT-PCR. Samples were obtained before PXO61 inoculation (ck) and at 2 and 4 d after inoculation. The expression level of each gene in D11UM7-2 was calculated relative to that in noninoculated wild-type plants. Circles indicate a significant difference ($P < 0.05$) between noninoculated and inoculated plants and asterisks indicate a significant difference ($P < 0.05$) between the D11UM7-2 and wild-type plants within the same treatment. Similar results were obtained in two independent biological experiments with independent transgenic lines (E and F). Bars represent mean (three replicates) ± standard deviation (E and F).
(G) Expression of *BIERF3* and *DREB1B* was induced in *SNAC1*-overpressing line (S19-2). Asterisks indicate a significant difference ($P < 0.01$, T-test) between the S19-2 and wild type.

(Schaller, 2001; Zhao et al., 2005) (Figure 4A). Our previous study showed that OsWRKY13 suppressed the expression of *LOX* and *AOS2* (Qiu et al., 2007). The present results further confirm that activation of OsWRKY13 inhibits JA synthesis (Figure 4B). Pathogen inoculation induced the accumulation of JA in rice leaves of both *OsWRKY13*-overexpressing and susceptible wild-type plants. However, the JA level was 2.4- to 12.8-fold lower in *OsWRKY13*-overexpressing plants than in wild-type plants before and after bacterial infection.

As expected, the genome-wide expression profiling showed that the "JA-mediated signaling pathway" subcategory of GO classification was enriched with down-regulated genes (Supplemental Table 4). All five genes (Os04g52090/*ERF4*, Os01g58420/*ERF3,* Os05g41780/*AP2-containing*, Os02g43790/

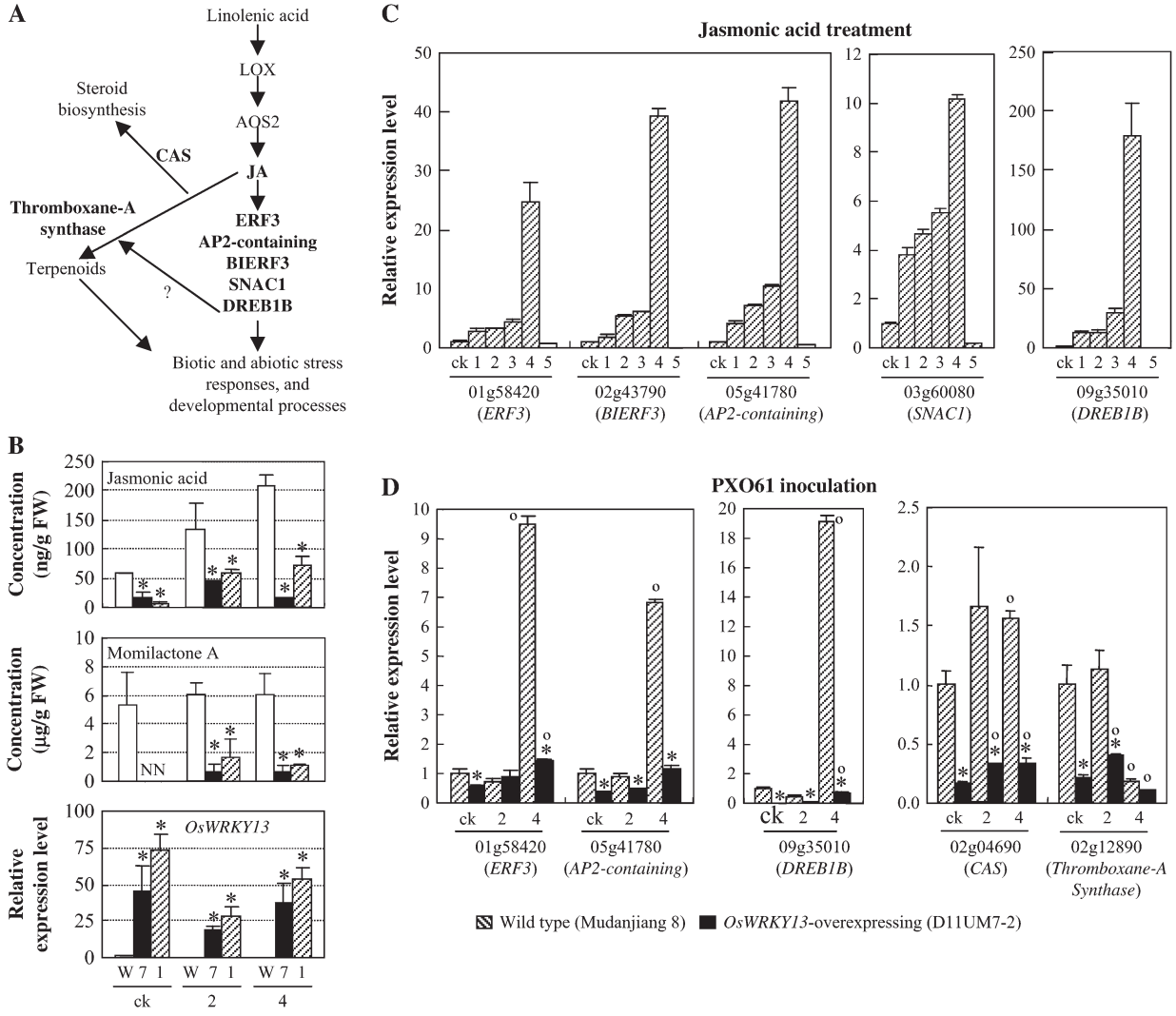

Figure 4. OsWRKY13 and pathogen infection influence the expression of genes putatively functioning in the JA signaling and terpenoid metabolism pathways.

Bars represent mean (three replicates) ± standard deviation (B, C and D).

(A) Schematic overview of the pathways. Question mark indicates that the functional position has yet to be confirmed.

(B) Effects of overexpressing *OsWRKY13* and pathogen infection on endogenous JA and momilactone A levels. W, wild-type Mundanjiang 8; 1 and 7, *OsWRKY13*-overexpressing lines, D11UM1-12 and D11UM7-2; FW, fresh weight; N, not detected. Asterisks indicate a significant difference ($P < 0.05$) between transgenic and wild-type plants. Samples were obtained before *Xoo* strain PXO61 inoculation (ck) and at 2 and 4 d after inoculation (B and D).

(C) Expression of OsWRKY13-suppressed genes after JA treatment in rice cultivar Minghui 63 analyzed by qRT-PCR. Samples were obtained before (ck) and at 0.5 (1), 2 (2), 4 (3), 8 (4), and 24 (5) h of JA treatment.

(D) Expression of OsWRKY13-induced genes in *OsWRKY13*-overexpressing and wild-type plants on *Xoo* infection analyzed by qRT-PCR. CAS, cycloartenol synthase. Circles indicate a significant difference ($P < 0.05$) between noninoculated and inoculated plants and asterisks indicate a significant difference ($P < 0.05$) between the *OsWRKY13*-overexpressing and wild-type plants within the same treatment. Similar results were obtained in two independent biological experiments with independent transgenic lines.

BIERF3, and Os03g60080/*SNAC1*) in this subcategory encode AP2/ERF (APETALA2/ethylene response factor) or SNAC types of transcription factors, which are mostly associated with developmental and growth processes as well as abiotic stress responses (Shinozaki et al., 2003; Olsen et al., 2005; Hu et al., 2006; Nakano et al., 2006). To confirm whether these genes were responsive to JA, the expression patterns of four of the five genes, *ERF3, AP2-containing*, *BIERF3*, and *SNAC1*, in response to JA treatment were examined. The transcript levels of the four genes were maximally increased 10- to 42-fold after JA treatment as analyzed by qRT-PCR (Figure 4C). These results also indicate that JA mediates the signal transduction of *SNAC1*-invovled abiotic stress pathway.

In addition to *SNAC1* and *BIERF3*, for which responses to pathogen infection are shown in Figure 3E, the expression of another two JA-responsive genes (*ERF3* and *AP2-containing*) was also analyzed following infection. The expression of the two genes was pathogen-responsive in wild-type plants (Figure 4D); they were induced at 4 d after infection. The expression levels of the two genes were significantly lower in *OsWRKY13*-overexpressing than in wild-type plants (Figure 4D).

Terpenoids are the secondary metabolites of JA (Creelman and Mullet, 1997) and these JA derivatives are related to plant developmental processes as well as biotic and abiotic stress responses (for a review, see Tholl, 2006) (Figure 4A). Accumulation of momilactone A, a major terpenoid phytoalexin of rice, enhances rice resistance to fungal pathogens (Dillon et al., 1997). The genome-wide expression profiling showed that seven genes in the "terpenoid metabolism" subcategory of GO classification were suppressed in *OsWRKY13*-overexpressing plants (Supplemental Table 4). The expression patterns of three of the seven genes, *Os02g12890* encoding thromboxane-A synthase, *Os02g04690* putatively encoding cycloartenol synthase (CAS), and *Os09g35010* encoding DREB1B transcription factor, were examined on pathogen infection. Thromboxane-A synthase is a member of cytochrome P450 family, which is involved in terpenoid synthesis (Chang et al., 2007). CAS catalyzes the synthesis of cycloartenol, which is the committed precursor of sterol biosynthesis, using oxidosequalene as the template from the isoprenoid pathway for terpenoid synthesis (Chappell, 2002; Qi et al., 2006). *CAS* was induced and *thromboxane-A synthase* was suppressed in susceptible wild-type plants after pathogen infection (Figure 4D). The expression levels of the two genes were significantly lower in *OsWRKY13*-overexpressing than in the wild-type plants. Accompanied by the suppression of thromboxane-A synthase, the level of momilactone A was significantly lower in *OsWRKY13*-overexpressing than in wild-type plants, although pathogen infection induced the accumulation of this phytoalexin in the transgenic plants (Figure 4B). *DREB1B* showed the same expression pattern as the JA-responsive genes *ERF3* and *AP2-containing* on pathogen infection (Figure 4D). Its transcript level increased 179-fold after JA treatment, suggesting that the function of DREB1B is controlled by JA signaling (Figures 4A, C). The expression of *DREB1B* was also significantly induced in *SNAC1*-overexpressing plants, indicating that it also functions downstream of SNAC1 (Figures 3A, F).

In addition to being more susceptible to abiotic stresses, the growth and development of *OsWRKY13*-overexpressing plants was also influenced. Analysis of two *OsWRKY13*-overexpressing lines (D11UM 1-1 and D11UM 7-2) with 10 plants in each line showed that the average booting date of these plants was delayed 7.0 $\pm$ 1.5 d and average plant height was reduced 5.95 $\pm$ 0.56 cm as compared to wild-type Mudanjiang 8. The retarded growth and development could be associated with the suppression of JA signaling and modified expression of terpenoid synthesis-related enzymes, although further study is needed to elucidate the molecular mechanisms. These results suggest that OsWRKY13 may regulate the JA-dependent pathway at multiple sites by suppressing JA synthesis, JA-responsive gene actions, and JA-dependent secondary metabolite synthesis. These results also suggest that the terpenoid phytoalexins are not involved the OsWRKY13-mediated blast resistance.

CONCLUSIONS

The present results suggest that OsWRKY13, an activator in rice resistance to bacterial blight and fungal blast diseases, appears to be an important regulator that directly or indirectly modulates the expression of a large number of genes. The genome-wide expression profiling provides information that allows exploration of the putative functions and signaling pathways mediated by OsWRKY13. Comparing the expression patterns of some differentially expressed genes identified by microarray analysis and the phenotypes between *OsWRKY13*-overexpressing and wild-type plants after pathogen infection, our data suggest that OsWRKY13 is also a regulator of other physiologic pathways during disease resistance by functioning at the convergent points of crosstalk among multiple pathways. The OsWRKY13-associated disease resistance pathway appears to positively interact with the glutathione/glutaredoxin system and flavonoid biosynthesis pathway via OsWRKY13 to monitor redox homeostasis and to putatively enhance the biosynthesis of flavonoid phytoalexins, respectively. This synergistic crosstalk should further enhance disease resistance. In addition, the OsWRKY13-associated disease resistance pathway appears to negatively interact with the SNAC1-involved abiotic stress defense pathway, JA signaling pathway, and terpenoid metabolism pathway via OsWRKY13 to suppress salt and cold stress responses and to putatively retard rice growth and development. This antagonistic crosstalk results in adaptive responses. However, the present results cannot exclude the possibility that some of the differentially expressed genes resulted from nonphysiological overexpression of OsWRKY13. Further comparative analysis of the expression patterns of these genes between *OsWRKY13*-overexpressing and wild-type plants on pathogen infection may answer whether they are responsive in disease resistance in natural conditions.

METHODS

Microarray Materials and Data Analysis

Leaves of a pool of 20 different plants were harvested from the 4-week-old uninfected wild-type Mudanjiang 8 (*Oryza sativa* ssp. *japonica*) and *OsWRKY13*-overexpressing independent homozygous transgenic lines, D11UM1-1 and D11UM7-2 (Qiu et al., 2007), growing in the greenhouse at 26°C with 12 h of daylight. D11UM1-1 and D11UM7-1 carry two and one transgene, respectively, and the two lines have more than 20-fold higher *OsWRKY13* transcript levels than wild type with or without pathogen infection (Fig. 4B). The experiments for each transgenic line and wild type were repeated three times by three independently grown sets of plants for preparing RNA samples used for independent hybridization to the microarrays. Total RNA was extracted with TRIZOL reagent (Invitrogen, Gaithersburg, MD) and was further purified with Nucleospin RNA Clean-up Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The RNA quality was assessed by formaldehyde agarose gel electrophoresis and concentration was quantitated by spectrophotometric analysis. Biotin-labeled cRNA was produced with MessageAmp II-Biotin Kit (Ambion, Houston, TX) with 5 μg total RNA as starting material for each sample reaction. Fifteen micrograms of the resulting biotin-tagged cRNA was fragmented to strands of 35 to 200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). Subsequently, 10 μg of this fragmented target cRNA was hybridized at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 320) to probe sets on an Affymetrix GeneChip Rice Genome Array (http://www.affymetrix.com/products/arrays/specific/rice.affx). This chip contains 57,381 probe sets to query 51,279 transcripts, which was designed based on the 59,712 gene predictions of TIGR's osa1 version 2.0 release. The chip was washed and then stained with streptavidin-phycoerythrin on an Affymetrix Fluidics Station 400, followed by scanning on a GeneChip Scanner 3000 (Affymetrix). The results were quantified and normalized using MicroArray Suite 5.0 (GCOS1.4) software (Affymetrix). The genes with probe set signal lower than 100 on the repeated six arrays for *OsWRKY13*-overexpressing lines D11UM1-1 and D11UM7-2 were considered as nonexpression and filtered out (Hu and Ma, 2006), resulting in 24,283 expressed genes in rice line D11UM1-1 and 24,293 expressed genes in D11UM7-2 for further analysis. The Significance Analysis of Microarrays (SAM) Excel Add-in (Tusher et al., 2001) was used to identify significantly differentially expressed genes between the control and *OsWRKY13*-overexpressing plants. The number of permutation for SAM analysis was 100. The delta value in the SAM was adjusted so that the estimated false discovery rate (FDR) was less than 5% for significant genes. The cutoff value of expression fold change was set as 2.0, which was commonly used for microarray analysis to further increase the stringency (Hu and Ma, 2006). Microsoft's Excel program was used to merge the overlapping significant genes from two transgenic lines. Annotations from Affymetrix for significant genes were checked in NCBI public database using Blastx program (Altschul et al., 1997) and in TIGR Rice Genome Annotation version 4.0 (http://rice.tigr.org, Yuan et al., 2005) and corrected if necessary.

Classification of Differentially Expressed Genes

To classify the genes according to their putative function, the Gene Ontology (Gene Ontology Consortium, 2001) term was used to perform a classification analysis. The GoMiner software was also used for this analysis to classify genes to different categories and subcategories in GO terms "biological process," "molecular function," and "cellular component" (Zeeberg et al., 2003). To perform this analysis, we first associated the relationship among Affymetrix rice gene/probe sets, TU annotation of TIGR rice genes, and their related GO information using MySQL (version 5.0, www.mysql.cn), and then finished the localization and customization of those data into the GoMiner database. Statistical significance was determined by Fisher's exact test (Zeeberg et al., 2003).

Pathogen Inoculation

Plants growing in the greenhouse at 25°C with 12 h of daylight were inoculated with Philippine *Xoo* strain PXO61 (provided by the International Rice Research Institute) at the booting stage by the leaf clipping method (Qiu et al., 2007). Mock-inoculated (control) plants were treated under the same condition except that the pathogen suspension was replaced with water.

Chemical and Abiotic Treatments

Rice plants for paraquat or JA treatment were grown in a greenhouse at 25°C, 70% relative humidity, and a 12-h photoperiod for 21 to 28 d. For plant response to oxidative stress, leaves of 28-d seedlings were exposed to a surface application of different concentrations of N,N′-dimethyl-4,4′-bipyridinium dichloride (also known as paraquat, Sigma, St. Louis, MO, USA) in 0.1% Tween-20 solution in the greenhouse as reported previously (Yang et al., 2004). Each site of the leaves was treated with 50 μL paraquat solution. For plant response to JA, the 21-day-old seedlings from rice cultivar Minghui 63 were sprayed with a solution containing 100 μM JA (Sigma) and 0.02% Tween 20 in the greenhouse at 25°C.

Plants for salt or cold stress were grown under 14 h light/10 h darkness at 25°C following the procedures of Hu et al. (2006). For cold stress, transgenic and wild-type plants at the four-leaf stage were transferred into a growth chamber under 14 h light/10 h darkness at 4°C for 2 d; the plants were then recovered by the following 6 d at 25°C. For salt stress, transgenic and wild-type plants germinated and grown on MS medium for 3 d were transferred to the MS medium containing 150 mM NaCl and grown under 14 h light/10 h darkness at 25°C for 15 d.

Quantitative Reverse Transcription-PCR

Total RNA was treated for 30 min with DNase I (Invitrogen) to remove contaminating DNA and used for RT-PCR. The RNA sample for *OsWRKY13*-overexpressing (D11UM7-2, T_3 family) or wild-type (Mudanjiang 8) plants was a mixture isolated from 20 leaves of 10 plants. RT-PCR was conducted as described by Wen et al. (2003).Quantitative PCR was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Supplemental Table 5 lists the PCR primers for all the genes. The expression level of actin was used to standardize the RNA sample for each qRT-PCR. The expression level of each gene in transgenic or treated plants was calculated relative to that in wild-type or untreated plants. The qPCR assay performed in a 25-μL volume containing 1 μL diluted reverse transcription product, 12.5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), and 0.32 μM of each primer. For each analysis, qRT-PCR assays were repeated at least twice with each repetition having three replicates; similar results were obtained in repeated experiments.

Quantification of JA and Phytoalexins

Each sample was harvested from six to eight plants growing in the greenhouse at 25°C with 12 h of daylight at maximum tillering stage. For quantification of endogenous JA, each JA sample was extracted from 1 g leaves. The samples were prepared and quantified using the high-performance liquid chromatography (HPLC)/ESI-MS/MS system as described by Ding et al. (2008). Quantification of the flavonoid phytoalexin, sakuranetin, and terpenoid phytoalexin, momilactone A, was following the procedures of Jung et al. (2005). In brief, leaves were cut into small pieces (1–2 cm), transferred to a glass test tube containing 10 mL of 80% aqueous methanol, and boiled for 10 min. Five microliters of the crude extract was injected into the same HPLC/ESI-MS/MS system used for JA quantification. Sakuranetin and momilactone A were monitored at a combination of m/z (mass-to-charge ratio) 287/287 and 315/271, in a positive-ion mode and multiple reaction monitoring (MRM) mode, respectively.

SUPPLEMENTAL DATA

Supplemental Table 1. The 236 genes showing more than 2-fold enhanced expression in *OsWRKY13*-overexpressing plants compared with wild-type Mudanjiang 8.

Supplemental Table 2. The 273 genes showing more than 2-fold suppressed expression in *OsWRKY13*-overexpressing plants compared with wild-type Mudanjiang 8.

Supplemental Table 3. Genes involved in the GO subcategories enriched in up-regulated genes in *OsWRKY13*-overexpressing plants.

Supplemental Table 4. Genes involved in the GO subcategories enriched in down-regulated genes in *OsWRKY13*-overexpressing plants.

Supplemental Table 5. PCR primers for qRT-PCR analysis.

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