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ORIGINAL RESEARCH

Rice *OsOPRs*: Transcriptional Profiling Responses to Diverse Environmental Stimuli and Biochemical Analysis of OsOPR1

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Abstract The 12-oxo-phytodienoic acid reductase (OPR) is a key enzyme in jasmonic acid (JA) biosynthesis. Previously, we reported the presence of 13 *OPR* isogenes (*OsOPR1-13*) in rice. OsOPRs phylogenetically belong to two subgroups, OPRI and OPRII. OsOPR13 is assigned to the second subgroup, which is involved in JA biosynthesis, while the others are found in the first subgroup. Here, we systematically investigated transcript levels of *OsOPRs* in various tissues and against diverse environmental stresses. Each gene was differentially involved in flower maturation, showing a tissue-specific response. *OsOPR1*, *OsOPR2*, and *OsOPR13* were also active in responses to wounding, a fungal elicitor (chitosan), salt, UV-C irradiation, H_2O_2, and ozone exposure. In the case of JA-responsive *OsOPRs* (*OsOPR1*, *OsOPR2*, *OsOPR6*, *OsOPR10*, and *OsOPR13*), co-application of JA and SA suppressed jasmonate-induced transcript levels and delayed *OsOPR10* expression. We also investigated the biochemical properties of OsOPR1 and found a flavin cofactor with optimal activity at pH7.8 and values of 0.048 min^{-1} k_{cat} and 8.33 μM K_m for (9S,13*R*)-12-oxo-phytodienoic acid. Here, we discuss the role of *OsOPRs* in stress responses and floral development.

Keywords Defense/stress response · Jasmonic acid (JA) · *Oryza sativa* · 12-oxo-phytodienoic acid reductase (OPR) · Oxylipin

The octadecanoid biosynthesis pathway in plants leads to the production of oxylipins, including 12-oxo-phytodienoic acid (OPDA) and jasmonic acid (JA), which are involved in immune responses to biotic and abiotic stresses as well as plant growth and development (Beale and Ward 1998; Howe 2001; Wasternack and Hause 2002; Weber 2002; Wasternack 2007). JA biosynthesis is initiated by the release of α-linolenic acid (LNA) from the chloroplast

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membrane by a lipase and 13(*S*)-hydroperoxydation of LNA by 13-lipoxygenase. This is then terminated by substantial reactions, e.g., the formation of (9*S*,13*S*)-OPDA by allene oxide synthase (AOS) and allene oxide cyclase (AOC), the reduction of (9S,13*S*)-OPDA to the corresponding 3-oxo-2-(2′-pentenyl)-cyclopentane-1-octanoic acid stereoisomer ((9*S*,13*S*)-OPC-8:0) by OPDA reductase (OPR), and three rounds of β-oxidation (Vick and Zimmermann 1983; Hamberg and Gardner 1992; Schaller 2001; Wasternack and Hause 2002). Because this process depends on the substrate stereoselectivity of OPRs, their characteristics have been examined in several plants, such as *Arabidopsis thaliana* (three AtOPRs (AtOPR1-3: Biesgen and Weiler 1999; Schaller et al. 2000)), tomato (three LeOPRs (LeOPR1-3: Strassner et al. 2002)), and pea (six PsOPRs (PsOPR1-6: Ishiga et al. 2002; Matsui et al. 2004)). Stereospecific assays have shown that AtOPR1/2 and LeOPR1 can catalyze the reduction of (9*R*,13*R*)-OPDA but not (9*S*,13*S*)-OPDA, a natural precursor of JA (Schaller et al. 1998, 2000; Strassner et al. 1999, 2002). By comparison, AtOPR3 and LeOPR3 effectively reduce four stereoenantiomers of OPDA to OPC-8:0, are localized to the peroxisomes, and are related to JA biosynthesis (Schaller et al. 1998, 2000; Strassner et al. 1999, 2002).

Previously, we searched the rice full-length KOME (http://cdna01.dna.affrc.go.jp/cDNA) database and reported octadecanoid pathway-related genes from a genomic viewpoint (Agrawal et al. 2004). Therein, 13 *OsOPRs* (OsOPR1–13) are divided into four subgroups—C, D, E, and F. OsOPR13 is highly homologous to AtOPR3 and LeOPR3 involved in JA biosynthesis (F group), while OsOPR3 is similar to AtOPR1/2 and LeOPR1, which catalyze the reduction of (9*R*,13*R*)-OPDA (D group) but not (9*S*,13*S*)-OPDA. Other OsOPRs are assigned to two subgroups: E and those that are highly homologous to members of the OsOPR1 C group. Earlier transcript profiling of *OsOPR1* against diverse environmental stresses has proved its relevance in rice defense responses and plant development (Agrawal et al. 2003a). Tani et al. (2008) have now demonstrated that OsOPR13 (termed OsOPR7) also reduces both enantiomers of *cis*-OPDA, is localized to the peroxisomes, and is involved in JA biosynthesis. Although the biological functions of most OsOPRs remain obscure, the presence of a large number (13) of *OPR* isogenes in rice suggests that each responds variously on developmental stages, different signal molecules, and under stress. Thus, in the present study, we have focused on the transcriptional profiling of all *OsOPR* genes in different tissues and against diverse environmental factors, such as wounding by cutting or the fungal elicitor chitosan, as well as salt, cadmium, drought, UV-irradiation, or exposure to hydrogen peroxide or ozone. We have also investigated the *OsOPR* response to JA, salicylic acid (SA), the protein synthesis inhibitor cycloheximide, and combinations of these stressors.

Materials and Methods

Plant Material and Treatments

Rice seeds (*Oryza sativa* L., japonica-type cv. Nipponbare) were obtained from the National Institute of Agrobiological Sciences, Tsukuba, Japan. They were cultured under white fluorescent light (wavelength 390 to 500 nm, 150 μmol m^{-2} s^{-1}, 12-h photoperiod) at 25°C and 70% relative humidity. The middle portions (2 cm long) of fully expanded leaves from 2-week-old seedlings were used for all treatments under continuous light, as described previously (Agrawal et al. 2003a, b). Leaf segments floated on Milli Q (MQ) water in covered Petri dishes served as the wounding-by-cutting treatment (labeled as CUT in figures). The drought treatment entailed placing segments in open Petri dishes without MQ water. Ultraviolet light (UV-C, 254 nm) was used to irradiate other segments with a Hitachi (Japan) germicidal lamp at a 15-cm distance (Agrawal et al. 2003a, b; Lee et al. 2008). Intact seedlings were fumigated with ozone (O_3; 0.2 ppm) as described previously (Rakwal et al. 2001). Panicles (from three mature plants), leaves (30 from whole seedlings), and leaf segments (30 total) were sampled at times indicated in the figures, then pooled from two independent experiments and stored at –80°C. These tissues were subsequently ground to a very fine powder in a chilled mortar and pestle with liquid nitrogen, and stored at –80°C until further analysis.

Chemicals

Jasmonic acid (±JA), SA, cycloheximide (CHX), SIGMA-SIL-A, NADPH, 2-cyclohexen-1-one (2-CyHE), and Q-Sepharose® Fastflow resin (bead size 45–156) were purchased from Sigma (MO, USA); (9*S*,13*R*)-12-oxo-phytodienoic acid ((9*S*,13*R*)-OPDA), from Cayman Chemical (MI, USA); and sodium chloride (NaCl), hydrogen peroxide (H_2O_2), cadmium chloride ($CdCl_2$), and the fungal elicitor CT (water soluble, MW 3,000 to 30,000), from Wako Pure Chemicals (Japan). Stock solutions were prepared as described previously (Agrawal et al. 2003a, b).

Total RNA Extraction

Total RNA was extracted from healthy and treated tissue powders with the QIAGEN RNeasy Plant Maxi Kit (QIAGEN, MD, USA). The extracted RNA purity and yield were determined spectrophotometrically (NanoDrop, DE, USA) and visually confirmed via formaldehyde-agarose gel electrophoresis.

Transcript Profiles of *OsOPRs* by Semi-Quantitative RT-PCR

Total RNA samples were DNase-treated with an RNase-free DNase (Stratagene, CA, USA). First-strand cDNA was synthesized in a 50 μl reaction mixture with a Strata-Script™ RT-PCR Kit (Stratagene), according to the manufacturer's protocol, using 10 μg of total RNA isolated from healthy and treated tissue powders. This mixture (in 1× buffer recommended by the polymerase manufacturer) contained 200 mM dNTPs, 10 pmol of each primer set, and 0.5 U of Taq polymerase (TaKaRa Ex Taq Hot Start Version; TaKaRa Shuzo Co. Ltd., Japan). Specific primers were designed from the 3′-UTR regions (Table 1) of each *OsOPR* by comparing and aligning them with all available, related genes in the NCBI and KOME (http://cdna01.dna.affrc.go.jp/cDNA/) databases. Thermal-cycling parameters were as follows: after an initial denaturation at 97°C for 5 min, samples were subjected to a regime of 25 to 30 cycles at 95°C for 45 s, 55°C for 45 s, and 72°C for 1 min. After the final cycle, an additional extension step was carried out for 10 min at 72°C (TaKaRa PCR Thermal Cycle Dice, Model TP600; Japan). After PCR was completed, the total reaction mixture was mixed with 2.0 μl of 10× loading buffer and vortexed; 10 μl was then loaded into the wells of a 1.5% agarose gel (Agarose ME; Iwai Chemicals, Japan). Electrophoreses were performed for ca. 30 min at 100 V in 1× TAE buffer, using a Mupid-ex electrophoresis system (ADVANCE, Japan). Gels were stained (20 μl of 50 mg ml^{-1} ethidium bromide in 100 ml of 1× TAE buffer) for 10 min. The stained bands were visualized with a UV-transilluminator (ATTO, Japan), and their intensities were calculated using a band and spot analyzer program (ATTO). The extent of altered levels of *OsOPRs* transcripts was defined as the ratio of their intensities in treated leaves to those in healthy leaves. Values represented the mRNA expression levels of each *OsOPR* minus the wounding-by-cut effect, and all results were the average of repeated RT-PCR experiments. Actin (Accession Number AK100267) gene expression served as a loading control.

Overexpression and Purification of OsOPR1 in *Escherichia coli*

Two primers were designed to introduce an *Nco*I site (underlined) at the 5′ end (5′-CCTAAGCCAACCATGGTG CACCACGCAC-3′) and a *Hind*III site (underlined) at the 3′ end (5′-TTTAGGTGACAAGCTTGCATAAGTA-3′) of the *OsOPR1* cDNA sequence (GenBank Accession No. AJ557138). The PCR product was cloned into the *Nco*I/*Hind*III sites of pET28b to produce a recombinant plasmid,

Table 1 Primer combinations used for RT-PCR

Accession (Gene)	Forward Primer		Reverse Primer		Product size (bp)
	Primer name	Nucleotide sequence (5′-3′)	Primer name	Nucleotide sequence (5′-3′)	
AJ557138 *OsOPR1*	RJSR239	GAGGAAGGGAACAAGGTGGT	RJSR240	TTCATGCTTGTCCGTATCACAT	300
AJ557139 *OsOPR2*	RJSR241	GCACCATTGAACAGGTATGACA	RJSR242	TCGAATACATCTTTCTCGAACA	286
AK102440 *OsOPR3*	RJSR243	GGTGGCTACAATAAGGAGGATG	RJSR244	AATGGCCATATTTCTTCAATCC	312
AK108079 *OsOPR4*	RJSR245	GCCTATGGGAGGCTCTTCTT	RJSR246	TGTAGCGGTAAATAGTGAAATCG	274
AK106409 *OsOPR5*	RJSR247	CGACCTCGTCGTCTACGG	RJSR248	TTAAAGCTTAAGCAGCAGCAAA	297
AK061212 *OsOPR6*	RJSR249	AGGTGATCGAAAATGGCTACAC	RJSR250	TTGCACGCAGGCATATACTAAC	294
AK059887 *OsOPR7*	RJSR251	ATGGCACTTCAGGAAAGTGTTT	RJSR252	AAGAGCTCTGAGGAATGGATTG	305
AK067218 *OsOPR9*	RJSR253	GATGTTCATGGTCGGAGGAG	RJSR254	ACCCAAACACACAAAGATCACA	310
AK105590 *OsOPR10*	RJSR255	AGCTGTTCAATGGCACTTTCAT	RJSR256	TGCACATTACTAAACCGAACATTT	307
AK100034 *OsOPR12*	RJSR257	TCATGGTGAACGGTGGGTA	RJSR258	CACAAATGTCCAAGCTAGATGC	318
AK104843 *OsOPR13*	RJSR259	ACGTACCAGGGCACATTCAT	RJSR260	CGTTGCCCTTTATTTTCCTTAC	310
AK100267 *Actin*	RJSR43	CTCCTAGCAGCATGAAGATCAA	RJSR44	ATGATAACAGATAGGCCGGTTG	294

pET28b/OsOPR1. OsOPR1 protein was overexpressed from transformants of that recombinant plasmid in BL21(DE3). Recombinant cells were grown in an LB medium (containing 30 μg ml^{-1} of kanamycin) with vigorous shaking at 30°C, followed by the addition of IPTG (1 mM) when the OD_{600} of the culture medium reached 0.6. Cells were grown for another 5 h and harvested by centrifugation (3,000×*g*, 15 min, 4°C). They were washed with 50 mM sodium phosphate (pH7.5) and resuspended in the same buffer containing 0.2% Tween 20 and 0.2 mM PMSF. Those resuspended cells were sonicated and centrifuged (13,000×*g*, 1 h, 4°C). The supernatant was loaded onto a Q-Sepharose column that had been equilibrated with 20 mM sodium phosphate (pH7.8). Overexpressed OsOPR1 protein was eluted with 20 mM sodium phosphate containing 100 mM NaCl (pH7.8). Purified OsOPR1 was separated on 10% SDS-PAGE gels using a discontinuous buffer system and a MINI-PROTEAN II Cell electrophoresis kit (Bio-Rad, CA, USA). Samples were mixed with 5× loading buffer (0.25 M Tris-HCl buffer (pH6.8) containing 10% SDS (*w*/*v*), 50% glycerol (*v*/*v*), 0.5% Bromophenol Blue (*w*/*v*), and fresh β-mercaptoethanol) and boiled for 2 min. The gel was run at 140 V for 2 h, then stained by Coomassie Brilliant Blue R-250.

Ph-Profiling Experiments
This reaction was carried out at 25°C in a 0.5-ml reaction mixture consisting of 0.04 mM NADPH, 0.2 mM substrate, and 8.7 μg of the OsOPR1 protein. Buffers over the tested pH range of 4.5 to 9.5 included the following: (1) 50 mM sodium acetate (pH4.5 to 5.5), (2) 50 mM sodium phosphate (pH6.0 to 7.8), (3) 50 mM Tris-HCl (pH8.0 to 8.5), or (4) 50 mM boric acid (pH9.0 to 9.5).

Assay for OsOPR1 Activity

The reaction for OsOPR1 activity was carried out at 25°C. A 0.5-ml reaction mixture consisted of 20 mM sodium phosphate buffer (pH7.8), 0.2 mM NADPH, 0.2 mM substrate ((9*S*,13*R*)-OPDA or 2-cyclohexen-1-one (2-CyHE)], and purified OsOPR1 (10 μg). Activity was determined spectrophotometrically by monitoring NADPH consumption at 366 nm (ε=3,200 M^{-1} cm^{-1}) for 5 min (Schaller and Weiler 1997a, b; Schaller et al. 1998, 2000; Rohde et al. 1999; Ishiga et al. 2002; Matsui et al. 2004). This reaction was stopped by adding acetic acid, and the reaction product was extracted with one volume of diethyl ether and then evaporated. The residual pellet was dissolved in mixed solvent containing *n*-hexen/2-propanol/acetic acid (98.00/1.61/0.11, *v*/*v*/*v*) and injected onto an HPLC system (LC-10A; Shimazu, Japan) equipped with a Mightysil Si 60 column (250×4.6 mm, 5 um particle size; Kanto Chemical, Japan). The reaction products were eluted with *n*-hexen/2-propanol/acetic acid (98.00/1.61/0.11, *v*/*v*/*v*) at a flow rate of 1 ml min^{-1}. Their absorbances at 221 nm for OPDA and 205 nm for OPC-8:0 were recorded simultaneously (Schaller and Weiler 1997a, b; Stelmach et al. 1999). Fractions of OPDA and OPC-8:0 were collected and evaporated. The residual dry pellet was trimethylsilylated with 10 μl of SIGMA-SIL-A at 85°C for 5 min for GC-MS analysis (Crarus 500; Perkin-Elmer, CT, USA). Trimethylsilylated OPDA or OPC-8:0 (1 μl) was injected onto an HP-5MS (30 m×0.25 mm, film thickness 0.25 μm). The injector temperature was set at 280°C. Programming of the column was a step gradient of 80°C (hold for 3 min), 80°C to 230°C (20°C min^{-1}), 230°C to 280°C (3°C min^{-1}), 280°C to 300°C (5°C min^{-1}), and 300°C (hold for 3 min).

Results

Tissue-Specific and Developmental Regulation of OsOPRs

To understand the expression profiles of rice *OsOPR* genes, we designed specific primers for all except *OsOPR8* and *OsOPR11* because their full sequences were unavailable. As a first step, we observed the transcript levels of *OsOPRs* in the first leaf, flag leaf, panicles before and after heading, and at maturity to determine their developmental and tissue-specific regulation (Fig. 1). Transcripts of *OsOPR1*, *OsOPR2*, *OsOPR4*, *OsOPR5*, *OsOPR6*, and *OsOPR12* were higher in the leaves than in the panicles. *OsOPR3* and *OsOPR7* transcripts were constitutively expressed regardless of the degree of floral maturity and were induced at significantly higher levels in all panicle types than in the first and flag leaves. Depending on maturity, *OsOPR10* and *OsOPR13* had enhanced expression while that of *OsOPR9* was suppressed. Our results suggested a role for *OsOPR9*, *OsOPR10*, and *OsOPR13* in flower maturation whereas differential expression levels of the other *OsOPRs* indicated their tissue specificities.

Effect of Wounding and Fungal Elicitor Treatments on *OsOPR* Expression

Oxylipins involved in defense responses to mechanical injury, herbivory, pathogen attack, and other environmental and developmental inputs are synthesized from the octadecanoid pathway (Beale and Ward 1998; Howe 2001; Wasternack and Hause 2002; Weber 2002; Wasternack 2007). Those pathway genes, such as *OsAOS1*, *OsAOC1*, and *OsOPR1*, are differently regulated when exposed to diverse stimuli in 2-week-old rice seedlings (Agrawal et al. 2003a, b, 2004). Here, we first examined the transcript levels of *OsOPR* genes in healthy leaves. RT-PCR analysis

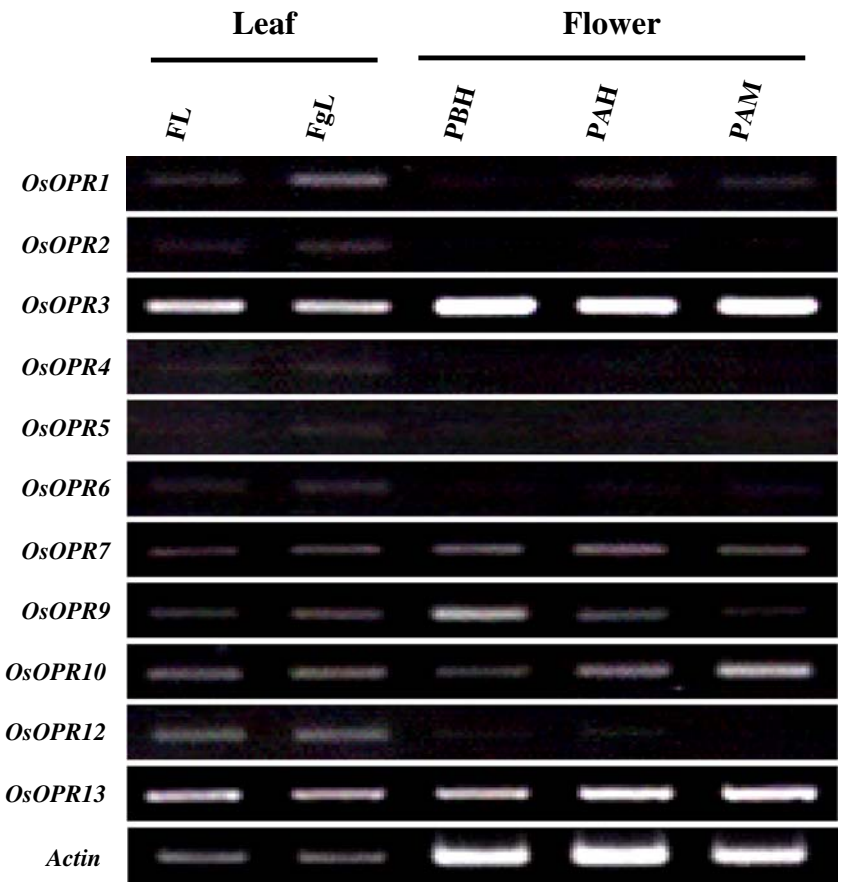

Fig. 1 *OsOPR* expression in first leaf (*FL*), flag leaf (*FgL*), and panicles before heading (*PBH*), after heading (*PAH*), and at maturity (pollination stage, *PAM*) from mature rice (cv. Nipponbare) plants. Each *OsOPR* gene was compared and aligned with all available, related genes in NCBI and KOME (http://cdna01.dna.affrc.go.jp/cDNA/) databases. Actin gene expression served as loading control

revealed that all except *OsOPR3* had very low constitutive expression (Fig. 2). We also checked the intensities of wound-induced transcript levels for *OsOPRs* relative to their basal readings. Transcripts for *OsOPR1*, *OsOPR2*, *OsOPR3*, *OsOPR10*, and *OsOPR13* were transiently induced by eight-, four-, 1.5-, two-, and twofold, respectively, at 30 min after wounding. Those of *OsOPR10* at 60 min and of *OsOPR2* and *OsOPR3* at 90 min, reverted to their basal levels, whereas those of *OsOPR1* and *OsOPR13* were maintained at higher-than-basal readings until 120 min (Fig. 2b). Furthermore, after subtracting the wounding effect (on *OsOPR* transcripts), we found that levels of *OsOPR1*, *OsOPR2*, and *OsOPR13* were dramatically increased, over ten-, seven-, and fivefold, at 60 min after CT treatment and were maintained at similarly high levels until 120 min (Fig. 2c).

Effect of Signal Molecules and a Protein Synthesis Inhibitor on *OsOPR* Expression

Previously, we reported the regulation of octadecanoid pathway genes by treatment with a protein synthesis inhibitor or signal molecules, including JA and SA (Agrawal et al. 2003a, b). Here, we observed that jasmonic and salicylic acids induced the expression of *OsOPR1*, *OsOPR2*, *OsOPR6*, *OsOPR10*, and *OsOPR13*, and that CHX enhanced the expression of *OsOPR1*, *OsOPR2*, *OsOPR4*, *OsOPR6*, *OsOPR10*, and *OsOPR13* (Fig. 3). After the jasmonate treatment, transcripts were maximal for *OsOPR6* and *OsOPR10* at 30 min, *OsOPR2* at 60 min, and *OsOPR1* and *OsOPR13* at 90 min. That of *OsOPR6* reverted to its basal level at 90 min, whereas *OsOPR1*, *OsOPR2*, *OsOPR10*, and *OsOPR13* transcripts were maintained above their basal readings until 120 min (Fig. 3b). Salicylic acid caused the greatest increases in expression, i.e., *OsOPR10* at 30 min, *OsOPR2* and *OsOPR6* at 60 min, *OsOPR13* at 90 min, and *OsOPR1* until 120 min. *OsOPR6* transcript reverted to its basal level at 90 min whereas those of *OsOPR2*, *OsOPR10*, and *OsOPR13* remained higher than their basal levels until 120 min (Fig. 3c). With CHX treatment, transcript levels were highest for *OsOPR10* at 60 min and *OsOPR*, *OsOPR2*, *OsOPR6*, and *OsOPR13* at 90 min; these were maintained there until 120 min. The *OsOPR4* transcript level showed an increase up to 120 min (Fig. 3d). These results suggested that the expression of *OsOPR1*, *OsOPR2*, *OsOPR4*, *OsOPR6*, *OsOPR10*, and *OsOPR13* is negatively regulated by a de novo synthesized protein factor.

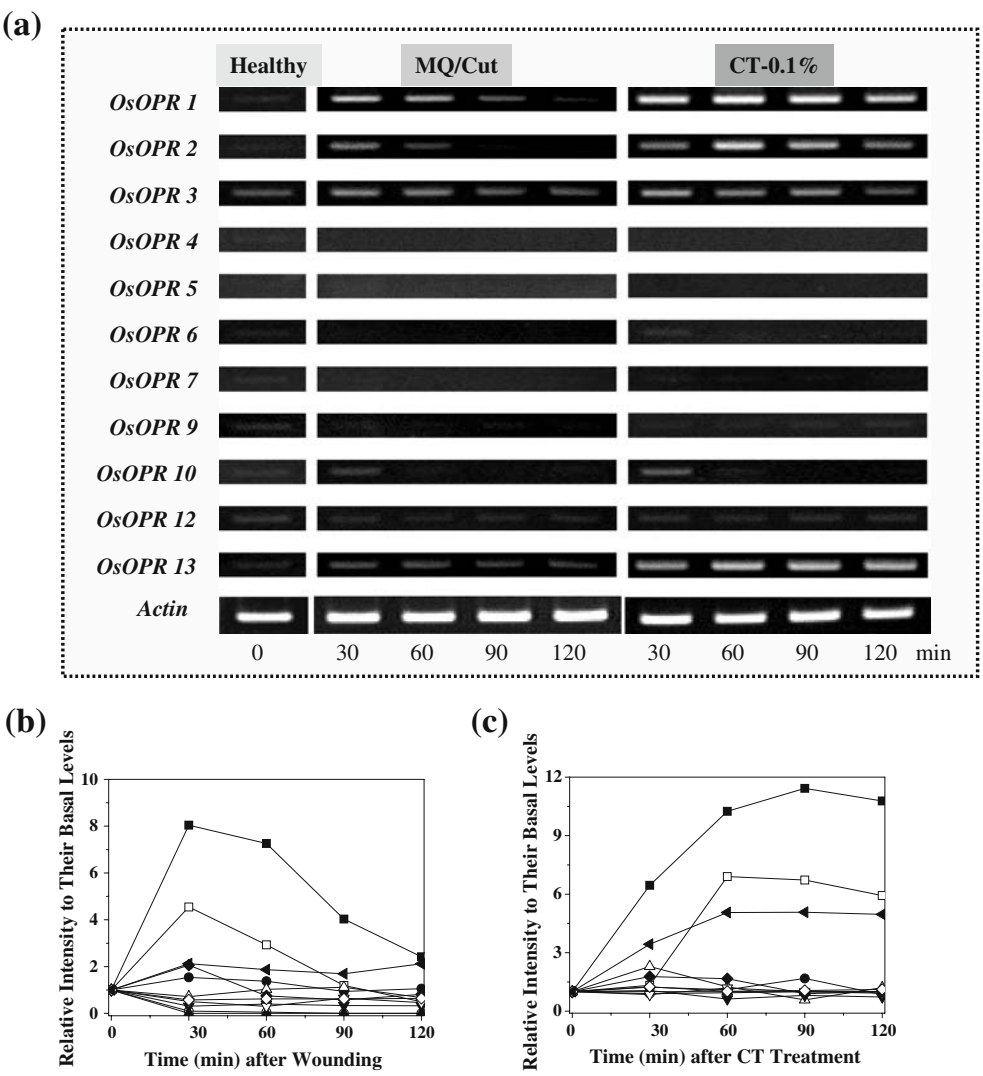

Fig. 2 Transcriptional kinetics of *OsOPRs* against wounding. **a** Leaf segments from 2-week-old seedlings were cut (*CUT*) or treated with *0.1% CT* (fungal elicitor chitosan), under continuous light (150 μmol m^{-2} s^{-1}). **b** Transcript levels were defined as ratios of *OsOPR* intensities for stressed versus control (healthy) leaves. Actin gene expression served as loading control. *OsOPR1* (*filled squares*), *OsOPR2* (*empty squares*), *OsOPR3* (*filled circles*), *OsOPR4* (*empty circles*), *OsOPR5* (*filled triangles*), *OsOPR6* (*empty triangles*), *OsOPR7* (*filled inverted triangles*), *OsOPR9* (*empty inverted triangles*), *OsOPR10* (*filled diamond*), *OsOPR12* (*empty diamond*), and *OsOPR13* (*filled left triangle*)

Effect of Co-applications between JA and SA or JA and CHX on *OsOPR* Expression

Cross-talk can occur between signal components (such as JA and SA) and their involvement in defense responses (Schenk et al. 2000; Agrawal et al. 2003a, b). Here, we examined how *OsOPR* transcript levels were influenced by the co-application of either JA and SA (Fig. 4a, b) or and JA and CHX (Fig. 4a, c). Both combinations increased the transcripts of five *OsOPRs*—*OsOPR1*, *OsOPR2*, *OsOPR6*, *OsOPR10*, and *OsOPR13*—above their basal readings up to 120 min. We also compared the transcript levels of co-application-induced *OsOPRs* with those of genes that had been induced by treatments with JA, SA, or CHX alone (Fig. 5). When JA was combined with SA, the expression kinetics of the co-application-induced *OsOPR1* transcript were similar to those with JA alone but less than the JA-induced transcript level (Fig. 5a). However, expression kinetics of the co-application-induced *OsOPR2* transcript was similar to those of SA as well as the SA-induced transcript level (Fig. 5b). Moreover, we observed that the kinetics of the co-application-induced *OsOPR6* and *OsOPR13* transcripts differed from those seen with JA and SA, and their levels were lower than the JA- and SA-induced transcript levels (Fig. 5c, e). Interestingly, this co-application treatment seemed to delay the expression of *OsOPR10* (Fig. 5d).

We also compared *OsOPR* expression between co-application of JA/CHX and the CHX- or JA-induced treatments (Fig. 6). Transcripts of the co-application-induced *OsOPR1*, *OsOPR2*, *OsOPR6*, and *OsOPR10* gradually increased until 120 min and were higher than CHX-induced levels but lower than JA-induced levels at the early time points (Fig. 6a, b, d, e). This suggested that expression of *OsOPR1*, *OsOPR2*, *OsOPR6*, and *OsOPR10* was regulated by a de novo synthesized negative factor(s) and a JA-induced activator(s). In the case of *OsOPR4* and *OsOPR13*, transcripts induced by the co-application were lower than those of CHX-induced levels and higher than the JA-induced levels (Fig. 6c, f). This again implied that expression of

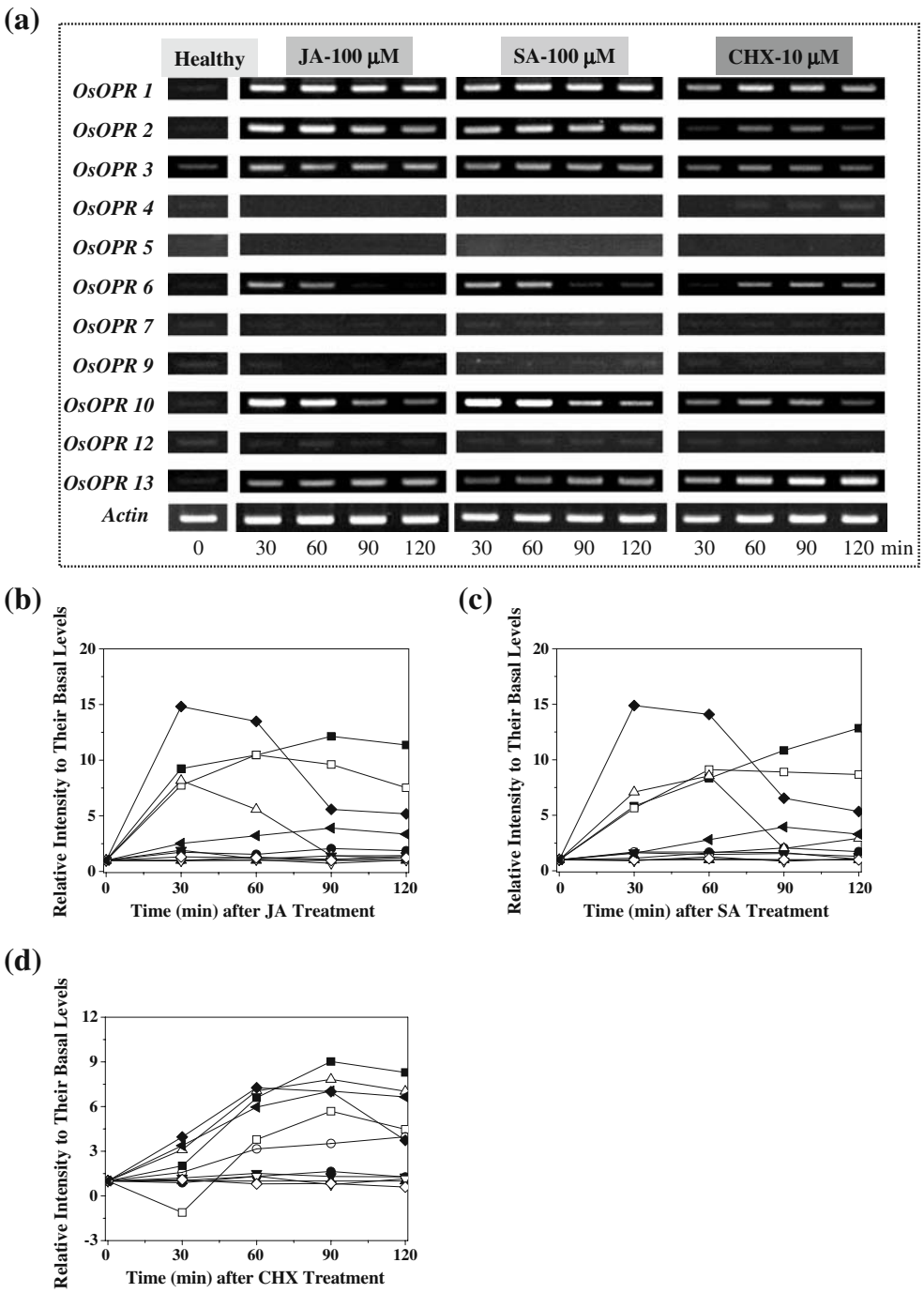

Fig. 3 a–d Transcriptional kinetics of *OsOPRs* against JA, SA, and protein synthesis inhibitor CHX. **a** Leaf segments were treated with 100 μM of *JA*, *SA*, or *CHX*. **b** Transcript levels were defined as ratios of *OsOPR* intensities for stressed versus control (healthy) leaves. Actin gene expression served as loading control. Intensities were calculated by subtracting wounding effect in treated segments. *OsOPR1* (*filled squares*), *OsOPR2* (*empty squares*), *OsOPR3* (*filled circles*), *OsOPR4* (*empty circles*), *OsOPR5* (*filled triangles*), *OsOPR6* (*empty triangles*), *OsOPR7* (*filled inverted triangles*), *OsOPR9* (*empty inverted triangles*), *OsOPR10* (*filled diamonds*), *OsOPR12* (*empty diamonds*), and *OsOPR13* (*filled left triangles*)

OsOPR4 and *OsOPR13* was regulated by a de novo synthesized negative factor(s) and a JA-induced repressor(s).

Differential Regulation of *OsOPRs* in Response to Environmental Cues

OsOPR transcript levels were monitored in their response to the environmental stresses of high salt, drought, cadmium, UV-C irradiation, H_2O_2 (Grant and Loake 2000; Agrawal et al. 2003a, b), and O_3 (Fig. 7a, b; Agrawal et al. 2003a, b). Treatment with NaCl increased the expression of *OsOPR1* until 60 min, *OsOPR13* until 90 min, and *OsOPR2* until 120 min, whereas transcripts of *OsOPR1* and *OsOPR13* were reduced until 120 min (Fig. 7a, c). Cd application transiently increased the levels of *OsOPR1* and *OsOPR2* at 60 min. Those of *OsOPR1* and *OsOPR2* were maintained at higher-than-basal levels until 120 min while that of *OsOPR13* was slightly increased over the same time period (Fig. 7e). However, drought stress did not result in any clear change in *OsOPR* transcript levels (Fig. 7d).

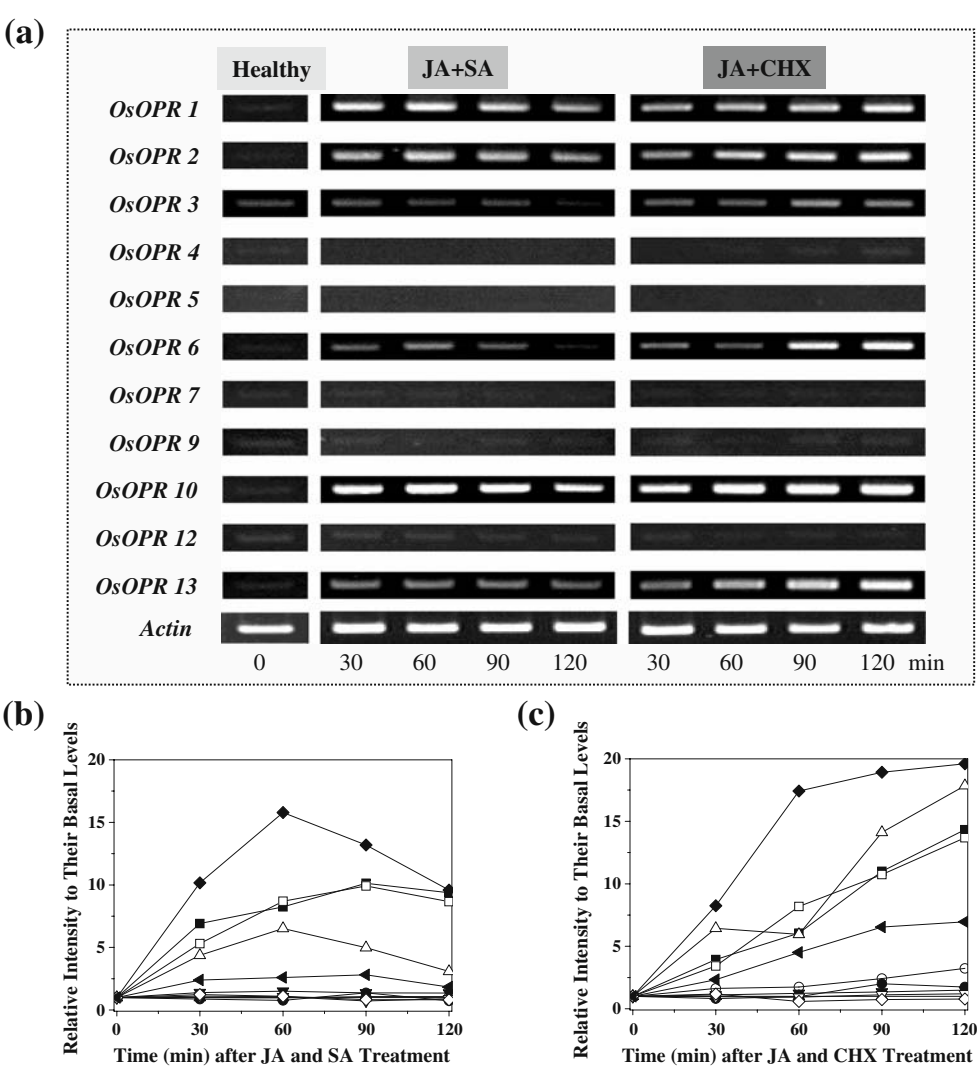

Fig. 4 **a**–**c**Transcriptional kinetics of *OsOPRs* in co-application treatments. **a** Leaf segments were treated with 100 μM each of JA and SA, or 100 μM each of JA and CHX. **b** Transcript levels were described as ratios of *OsOPR* intensities for stressed versus control (healthy) leaves. Actin gene expression served as loading control. Intensities of *OsOPRs* were calculated by subtracting wounding effect in JA plus SA- or JA plus CHX-treated segments. *OsOPR1* (*filled squares*), *OsOPR2* (*empty squares*), *OsOPR3* (*filled circles*), *OsOPR4* (*empty circles*), *OsOPR5* (*filled triangles*), *OsOPR6* (*empty triangles*), *OsOPR7* (*filled inverted triangles*), *OsOPR9* (*empty inverted triangles*), *OsOPR10* (*fillled diamonds*), *OsOPR12* (*empty diamonds*) and *OsOPR13* (*filled left triangles*)

Under UV-C irradiation, levels of *OsOPR1*, *OsOPR2*, *OsOPR6*, and *OsOPR10* gradually rose until 120 min. That of *OsOPR13* reached its maximum at 90 min where it was maintained until 120 min (Fig. 7f). Application of hydrogen peroxide increased transcripts of *OsOPR10* until 30 min, *OsOPR2* and *OsOPR13* until 60 min, and *OsOPR1* up to 120 min. Expression of *OsOPR10* returned to its basal level at 90 min while that of *OsOPR2* and *OsOPR13* was maintained at higher-than-basal readings until 120 min (Fig. 7g). Exposure to O_3 resulted in the greatest expression of *OsOPR1*, *OsOPR2*, and *OsOPR13* at 90 min, and of *OsOPR6* at 120 min; levels of those first two remained high until 120 min (Fig. 7h).

Biochemical Properties of OsOPR1

Among all *OsOPRs* examined here, *OsOPR1*, *OsOPR2*, and *OsOPR13* were involved in responses to almost all of the applied/tested stimuli. Based on alterations in transcript levels against these, we concluded that OsOPR1 had a particularly important role in such defense responses so we examined its biochemical characteristics. *OsOPR1* cDNA was inserted into the pET28b plasmid and overexpressed as a soluble protein in *E. coli*. This overexpressed His-tagged protein was purified to homogeneity by Q-Sepharose (Fig. 8a). Its UV-visible spectrum showed strong absorptions at 375 and 450 nm (Fig. 8b), indicating the presence of FMN, as was previously observed in other OPR enzymes (Strassner et al. 1999). OsOPR1 activity was assayed by monitoring NADPH consumption, with optimal activity occurring at pH7.8 (Fig. 8c). The respective OsOPR1 k_{cat} and K_m were 0.062 min^{-1} and 460 μM for 2-cyclohexen-1-one (2-CyHE; Fig. 8d), and 0.048 min^{-1} and 8.33 μM for OPDA (Fig. 8f).

To further confirm these OsOPR1 products, the reaction mixture of OsOPR1 and OPDA was subjected to SP-HPLC analysis. The resulting chromatogram provided two major peaks at retention times of 10.4 and 14.5 min, which were tentatively assigned as OPC-8:0 and OPDA, respectively (Fig. 9b) when compared with the chromatogram of standard OPDA (Fig. 9a). These putative OPDA and

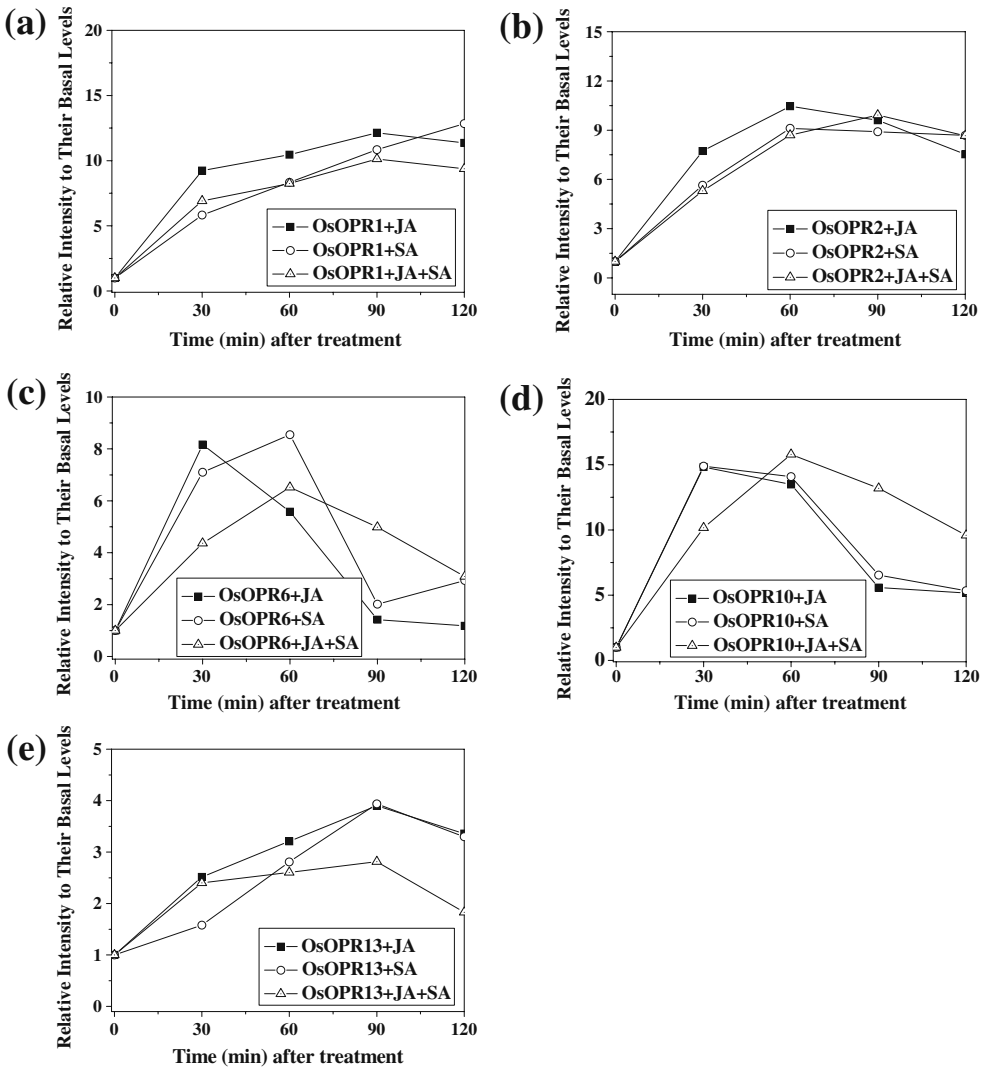

Fig. 5 Comparative analysis of transcriptional kinetics for JA-, SA-, or JA/SA-responsive *OsOPRs*. Transcriptional intensities of *OsOPR1* (**a**), *OsOPR2* (**b**), *OsOPR6* (**c**), *OsOPR10* (**d**), and *OsOPR13* (**e**) in JA (*filled squares*)-, SA (*empty circles*)-, or JA/SA (*empty triangles*)-treated leaves

OPC-8:0 were trimethylsilylated and analyzed via GC-MS. Our GC chromatogram of each compound showed two peaks with identical mass spectra. Their derivatives suggested that cis/trans isomerization of OPDA and OPC-8:0 at C-9 and C-13 occurred during trimethylsilylation. Mass spectra of these trimethylsilylated OPDA and OPC-8:0 are presented in Supplementary Fig. 1a, b, respectively. Analysis of fragmentation patterns verified the structures of trimethylsilylated OPDA (M^+ 364*m/z*, [M–CH_3] 349*m/z*, [M–C_5H_9] 296*m/z*, [M–$C_8H_{14}O_2$TMS] 149*m/z*) and trimethylsilylated OPC-8:0 (M^+ 366 m/z, [M–CH_3]: 351*m/z*, [M–C_5H_9] 298*m/z*, [M–$C_8H_{14}O_2$TMS] 151*m/z*). Therefore, our results clearly demonstrated that the OsOPR1 enzyme converted OPDA into OPC-8:0.

Discussion

OPRs assigned to a family of Old Yellow Enzyme (EC 1.6.99.1) catalyze the NADPH-dependent reduction of OPDA to OPC-8:0, and are involved in the biosynthesis of oxylipins such as JA, a signal molecule implicated in immune responses and plant growth and development (Vick and Zimmermann 1983; Hamberg and Gardner 1992; Schaller et al. 1998; Schaller 2001; Wasternack 2007). OPRs are classified into subgroup OPRI or OPRII based on their substrate selectivity (Schaller et al. 1998, 2000; Strassner et al. 1999, 2002). Those in that second subgroup, e.g., AtOPR3 and LeOPR3, efficiently reduce (9*S*,13*S*)-OPDA, a precursor of JA, but those in the OPRI subgroup, such as AtOPR1, AtOPR2, and LeOPR1, preferentially catalyze the reduction of (9*R*,13*R*)-OPDA rather than (9*S*,13*S*)-OPDA (Schaller et al. 1998, 2000; Strassner et al. 1999, 2002; Agrawal et al. 2004).

Phylogenetic analysis using the NCBI genome database of green algae (*Chlamydomonas reinhardtii*; http://www.ncbi.nlm.nih.gov/blast) has shown that each of these subgroups is evolutionally separated (Supplementary Fig. 2). Our current results indicate that, out of 13 OsOPRs, OsOPR13, homologous to AtOPR3 and LeOPR3 (OPRII

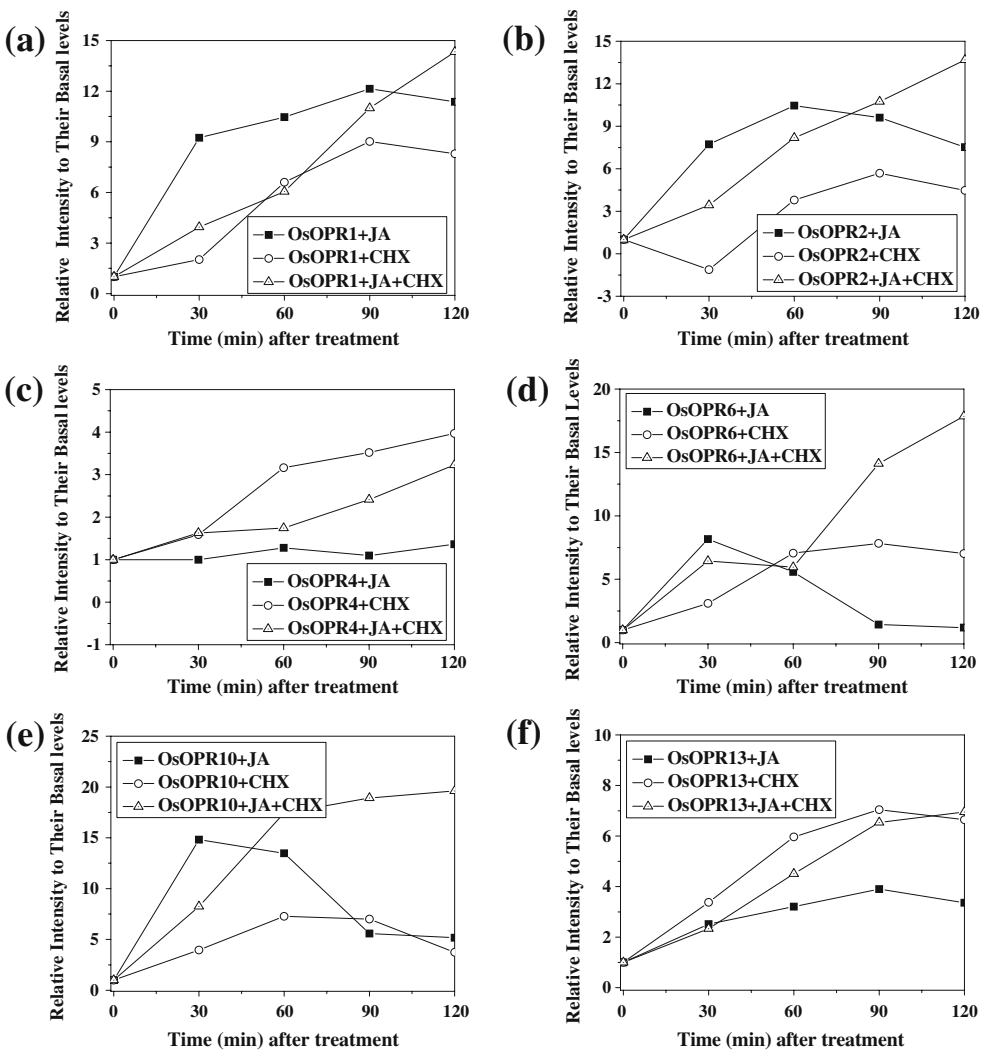

Fig. 6 Comparative analysis of transcriptional kinetics for JA-, CHX-, or JA/CHX-responsive *OsOPRs*. Transcriptional intensities of *OsOPR1* (**a**), *OsOPR2* (**b**), *OsOPR4* (**c**), *OsOPR6* (**d**), *OsOPR10* (**e**), and *OsOPR13* (**f**) in JA (*filled squares*)-, CHX (*empty circles*)-, or JA/CHX (*empty triangles*)-treated leaves

subgroup), is involved in JA biosynthesis. Most OsOPRs could be assigned to OPRI but are evolutionally separated with the dicot OPRI subgroup homologous to OsOPR3. The amino acid sequences of OsOPRs in the OPRI subgroup have high similarity (54% to 100%) with OsOPR1, which is involved in multiple defense or stress responses (Agrawal et al. 2003a). Biochemical analysis of our purified OsOPR1 revealed the presence of FMN as a cofactor, as well as an optimal pH and kinetic constants for (9*S*,13*R*)-OPDA (Fig. 8). However, we could not demonstrate its substrate stereoselectivity. Tani et al. (2008) have reported that OsOPR1 preferentially reduces (9*R*,13*R*)-OPDA over (9S,13*S*)-OPDA. Our findings, therefore, suggest that the OsOPRs in that OPRI subgroup are not involved in JA biosynthesis but may possess other biological functions. Breithaupt et al. (2001) have proposed that OPRI is involved in either (1) the removal of (9*R*,13*R*)-OPDA to maintain a (9S,13*S*)-OPDA pool for JA biosynthesis (Schaller et al. 1998) or (2) the activation of a pathway parallel to JA biosynthesis leading to various oxylipins that function in response to biotic or abiotic stresses and plant growth processes (Bate and Rothstein 1998; Kramell et al. 2000; Weber 2002).

Transcript profile data for *OsOPRs* on tissue specificity and panicle maturity showed that *OsOPR1*, *OsOPR2*, *OsOPR4*, *OsOPR5*, *OsOPR6*, and *OsOPR12* are specifically expressed in the leaves, whereas *OsOPR3* expression is strongly enhanced in panicles rather than leaves. Furthermore, *OsOPR10* and *OsOPR13* transcript levels are induced, depending on flower maturity, while *OsOPR9* expression is suppressed. These results imply that *OsOPR9*, *OsOPR10*, and *OsOPR13* may have roles in floral maturation.

Transcript profiling of *OsOPRs* against diverse stresses revealed that *OsOPR1*, *OsOPR2*, and *OsOPR13* are active in responses to wounding, a fungal elicitor, salt, UV-C irradiation, H_2O_2, and O_3 exposure. In the case of drought stress especially, the expression profiles we observed with RT-PCR do not match a previous report of *OsOPR1* expression via northern blot analysis, perhaps because a full-length cDNA probe was used in that earlier study

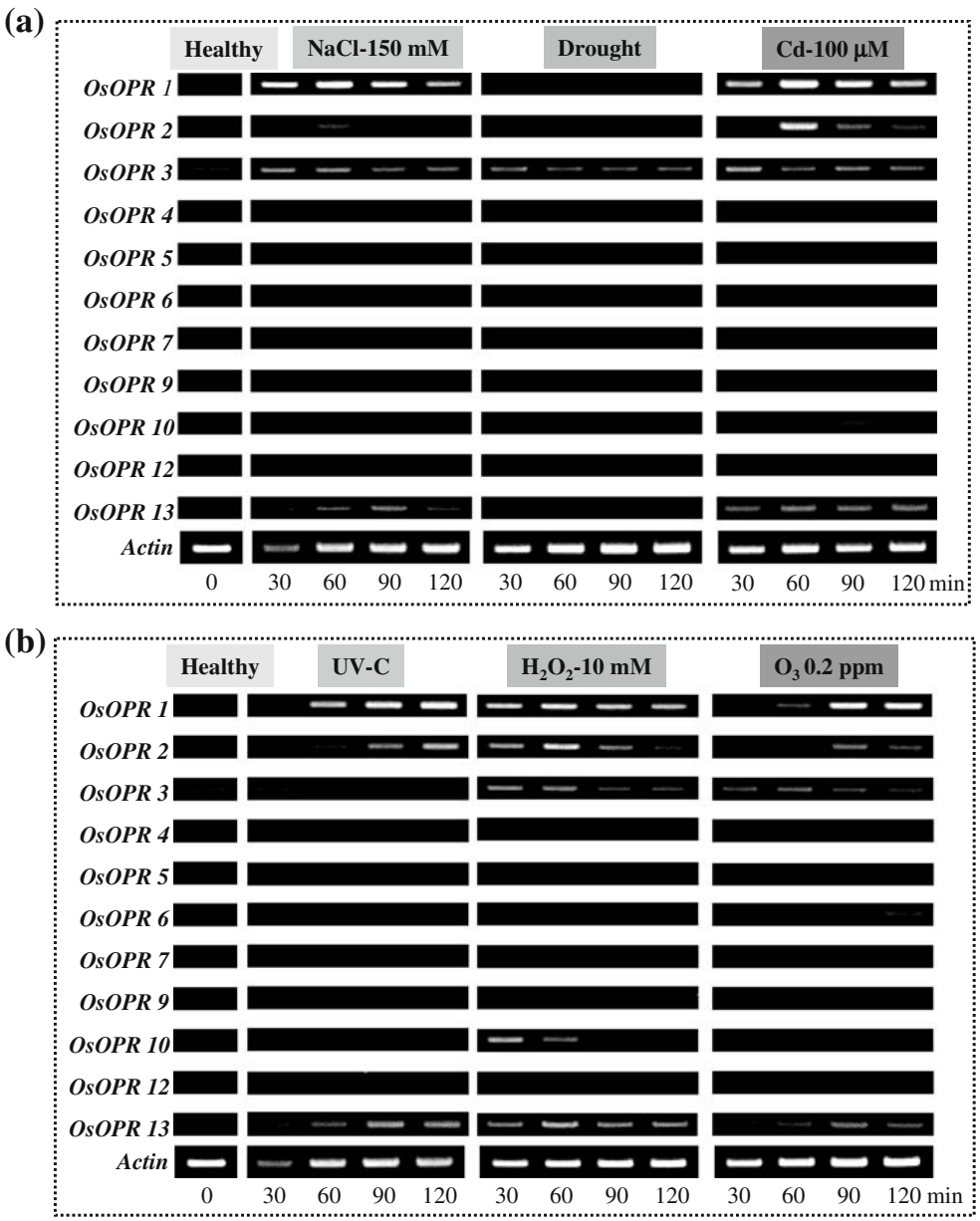

Fig. 7 Transcriptional kinetics of *OsOPRs* against environmental stressors. **a** Leaf segments were treated with NaCl (150 mM), drought, Cd (100 µM), UV-C, or 10 mM H_2O_2 (Agrawal et al. 2003a, b). For in vivo experiments, pots containing 2-week-old seedlings were exposed to ozone (O_3, 0.2 ppm) in controlled fumigation chamber. Treatments were done under continuous light (150 or 350 µmol m^{-2} s^{-1} for O_3; *CON* refers to clean air control). **b** Transcript levels were defined as ratios of *OsOPR* intensities for stressed versus control (healthy) leaves. Actin gene expression served as loading control. Intensities were calculated by subtracting wounding effect in treated segments

(Agrawal et al. 2003a). Therefore, this point must be clarified by future research. Additionally, we can suggest a role for *OsOPR3* in wounding; *OsOPR6* in UV-C irradiation and O_3 exposure; and *OsOPR10* in wounding, UV-C irradiation, and H_2O_2. We can also infer that *OsOPR13* in the OPRII subgroup and *OsOPRs* in the OPRI subgroup are involved in floral development and responses to biotic and abiotic stresses. Tani et al. (2008) have reported that *OsOPR13* functions in the biosynthesis of JA, which acts as a signal against biotic and abiotic stresses, as well as in growth inhibition, flower production, and plant senescence (Wasternack 2007). OsOPR1 is also proposed to reduce the double bond of α,β-unsaturated carbonyl compounds such as 9-/13-ketooctadecadienoic acid (9-/13-KODE) (Tani et al. 2008).

Strassner et al. (1999) have shown that LeOPR1 can decrease the amount of aldehydes, e.g., *trans*-hex-2-enal, cinnamaldehyde, and *trans*-dodec-2-enal. Several enzymes (AOS, divinyl ether synthase, hydroperoxide lyase, peroxygenase, and epoxy alcohol synthase) take part in converting hydroperoxylinole(n)ic acid into oxylipins, such as JA, OPDA, aldehydes, ketones, diols, triols, ketols, and oxoacids, which are regulators of plant defense-gene expression (Penninckx et al. 1996; Bate and Rothstein 1998; Weichert et al. 1999; Vollenweider et al. 2000; Alméras et al. 2003; Glazebrook 2005) and are involved in plant growth and fertility (Sanders et al. 2000; Stintzi and Browse 2000; Vellosillo et al. 2007; Wasternack 2007). Therefore, all of these results imply that the natural products of OsOPRs in the OPRI subgroup are involved

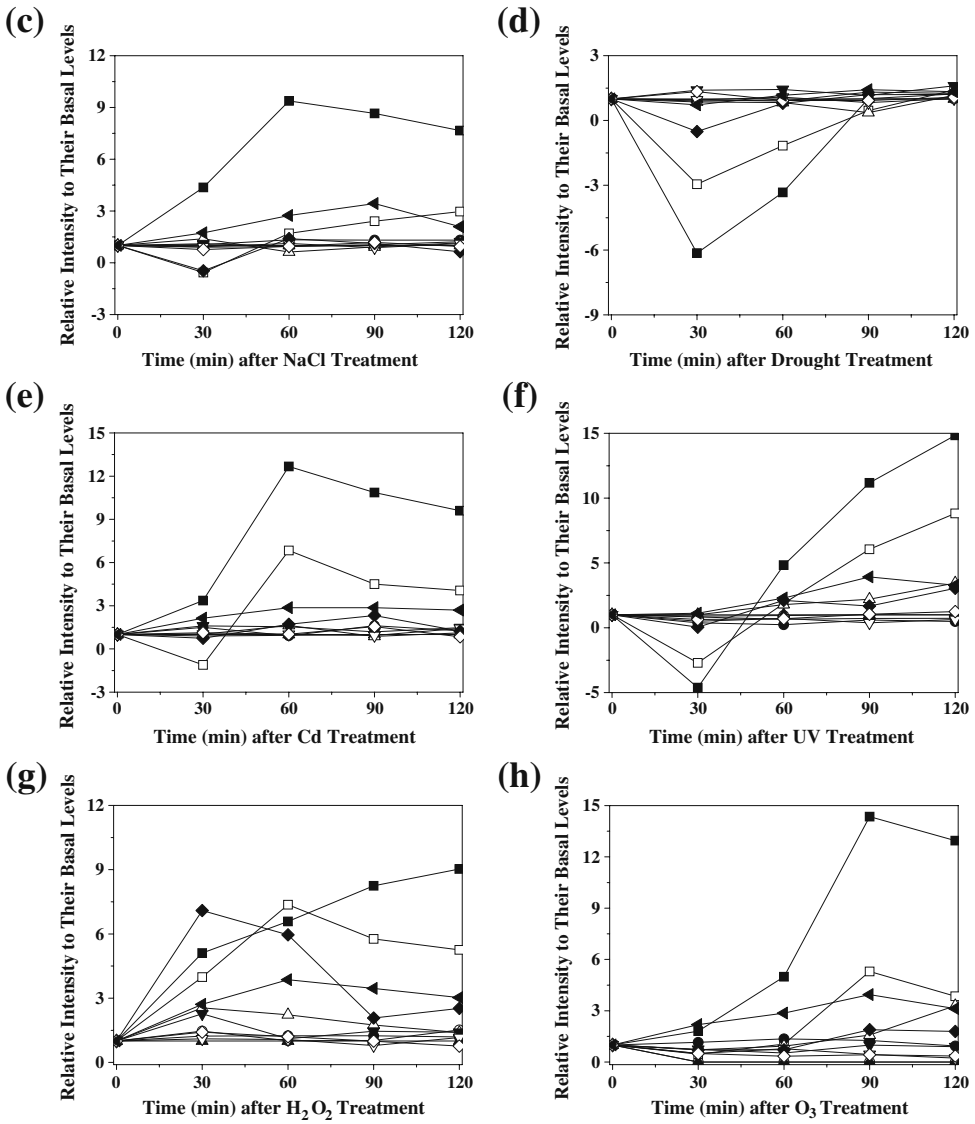

Fig. 7 (continued)

in plant development and stress responses (Bate and Rothstein 1998; Kramell et al. 2000; Weber 2002).

The co-application of JA and SA here did not render a synergistic effect on JA- and SA-induced *OsOPRs* (i.e., *OsOPR1*, *OsOPR2*, *OsOPR6*, *OsOPR10*, and *OsOPR13*). Conversely, the transcript levels of *OsOPR1*, *OsOPR2*, *OsOPR6*, and *OsOPR13* were lower than those induced by JA alone; for *OsOPR10*, co-application appeared to delay its expression (Fig. 5). However, a previous experiment with an entire *OsOPR1* full-length cDNA probe showed that co-application led to a synergistic effect on *OsOPR1* expression (Agrawal et al. 2003a), perhaps because of nonspecificity by that probe. Furthermore, the above results suggest that the SA signaling pathway in some way affects the JA signaling pathway in regulating the expression of *OsOPR1*, *OsOPR2*, *OsOPR6*, *OsOPR10*, and *OsOPR13*. Evidence of negative crosstalk between those pathways is accumulating in the literature (Reymond and Farmer 1998; van Poecke and Dicke 2002; Lee et al. 2004). NPR1, MPK4, COI1, and WRKY70 are considered critical components for mediating this antagonistic effect in *Arabidopsis* (Spoel et al. 2003; Li et al. 2004; Balbi and Devoto 2008). Salicylic acid is proposed to stimulate the conversion of hydroperoxylinole(n)ic acid into hydroxylinole(n)ic acid, which blocks JA biosynthesis (Weichert et al. 1999). Mao et al. (2007) also have shown that the co-application of MeJA and SA synergistically induces AtWRKY62 expression, which suppresses JA-responsive gene expression. Therefore, the results of co-application with JA and CHX suggests that the JA signal elicits the

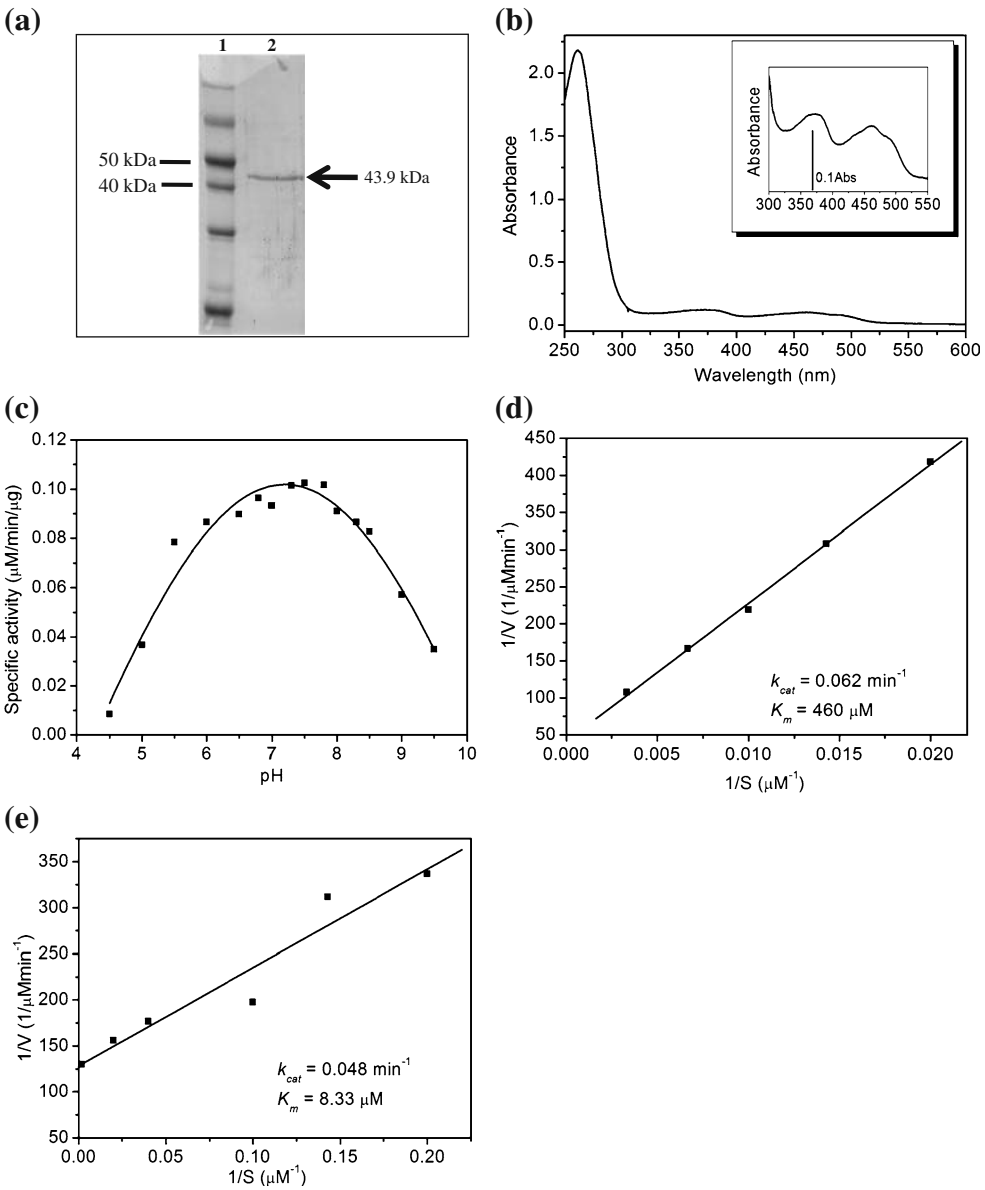

Fig. 8 Characterization of recombinant OsOPR1 protein. **a** Purification by ionic exchange chromatography, using Q-Sepharose. *Lane 1*, MW marker; *Lane 2*, purified OsOPR1 protein. **b** UV-visible spectrum of purified protein was measured from 250 to 600 nm. *Inset* shows enlarged spectrum from 300 to 550 nm, indicating presence of FMN cofactor at 375 and 450 nm. **c** pH-profiling experiment (range 4.5 to 9.5) using 2-CyHE as substrate. Protein activity was determined spectrophotometrically by monitoring consumption of NADPH at 366 nm. Lineweaver-Burk plots of OsOPR1 with either 2-CyHE (**d**) or (9S,13*R*)-OPDA (**e**) as substrate

expression of an activator and the activation of a positive regulator to control the transcription of *OsOPR1*, *OsOPR2*, *OsOPR6*, and *OsOPR10*, whereas JA induces the activation of a positive regulator and the expression of a repressor to regulate *OsOPR4* and *OsOPR13* (Fig. 6). In *Arabidopsis*, jasmonate activates SCF^{COI1}-dependent proteasomes, degrades the JAZ-MYC2 complex, and then releases MYC2, leading to the transcriptional activation of JA-

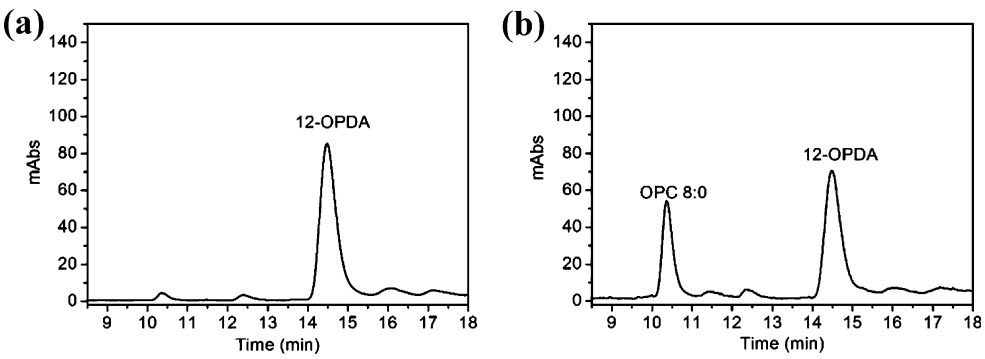

Fig. 9 SP-HPLC analysis of OsOPR1 reaction products. Chromatogram of **a** 12-OPDA standard at 221 nm and **b** products at 205 nm. Substrate ((9S,13*R*)-OPDA) was incubated with OsOPR1 for analyzing mixture

responsive genes (Chini et al. 2007; Balbi and Devoto 2008). MAPKs (mitogen-activated protein kinases) also are intricately involved in JA-signaling in *Arabidopsis* (Petersen et al. 2000), tobacco (Seo et al. 2007), tomato (Holley et al. 2003), and rice (Agrawal et al. 2003c; Lee et al. 2008), thus, further demonstrating the relevance of phosphorylation of protein kinases and MYC2 to the regulation of *OsOPR* expression.

Conclusion

In summary, accumulating evidence demonstrates the importance of JA and other oxylipins, including substrates of enzymes from the OPRI subgroup, in plant development, and stress responses. Transcript profiling of *OsOPRs* in various rice tissues and against diverse stresses has revealed that each gene is differently regulated, being dependent upon tissue specificity, the maturity of panicles, and the influence of signal molecules and stressors. Our results not only provide new insight into the mRNA expression levels of different *OsOPRs* among tissue types and in response to environmental stressors, but also present crucial cues for further understanding of the oxylipin pathways in that crop.

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