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# Structure and expression of 12-oxophytodienoate reductase (OPR) subgroup I gene in pea and oxidoreductase activity of their recombinant proteins

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Footnotes: The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank/EMBL databank with accession number(s) AB095738 to AB095742 for genomic DNAs, and BAB40340 and AB104738 to AB104742 for cDNAs of *PsOPR* genes. [§] The first two authors contributed equally to this work.

**Abstract**

Recently, we observed that expression of a pea gene encoding oxophytodienoic acid reductase (OPR), *S64*, was induced by the suppressor of a pea pathogen, *Mycosphaerella pinodes,* which blocks pea defense responses. Because it is known that genes for OPR consist of several homologous members, we isolated genomic and cDNA clones encoding a member of the OPR subfamily. We isolated five members of the putative *OPR* gene family from a pea genomic DNA library and amplified six cDNA clones including *S64* by a Reverse transcriptase-polymerase chain reaction (RT-PCR) strategy. Sequencing analysis revealed that *S64* corresponds to *PsOPR2,* and the deduced amino acid sequences for the six *OPR*-like genes shared more than 80% identity with each other. According to their sequence similarity, all *OPR*-like genes isolated belong to OPR subgroup I, which does not involve the enzymes for jasmonic acid biosynthesis. However, they varied in exon/intron organizations and promoter sequences. To investigate the member-specific expression of *OPR*-like genes, RT-PCR was used with member-specific PCR primers. The results indicated that the *OPR*-like gene most strongly induced by the inoculation of a compatible pathogen and by treatment with the suppressor was *PsOPR2*. Further, the ability of the oxidoreductases of the six recombinant OPR-like proteins to reduce a model substrate, 2-cyclohexen-1-one (2-CyHE), was investigated. The results indicated that PsOPR1, 4 and 6 have a strong and PsOPR2 has a most remarkable ability to reduce 2-cyclohexen-1-one, whereas PsOPR3 has little and PsOPR5 has no ability to reduce it. Thus, the six OPR-like proteins could be classified into four types. Interestingly, the gene structures, expression profiles, and enzymatic

activities used to classify each member of the pea *OPR*-like gene family clearly concurred with each other, indicating that each OPR-like member possesses distinct functions.

**Key words** Coronatine • Flavoproteins • Jasmonic acid • Oxophytodienoic acid reductase • OPR • Suppressor

## Introduction

The pea fungal pathogen *Mycosphaerella pinodes* is known to produce both a glycoprotein elicitor and a glycopeptide suppressor in its germination fluid (Shiraishi et al. 1994, 1999). Elicitor-induced plant defense responses include phytoalexin accumulation (Shiraishi et al. 1978; Yamada et al.1989), activation of genes coding for phytoalexin biosynthetic enzymes (Yamada et al. 1989) and PR proteins (Yoshioka et al. 1992), and superoxide generation (Kiba et al. 1996). On the other hand, the suppressor inhibits or delays the elicitor-induced plant defense responses described above (Shiraishi et al. 1978; Yamada et al. 1989; Yoshioka et al. 1992; Kiba et al. 1996). Recently, we determined that a suppressor not only inhibits active plant defense responses, but also induces particular plant gene(s) (Ishiga et al. 2002). One example is *S64*; the cDNA clone was isolated from suppressor-treated pea epicotyls by differential screening. Northern blot analysis revealed that the expression of *S64* was strongly induced by treatment with a suppressor and inoculation of a compatible pathogen of pea. Further, the deduced amino acid sequence indicates that *S64*

potentially encodes 12-oxophytodienoic acid reductase (OPR), which leads to the biosynthesis of jasmonic acid (JA) or related compounds, and that S64 recombinant protein produced in *Escherichia coli* possesses NADPH-dependent reductase activity for 2-CyHE and a racemic mixture of 12-oxophytodienoic acid (OPDA) (Ishiga et al. 2002). In *Arabidopsis thaliana*, there are three *OPR* genes, *AtOPR1, 2* and *3*. *AtOPR1* and *AtOPR2* were reported to be induced by wounding (Biesgen and Weiler 1999), and their protein products do not catalyze the reduction of *cis*(+)-OPDA, a precursor of JA (Schaller et al. 2000). On the other hand, AtOPR3 catalyzes the reduction of *cis*(+)-OPDA to 3-oxo-2-(2(Z')-petenyl)-cyclopentane-1 octanoic acid (OPC 8: 0) (Schaller et al. 2000). Therefore, AtOPR3 regulates the JA biosynthetic pathway. JA is widely distributed in plants and affects a variety of processes, including fruit ripening, production of viable pollen, root growth, tendril coiling, plant responses to wounding and abiotic stresses and defenses against insects and pathogens (Creelman and Mullet 1997). DNA macroarray analysis revealed that the expression of all three *AtOPRs* was induced by methyl jasmonic acid (MeJA) (Sasaki et al. 2001). Thus, JA biosynthesis seems to be regulated by JA itself. The *OPR* gene family is also known in tomato, where it consists of at least three members, *LeOPR1, 2* and *3*. Among them only LeOPR3 participates directly in jasmonic acid biosynthesis (Straßner et al. 2002). LeOPR3 and AtOPR3 are shown to localize in peroxisome, while other OPRs are cytosolic enzymes (Straßner et al. 2002). Thus the role of *LeOPR1*, which is most closely related to *AtOPR1*, in the octadecanoid pathway is also still unclear (Straßner et al. 1999, 2002). Very recently, the *OPR* gene was also isolated from a monocot plant, rice, and designated *OsOPR1*. Like AtOPR1, OsOPR1

preferentially reduced *cis*(-)-OPDA but not *cis*(+)-OPDA (Sobajima et al. 2003). The authors classified plant OPRs into two subgroups in terms of specificity against stereoisomers of *cis*-OPDA; subgroup I consists of AtOPR1, AtOPR2, LeOPR1 and OsOPR1, and subgroup II, AtOPR3 and LeOPR3 (Sobajima et al. 2003). Only OPRs belonging to subgroup II catalyze *cis*(+)-OPDA, a precursor of JA, and are involved in JA biosynthesis, while subgroup I is not involved in JA biosynthesis, and its role is still obscure. The non-host-specific phytotoxin coronatine is produced by several pathovars of *Pseudomonas syringae* and induces leaf chlorosis, the production of ethylene and several proteins, e.g., proteinase inhibitors, and inhibits root growth (Bender et al. 1999). Coronatine has been shown to be an essential factor in the early stages of *Arabidopsis* infection and presumably acts by suppressing defense-related genes (Mittal and Davis 1995). On the other hand, coronatine seems to mimic several effects of octadecanoids like JA in plants and is thought to be an octadecanoid analog mediating and inducing defense reactions (Weiler et al. 1994). Therefore, the effect of coronatine on the expression of *OPR*-like genes was examined.

In this paper, we present the analysis of the *OPR*-like gene family in pea. Five members of the *OPR*-like gene family and six cDNA clones including *S64* were isolated from a pea genomic DNA library and cDNA library, respectively. To investigate the member-specific expression of *OPR*-like genes in response to biological, physiological and physical stresses, RT-PCR was used with member-specific PCR primers. We further measured the oxidoreductase activity of each OPR-like recombinant protein. From these structural and functional analyses, we discuss the

functional relevance and molecular evolution of the *OPR*-like gene family in pea.

**Material and methods**

Plant materials and fungal inoculation

Pea (*Pisum sativum* L. cv. Midoriusui) was grown in darkness as described (Yamada et al. 1989), and etiolated epicotyls were cut longitudinally and treated with chemicals or water, elicitor and/or suppressor prior to the RNA extraction. For the inoculation and dehydration experiments, pea plants were grown in the growth chamber with a 16 h photoperiod for one month. Leaves that had been detached at the petiole and preincubated in water for 6 h were inoculated with a virulent pathogen, *Mycosphaerella pinodes,* or a nonpathogen, *Ascochyta rabiei,* at a concentration of 250,000 spores/ml, or placed at room temperature without any water for the dehydration experiments. At each sampling, leaves were collected and frozen in liquid nitrogen for the RNA extraction.

Preparation of elicitor and suppressor

Elicitor and suppressor were prepared from pycnospore germination fluid of *M. pinodes* as described (Yamada et al. 1989) and used at the final concentrations of 500 μg/ml glucose-equivalent and 200 μg/ml BSA-equivalent, respectively.

Chemicals

Commercially available OPDA and MeJA were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and Wako (Tokyo, Japan), respectively. Coronatine was chemically synthesized as described previously (Nara et al. 1997). We analyzed the effects of chemicals on *OPR*-like gene expression in pea epicotyls. A CyHE was purchased from Sigma (Tokyo, Japan) and used for model substrate of recombinant proteins.

Cloning of *OPR*-like genes and corresponding cDNA clones

The *OPR*-like genes were isolated from a Lambda DASH II (Stratagene, La Jolla, CA, USA)-based genomic library of *Pisum sativum* L. cv. Alaska. For the screening of genomic phage clones possessing *OPR*-like genes, we used a digoxigenin (DIG)-labeled DNA probe corresponding to the *S64 (PsOPR2)* cDNA synthesized with DIG PCR Labeling Mix (Boehringer Mannheim, Mannheim, Germany). The plaque DNA was hybridized at 42˚C overnight in 0.75 M NaCl, 75 mM sodium citrate, 50% (v/v) formamide, 0.02% (w/v) SDS, 2% (w/v) blocking reagent (Boehringer) and 0.1% (w/v) lauroylsarcosine, then finally washed at 65˚C in 75 mM NaCl, 7.5 mM sodium citrate, 0.1% (w/v) SDS. The hybridized phage clones were further digested with appropriate restriction enzymes and subcloned into plasmid vectors, pBluescript II SK$^-$ (Stratagene) or pGEM 3Zf(+) (Promega, Madison, WI, USA).

To isolate cDNA clones corresponding to *OPR*-like genomic clones, we screened a cDNA library constructed from suppressor-treated pea epicotyls (Ishiga et al. 2002) by PCR.

Corresponding cDNA clones were also isolated by the RT-PCR method using total RNA prepared from pea epicotyls treated with 100 □M jasmonic acid or 12-OPDA for 3 h. PCR conditions are described in Table 1. After isolation of the candidates for *OPR*-like cDNAs, DNA sequencing was carried out to confirm the cloning. After DNA cloning of PCR products, all nucleotide sequences were verified no mutations.

DNA sequencing and homology search

The nucleotide sequences were determined by the dideoxy chain termination method with an ABI310 sequencer (PE Applied Biosystems, Chiba, Japan). Homologies at the DNA and deduced amino acid sequence levels were analyzed with the Blast search protocol on the Internet (http://www.blast.genome.ad.jp).

RNA extraction and semi-quantitative RT-PCR analysis

Total RNA from pea epicotyls or leaves was extracted by a single-step method (Chomczynski and Sacchi 1987) with slight modification. One microgram of total RNA was reverse-transcribed by AMV reverse transcriptase (Takara, Kyoto, Japan) using the poly dT20 primer. To detect member-specific transcripts and whole transcripts for *OPR*-like gene expression, specific and consensus (common sequence among the *OPR*-like gene family) PCR primers were designed, respectively. The primer sequences and amplification conditions for each *OPR*-like gene are indicated in Table 2. PCR amplification was performed with AmpliTaq Gold (Roche Diagnostics,

Tokyo, Japan) except for *PsOPR2*. The amplification for *PsOPR2* was carried out with SuperTaq (Sawady, Tokyo, Japan). For semi-quantitative PCR amplification, 1 μl of first-strand cDNA was used as the template for PCR. To avoid saturation of the PCR product, we repeated relatively fewer PCR cycles (20-23 cycles); 10 μl of PCR product was subjected to agarose gel electrophoresis. Amplified PCR products were visualized by staining with ethidium bromide or detected by Southern blot hybridization using a DIG-labeled *S64* cDNA probe.

Expression and purification of OPR-like recombinant isozymes

The region for the open reading frame (ORF) of *OPR*-like cDNAs was amplified by PCR with an *Nde*I site-linked 5' primer and a *Bam*HI site-linked 3' primer, as shown in Table 1, using respective cDNAs as templates. After cleavage of the amplified DNA fragment with *Nde*I and *Bam*HI, the ORF region was inserted into a pET expression vector 16b (Novagen, Madison, WI, USA) at the same restriction sites. The resultant plasmid was introduced into bacterial host strain BL21 (DE3) or BL21 (DE3) plysS, and recombinant OPR-like proteins with a His-tag sequence were produced. After 3 h exposure to isopropyl thio-β-D-galactoside at a final concentration of 1.0 mM at 37˚C, bacterial cells were harvested, then solubilized with 3 ml of B-PER® Reagent (Pierce, Rockford, IL, USA). After centrifugation for 10 min at 10,000 rpm, the supernatant was collected, then the recombinant protein was purified using MagExtractor™ (TOYOBO, Tokyo, Japan) as described in the manufacturer's specifications.

Measurement of NADPH-dependent oxidoreductase activity

Enzymatic activity of OPR-like recombinant proteins to reduce 2-CyHE was determined spectrophotometrically by monitoring the reduction of absorbance at 366 nm concomitant with the disappearance of NADPH ($\varepsilon_{366nm}$=3.3 $mM^{-1}$ $cm^{-1}$). The reaction was carried out at 25˚C in a 100 µl reaction mixture consisting of 50 mM potassium-phosphate buffer (pH 7.5), 0.4 mM NADPH, 0.4 mM substrate and 5 µg OPR-like recombinant protein.

**Results**

Isolation and structural analysis of genomic DNA for *OPR*-like genes in pea

By screening the pea genomic DNA library with DIG-labeled *S64* cDNA (Ishiga et al. 2002) as a probe, we obtained four independent positive clones (clones 6, 10-6, 6-2 and 4-10), as shown in Fig. 1A. Restriction mapping and southern blot hybridization revealed the location and orientation of each *OPR*-like gene in four phage clones. To identify the *OPR*-like gene structure, DNA fragments that hybridized to the *S64* cDNA probe were further subcloned into plasmid vectors, and nucleotide sequences were determined. Sequence analysis revealed that pea contains at least five *OPR*-like genes, designated *PsOPR1* to *5*, and that *S64* cDNA corresponds to *PsOPR2*. In phage clone 6-2, two *OPR*-like genes, *PsOPR3* and *4*, are organized with a tandem repeat in a cluster; however, *PsOPR4* lacks the 3'-part of the gene. Regions homologous to the *S64* sequence in each *OPR*-like gene are separated into three to four portions that constitute exons as shown in Fig. 1B. Thus the five *OPR*-like genes could be classified into two groups: the first group (*PsOPR1* and *2*) possesses

three exons, and the other group (*PsOPR3* and *5*) possesses four exons. The first intron of both groups occurred at the same position, but the positions of other introns varied in each group. Although we did not isolate the entire region of *PsOPR4*, we expect that *PsOPR4* probably belongs to the first group due to its extremely high sequence similarity to *PsOPR1* (98.3% at the DNA level).

To clarify gene expression and enzymatic characterization of recombinant proteins from *OPR*-like genes isolated from the genomic library, we isolated the corresponding cDNA clones for *PsOPR1, 3, 4* and *5* with their specific primers by PCR. Using the sequence information obtained from *OPR*-like genomic clones, we designed primers for RT-PCR to clone directly into the expression vector, pET16b (Novagen), with a Topo TA Cloning Kit (Stratagene) as shown in Table 1. We first used a cDNA library constructed from suppressor-treated pea epicotyls (Ishiga et al., 2002) as a template DNA, then isolated corresponding full-sized cDNAs for *PsOPR1*, *PsOPR4* and the novel *OPR*-like cDNA *PsOPR6*, but not for *PsOPR3* and *PsOPR5*. These two cDNA clones were isolated by the RT-PCR method using total RNAs prepared from pea epicotyls treated with 100 □M jasmonic acid for 3 h and *PsOPR3*- and *PsOPR5*-specific primers.

To obtain maximal identities, we aligned the deduced amino acid sequences of PsOPR1-6 as shown in Fig. 2A. The amino acids of OPR-like proteins vary from 362 to 371 amino acids in length; PsOPR2 is the longest polypeptide, with a 9-amino-acid extension at the C-terminus. All the *OPR*-like genes are highly homologous to each other with more than 80% and 85% identities at the nucleotide and amino acid sequence level, respectively. Among the six

*OPR*-like genes, *PsOPR1*, *4* and *6* are the most similar to each other with more than 95% identity at both nucleotide and amino acid sequence levels. In Table 3, we summarized the percent sequence similarities of six members of *OPR*-like gene family at the nucleotide and amino acid sequence levels.

OPR is a flavin mononucleotide (FMN)-dependent oxidoreductase in plants that belongs to the family of old yellow enzyme. Recently, the FMN binding site and the substrate-binding site of tomato LeOPR1 were investigated by X-ray analyses (Breithaupt et al. 2001). We found that all pea OPR-like proteins conserve putative FMN- and substrate-binding sites (Fig. 2A). As shown in Fig. 2B, the phylogenetic tree based on the amino acid sequences of pea OPR-like proteins and other plant OPRs revealed that all pea OPR-like proteins isolated are clustered with the OPR subgroup I, which includes AtOPR1 and AtOPR2 of *A. thaliana* (Biesgen and Weiler 1999), LeOPR1 and LeOPR2 of tomato (Straßner et al. 1999) and OsOPR1 of rice (Sobajima et al. 2003) and which are not involved in jasmonic acid biosynthesis. Thus, all pea OPR-like proteins constitute an independent cluster apart from the OPR subgroup II enzymes involved in the jasmonic acid biosynthesis, like *Arabidopsis* AtOPR3. AtOPR3 is reported to locate in peroxisome, while AtOPR1 and AtOPR2 are thought to be cytosolic enzymes (Straßner et al. 2002). The computer prediction for protein localization showed that six PsOPRs are cytosolic enzymes by PSORT program (http:// psort.nibb.ac.jp/, data not shown).

Further, promoter sequences of *PsOPR1* and *PsOPR4* showed high sequence similarity(92.6%) for about 800 bp of the upstream region (data not shown). However, the other

promoter sequences do not have significant sequence similarity to each other. Recently, two *cis*-regulatory elements, JASE1 (CGTCAATGAA) and JASE2 (CATACGTCGTCAA), in the promoter of *Arabidopsis AtOPR1* gene were reported. These *cis*-elements were thought to be involved in the regulation by senescence and JA (He and Gan 2001). We also found the JASE1 element in the promoter of *PsOPR1* and some potential *cis*-elements in all *PsOPR*-like genes (data not shown). However, the significance of these sequences is not clear at present.

*OPR* expression in response to fungal inoculation

To analyze each *OPR*-specific expression in response to various stimuli, we performed semi-quantitative RT-PCR. As the first step, we designed a set of specific primers for each *OPR*-like gene, as shown in Table 2 and Fig. 3A, and examined specific PCR amplifications in the presence of all members of *OPR*-like cDNAs as templates (Fig. 3B). Using the suitable conditions for semi-quantitative RT-PCR obtained, we first analyzed the expression of *OPR*-like genes in response to fungal inoculation (Fig. 4). Large amounts of *OPR*-like transcripts amplified with consensus primers were detected when inoculated with a pea pathogen, *M. pinodes*, whereas they were not detected in non-treated control leaves (Fig. 4, C2), and only slightly detected in the leaves that were treated with water or inoculated with a pea non-pathogen, *A. rabiei*. Member-specific RT-PCR for each *OPR*-like gene revealed that *PsOPR2* is a major transcript induced by the inoculation of *M. pinodes*. The expression of *PsOPR4* is also slightly induced by the inoculation of *M. pinodes*, but not by that of *A. rabiei*.

*OPR* expression in response to physical and chemical treatments

Because Northern blot analysis revealed that the expression of *S64* (*PsOPR2*) in pea leaves was induced by detachment and dehydration treatments (Ishiga et al. 2002), we investigated the expression of each *OPR*-like gene under these conditions. As shown in Fig. 4, detachment and dehydration induced *OPR*-like gene expression (see *PsOPR* consensus) in pea leaves, as we previously observed. Further member-specific RT-PCR revealed that *PsOPR2* is the strongest transcript among *OPR*-like genes in the above conditions. Further, the expression of *PsOPR1, 3* and *4* were weakly and *PsOPR6* was slightly induced by these physical stimuli. However, no *OPR*-like genes were detectable in the intact pea leaves (Fig. 4A, C1).

The analysis of *OPR*-like gene expression in etiolated pea epicotyls revealed that the *OPR*-like gene was induced by treatment with the *M. pinodes* suppressor, but the induction was not inhibited by concomitant application of the elicitor (Ishiga et al. 2002). As shown in Fig. 5B, the semi-quantitative analysis indicates that the suppressor strongly induced the expression of *PsOPR2* and *4*. The suppressor also weakly induced the expression of *PsOPR1* and *6*. Further, the elicitor did not interfere with the suppressor-induced expression of *PsOPR1, 2* and *6*. *PsOPR2*-transcripts were significantly detected in water- and elicitor-treated pea epicotyls; the level corresponds to the weak signal detected in Northern blot hybridization (Ishiga et al. 2002).

*OPR*-like gene expression was induced by treatment with not only the *M. pinodes*-suppressor, but also by methyl jasmonate and 12-OPDA (Ishiga et al. 2002). Therefore,

we investigated each *OPR* gene expression by semi-quantitative RT-PCR (Fig. 5A). Consequently, MeJA induced *PsOPR2* expression, and 12-OPDA did also but only weakly. In this study, we also examined the effect of the phytotoxin coronatine, a mimic of jasmonic acid produced by some phytopathogenic bacterial pathovars of *Pseudomonas syringae*. Coronatine strongly induced the expression of *PsOPR1, 2* and *4*, and weakly induced *PsOPR3* and *6*. These semi-quantitative analyses revealed that *PsOPR2* is the most common *OPR*-like transcript and that *PsOPR5* was little expressed under the conditions examined.

Production of recombinant OPR-like proteins and their NADPH-dependent oxidoreductase activity

To investigate the NADPH-dependent oxidoreductase activity of pea OPR-like proteins, we first produced their recombinant proteins using an expression system in *E. coli*. As shown in Fig. 6A, we successfully produced each recombinant protein, and the purity of the proteins was confirmed by major single bands in SDS-PAGE (Fig. 6A). NADPH-dependent oxidoreductase activity was investigated by the consumption of NADPH using CyHE (Fig. 6B) as a model substrate. Fig. 6C shows that recombinant PsOPR2 has the strongest reductase activity, and recombinant PsOPR1, 4 and 6 show similarly strong reductase activity. However, recombinant PsOPR3 shows only weak activity, while PsOPR5 shows no activity.

**Discussion**

In this study, we investigated the *OPR*-like gene family in pea. Genomic and cDNA clonings revealed promoter sequence, exon/intron organization and deduced amino acid sequence similarities in pea *OPR*-like genes (Figs. 1, 2 and Table 3). According to their homologies, all *OPR*-like genes isolated belong to OPR subgroup I, which is not involved in JA biosynthesis, (Fig. 2B). We screened the genomic clones to isolate those possessing the *OPR* gene using *S64* (*PsOPR2*) cDNA as a probe under highly stringent hybridization condition. This should be the reason we isolated only OPR subgroup I, because in *A. thaliana*, the DNA sequence homology between OPR subgroup I and OPR subgroup II was as low as 54%. Thus pea *OPR*-like gene family (OPR group I) comprises at least six members, indicating that pea *OPR*-like genes are more complicated comparing to *Arabidopsis* and tomato. The significance of the complexity of the genes is not clear at present. It should be clarified whether complicated existence of *OPR*-like genes in pea relate to the specific function of leguminous plants, like a symbiotic relationship with root nodule bacteria.

The number and position of the introns in the three *AtOPR* genes varied; the position of intron "B" is conserved in all *AtOPRs*, whereas intron "A" is conserved in *AtOPR2* and *3*. However, intron "C" is unique to *AtOPR3*, and intron "E" is conserved in *AtOPR1* and *2*. Thus, the organization of introns in the three *AtOPRs* is different. In the case of pea *OPR*-like genes, introns "A" and "D" are conserved in all *PsOPRs*, but only *PsOPR3* and *5* possess intron "E". Thus, the organization of introns in any *PsOPR* is not the same as in *AtOPR*. Although the deduced amino

acid sequences of PsOPRs showed high homology to each other, the varied structure of their introns indicates the differential molecular evolution of *PsOPR* genes. Further, the different patterns of *OPR* introns in pea and *A. thaliana* suggest that some introns might have appeared after differentiation of the leguminous and cruciferous families in plant evolution.

Analysis of member-specific expression of *PsOPR* genes suggested that *PsOPR2* is most and *PsOPR4* is the second important *OPR*-like proteins in pea under the conditions analyzed. *PsOPR2* is the most strongly and *PsOPR4* is also induced by the inoculation of a compatible pathogen of pea and suppressor treatment. *PsOPR2* and *PsOPR4* are also induced by the coronatine treatment and *PsOPR2* is strongly induced by two different physical stresses, detachment and dehydration (Figs. 4 and 5). Further recombinant PsOPR1, 4 and 6 showed a strong and PsOPR2 showed a most remarkable ability to reduce CyHE (Fig. 7). We did not detect any signals of the *PsOPR5* transcript by RT-PCR or oxidoreductase activities of the recombinant PsOPR5 protein under the conditions studied, indicating that *PsOPR5* might be a silenced pseudogene. However, PsOPR5 conserves the amino acids required for the oxidoreductase of flavoproteins (Fig. 2A). We observed that recombinant protein of PsOPR3 also showed little activity to reduce CyHE (Fig. 6C). The amino acid residues in PsOPR3 and 5 that differ from any residues of PsOPR1, 2, 4 and 6, including the N-terminal extension, might be a cause of the remarkable reduction of the enzymatic activity. Alternatively, PsOPR5 might require NADH and/or reduce other substrates under specified conditions.

The substrate for OPR subgroup I in plants is still unknown. Because *PsOPR2* was the

most strongly and *PsOPR4* is also induced by suppressor-treatment and inoculation with a virulent pathogen (Figs. 4 and 5), the gene products of *PsOPR2* and *PsOPR4* might contribute to the establishment of compatibility. In this respect, it is very interesting that a fatty acid containing the α, β-unsaturated carbonyl group powerfully induced the expression of the wide range of genes involved in defense, oxidative burst including glutathione-*S*-transferase and various other signaling/stress responses (Vollenweider et al. 2000; Alméras et al. 2003). PsOPR2 has strong and PsOPR1, 4 and 6 have moderate activity to reduce CyHE, a model substrate of α, β-unsaturated carbonyl compounds. Vollenweider et al. (2000) also reported that linoleic acid 9- and 13-ketodienes (KODEs) remarkably accumulate due to the inoculation of an avirulent strain of *P. syringae* pv. *tomato*; while the accumulation of these compounds was significantly lower in virulent bacteria-inoculated leaves in *A. thaliana*. These results indicate that KODEs biosynthesis was not strongly stimulated by the inoculation of virulent bacteria; otherwise, KODEs were effectively catalyzed by flavoproteins such as OPR subgroup I enzymes. CyHE is also reported to induce heat shock protein 70 with antiviral activity in mammalian cells (Rossi et al. 1996). Thus, the α, β-unsaturated carbonyl reactivity feature seems to be the key structure triggering the defense response in both animals and plants. Further investigation should be required.

Old yellow enzyme was initially isolated from brewer's bottom yeast and was shown to possess FMN as cofactor. Now proteins similar to those of old yellow enzyme have been discovered not only in fungal and plant species but also in bacterial species (French and Bruce 1995; Rohde et al. 1999). *P. syrignae* pv. *glycinea* expresses Ncr (NAD(P)H-dependent

2-cyclohexen-1-one reductase) at low temperatures, during which the bacteria exhibit high virulence. Ncr also belongs to the flavoprotein family and possesses CyHE reductase activity (Rohde et al. 1999). At present, a number of genes encoding flavoproteins have been discovered in animal and plant pathogenic bacteria by genome sequencing projects. To investigate the potential correlation of the virulence of putative bacterial flavoproteins, mutational analyses of OYE are required. Further, the transcriptional regulation of *PsOPR2* and *PsOPR4* should also be investigated. We are now producing transgenic plants carrying the *PsOPR2* promoter with a reporter gene to clarify the signal transduction pathway leading to the activation of *PsOPR2* gene expression in response to invasion of virulent pathogens and environmental stimuli.

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Figure legends

**Fig. 1** Structure of the *OPR* genomic clones. (A) Physical map of phage clones possessing *OPR*-like genes. The horizontal lines indicate genomic DNA in the phage clone, and the thick arrows indicate the position and direction of *OPR*-like genes. Some restriction sites used to subclone the DNA fragments into plasmid vectors are shown (S, *Sac*I; E, *Eco*RI; H, *Hin*dIII). *PsOPR3* and *PsOPR4* were included in phage clone No. 6-2. (B) Schematic comparison of exon/intron organization in *PsOPR* and *AtOPR* genes. Positions of introns in OPR-like proteins are indicated as vertical lines for five members of putative *OPR*-like genes in pea (*PsOPR1-5*) and three *OPR* members of *A. thaliana* (*AtOPR1-3*), and conserved positions were designated A to E as indicated. The downstream region from the second intron in *PsOPR4* was not isolated.

**Fig. 2** Alignment and phylogenetic analysis of deduced amino acid sequences of OPR-like proteins. (A) Alignment of the deduced amino acid sequences for PsOPR1-6. The deduced amino acid sequences are aligned and numbered from the putative N-terminal methionine. In the lines of PsOPR1 and PsOPR3-6, only amino acids that differ from the sequence of PsOPR2 are indicated. Dots and asterisks indicate identity to the PsOPR2 sequence and positions that are identical in all six putative OPR-like polypeptides, respectively. Amino acids shaded in black and grey correspond to residues in LeOPR1 that contribute to the bindings to NADPH and FMN, respectively. (B) Phylogenetic tree based on the amino acid sequences of pea OPR-like proteins and their homologous proteins. The phylogenetic tree was constructed using the UPGMA program of

Genetyx-Mac (Software Development, Tokyo, Japan). The names of proteins are indicated at the right of the tree with the accession number in parentheses.

**Fig. 3** Member-specific amplification of *PsOPR* by PCR. (A) PCR strategy for the member-specific amplification of *OPR*-like genes. Horizontal bars indicate cDNA sequences, and their sizes are shown on the right. The arrows and gray boxes indicate the positions of the member-specific PCR primers and amplified DNA fragments, respectively. "*PsOPRs* consensus" indicates the consensus primers that can amplify all six members of *PsOPRs*. (B) Member-specific amplification of *PsOPRs*. Ten nanograms of each cDNA plasmid was used as a template for PCR. Primer sequences and PCR conditions are described in Table 2. A one-tenth volume of the PCR product was subjected to 0.7% agarose gel electrophoresis and visualized by ethidium bromide staining. The sizes of amplified DNA fragments are indicated in the right.

**Fig. 4** RT-PCR analysis of *OPR*-like genes expression in pea leaves. Responses of *PsOPR* genes to physical stresses (A) and fungal inoculations (B) were analyzed. Intact leaves were cut from one-month-old pea plants at the petiole and immediately frozen in liquid nitrogen (C1), or preincubated for 3 h (dt) and 6 h (C2) with water supply. Alternatively, detached leaves were preincubated for 6 h with water supply, then held for 3 h at room temperature without any water supply (dh). In the inoculation experiment, preincubated leaves for 6 h after detachment were inoculated with a pea pathogen, *M. pinodes* (Mp), a nonpathogen, *A. rabiei 21* (Ar) or treated with

water (W) for 9 h. PCR cycles were repeated 23 times in (A) and 20 times in (B). Each total RNA used in RT-PCR was independently isolated and its quality was verified by staining with an ethidium bromide. Although the degree of staining was different in each RNA, the bands for rRNA were clearly observed.

**Fig. 5** RT-PCR analysis of *OPR*-like genes expression in pea epicotyls. Responses of *PsOPR* genes to treatment with chemical compounds and fungal signal molecules were analyzed. Etiolated pea epicotyls (C) were cut longitudinally and treated with water (W), elicitor (E), suppressor (S) or elicitor and suppressor (E+S) for 3 h. Alternatively, pea epicotyls were treated with 100 $\mu$M MeJA (J), a racemic mixture of 12-OPDA (O) or coronatine (Co) for 3 h. PCR cycles were repeated 23 times. Each total RNA used in RT-PCR was independently isolated and its quality was verified by staining with an ethidium bromide. Although the degree of staining was different in each RNA, the bands for rRNA were clearly observed.

**Fig. 6** Purification and NADPH-dependent reductase activity of PsOPRs. (A) SDS-PAGE analysis of purified recombinant PsOPRs. "M" indicates the positions of size marker proteins. Purified OPR-like proteins (1.1 □g, PsOPR1 to 6) were electrophoresed into lanes 1 to 6. (B) Structure of 2-cyclohexen-1-one, which was tested as a potential substrate of OPR-like proteins. (C) NADPH-dependent oxidoreductase activity of each OPR protein. NADPH-dependent oxidoreductase activity was measured by the consumption of NADPH with the potential substrate

CyHE.

**Fig. 7** Molecular relevance between structure, expression and enzymatic activity. Expression profiles and enzymatic activity were summarized with a phylogenetic tree based on the amino acid sequences of six members of PsOPR. The level of expression of individual members of the *PsOPR* gene family in response to environmental stimuli and enzymatic reduction with CyHE as a substrate is indicated by plus signs.

Table 1 PCR primers used for isolation and connection of *OPR* cDNAs into pET16b vector

| Gene | Specific primers | Primer sequence (5'-3') |
|---|---|---|
| *PsOPR1, 4* and *6* | *PsOPR146* pET5' | GGAATTCCATATGGGTGCTACCACTACTGA |
| | T7 primer | GTAATACGACTCACTATAGGGC |
| *PsOPR2* | *PsOPR2* pET5' | GGAATTCCATATGATGGGTGCTCCAATTGCCAA |
| | *PsOPR2* pET3' | CGGGATCCTCAAGCCTTGGATTCAACCG |
| *PsOPR3* | *PsOPR3* pET5' | GGAATTCCATATGAATCTCAATACGGATGC |
| | *PsOPR3* pET3' | CGGGATCCTCATTCAAGAAAAGGATAGT |
| *PsOPR5* | *PsOPR5* pET5' | GGAATTCCATATGGGTATCAAAAAAGTTGA |
| | *PsOPR5* pET3' | CCGCTCGAGTCAGTCGAGAAATGGGTAGT |

Underlined sequences CATATG, GGATCC and CTCGAG are restriction sites for *Nde*I, *Bam*HI and *Xho*I, respectively.

Table 2 Member specific primers for *OPR* genes and conditions used in semi-quantative RT-PCR analysis

| Gene | Specific primers | Primer sequence (5'-3') |
|---|---|---|
| *PsOPR1* | *PsOPR1,4&6* 5'sp | ACCAAGACGACTAAGGACAG |
| | *PsOPR1* 3'sp | AGAGAAGATTGAGAGGAATC |
| *PsOPR2* | *PsOPR2* cRT5' | CTGATACAGACCTTTGATCC |
| | *PsOPR2* cRT3' | TCAACCGGTGTTTCATCGTT |
| *PsOPR3* | *PsOPR3* 5'sp | TACGGATGCGGCTCTTACCA |
| | *PsOPR3* 3'sp | TCAACTGGATCAAAGGTATG |
| *PsOPR4* | *PsOPR1,4&6* 5'sp | ACCAAGACGACTAAGGACAG |
| | *PsOPR4* 3'sp | TCTTATTTAAGTGGCCAACT |
| *PsOPR5* | *PsOPR5* 5'sp | CATGCTAAAGGCGGTGTCAT |
| | *PsOPR5* 3'sp | GTGAGGACTATTATCAACCA |
| *PsOPR6* | *PsOPR6* 5'sp | GGAATTCCATATGGGTGCTACCACTACTGA |
| | *PsOPR6* 3'sp | GTTCCACCCTTGGATGCTAG |
| *PsOPR Consensus* | *PsOPR* cons 5'sp | ACAAGTGGAGGCATGGAAAC |
| | *PsOPR* cons 3'sp | ATTGGGATTGGAGTCTCCAC |

PCR was carried out under the following conditions: one cycle of 10 min at 95˚C, 30 cycles of 30 s at 95˚C, 30 s at respective annealing temperature (*PsOPR1*, 60˚C; *PsOPR2,* 55˚C; *PsOPR3,4* and *5,* 56˚C; *PsOPR6,*62˚C; *PsOPR consensus,* 48˚C), 1 min (*PsOPR1, 3, 4, 5*, and *PsOPR consensus*), 30 s (*PsOPR2*) or 1.5 min (*PsOPR6*) at 72˚C.

Table 3 Percentage homology of pea *OPR* genes between each other[a]

| | *PsOPR1* | *PsOPR2* | *PsOPR3* | *PsOPR4* | *PsOPR5* | *PsOPR6* |
|---|---|---|---|---|---|---|
| PsOPR1 (AB104738) | | 90.9 | 88.3 | 98.3 | 80.7 | 95.3 |
| PsOPR2 (AB044940) | 87.8 | | 90.2 | 90.9 | 80.7 | 90.6 |
| PsOPR3 (AB104739) | 90.3 | 91.1 | | 87.5 | 83.1 | 89.7 |
| PsOPR4 (AB104741) | 98.0 | 93.4 | 89.1 | | 79.9 | 95.6 |
| PsOPR5 (AB104740) | 85.6 | 85.2 | 85.2 | 85.4 | | 81.5 |
| PsOPR6 (AB104742) | 96.1 | 90.4 | 91.4 | 95.9 | 85.7 | |

[a] The upper part of the matrix shows the percentage homology at the level of the nucletide sequence in th coding region. The lower part shows the percentage of identity at the amino acid level based on the alignment in Fig. 2. The accession numbers in the DDBJ/GenBank/EMBL databank for the corresponding genes are shown in each parenthesis.

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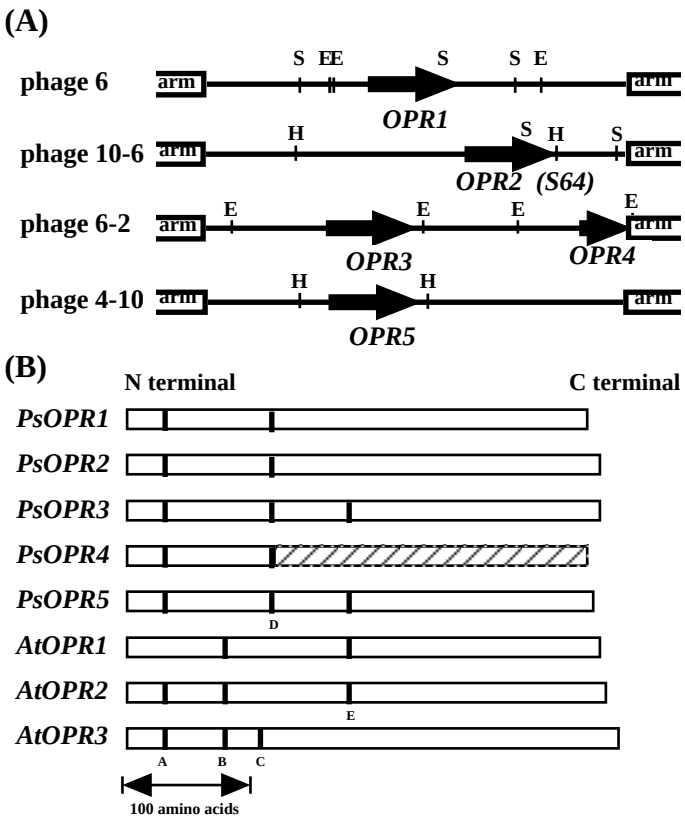
Fig. 1

up



Matsui et al
Fig. 2

up

**(A)**

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PsOPR1        MGATTTDP...L...N..K.K...........T.............S.....................L.D.......Q.........D.  94
PsOPR2  M----GAPI--ANAIPLITPYKMGNFNLSHRVVMAPLTRMRSYNNVPQPHAILYYAQRASQGGLLIAEATGVSDTAQGYPNTPGIWTKEQVEAWKPIVEA  94
PsOPR3  .NLNTD.ALTTSDP...L.............L.............I..N.........F.................V...D.........D. 100
PsOPR4        MGATTTDSV..L...N..K.K...........T.............S.....................L.D.......Q.........D.  94
PsOPR5  .GIK-KVEMSA.DP...V.............L.....T.............S.........T.............................D.  99
PsOPR6        MGATTTDSV..V...N..K.K...........T.............S.....................D.......Q.........D.  94
                     ** *** ** * ****** *****  ******** ** ** ******* ** ********** * *** *** ********* *

PsOPR1  ....GAI..C..........S......A.....................D.......................................... 194
PsOPR2  VHAKDSVFFLQIWHVGRVSNSIYQPNGQAPISSTDKAITSNDQQQFTAPRRLRTNEIPNIVNDFKLAARNAIEAGFDGVEIHGAHGYLLDQFMKDKVNDR 194
PsOPR3  ....G....C....A......I......A.......SL...EE..........D...D......I.....................V.......... 200
PsOPR4  ....GAI..C..........S......A.....................D.......................................... 194
PsOPR5  ....GG.IIC..........C......T....S..SL..SHA....P....S.D...D.....R.............I.........IE.....E.... 199
PsOPR6  ....G.I..C..........I......A.......S.............D...D...H.................................... 194
        ****      ***** ****** ****** **** **  **   **** **** * *** *** *  ************ ***** ***  ***** ****

PsOPR1  .............PL......V.....ER..........F.............V.................I.V......-...C............ 293
PsOPR2  TDEYGGSLENRCRFTFEVVEAVANEIGADKVGIRLSPFAEYAESGDSNPNALGLYMANALNKYNILYCHMVEPRLIQTFDPVE-TPHSLEPMRKAFNGTF 293
PsOPR3  .............ALQ............L.....................V.................H......-.............. 299
PsOPR4  .............PL......V.....ER..........F.....................I.........I.D......-.............. 293
PsOPR5  .......I......AL.....AV.................HS.C...S.KE.....V.......L.........IKSA.EV.DNS...LV.......... 299
PsOPR6  .............L......V.....ER..........F.....................V.....................-.............. 293
        ******* ******   ****  *****   *********  * *** *  ***** ******* *********    *  *   ** * **********

PsOPR1  ..........................................N.......................... 362
PsOPR2  MVAGGYNRQDGIKAIAENRADLVVYGRWFISNPDLPKRFALDAPLNKYNRETFYSSDPVIGYTDYPFLNDETPVESKA 371
PsOPR3  ............N.............L.LA.................H.........L......... 368
PsOPR4  ..........................................N.............E............ 362
PsOPR5  I.....D.....N.V...KT.......L.LA.....................T....L.........D 368
PsOPR6  ............N..D...........L.............N.................L......... 362
         *****  ***** *  **  *******  *  ********** ****** *****  * ** ******** 
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**(B)**

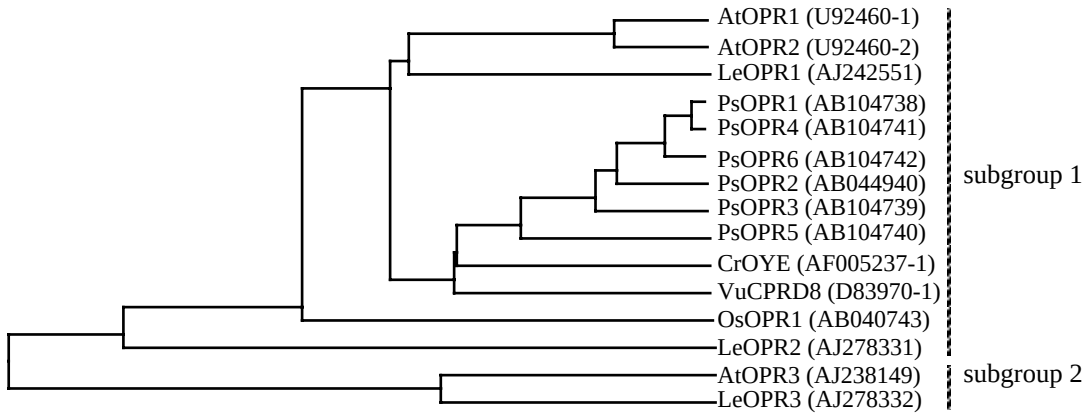

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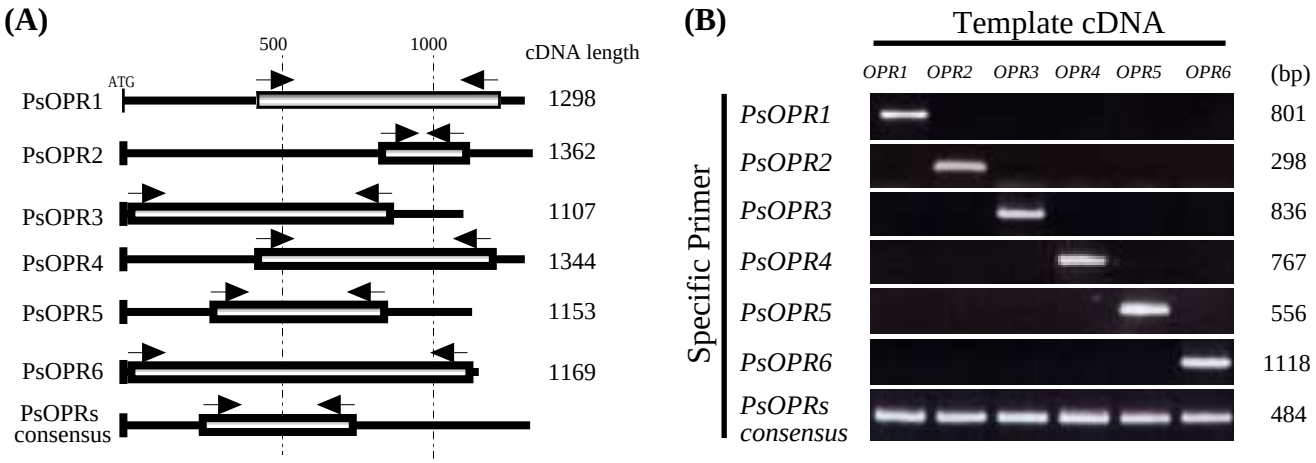
Fig. 3

up



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up

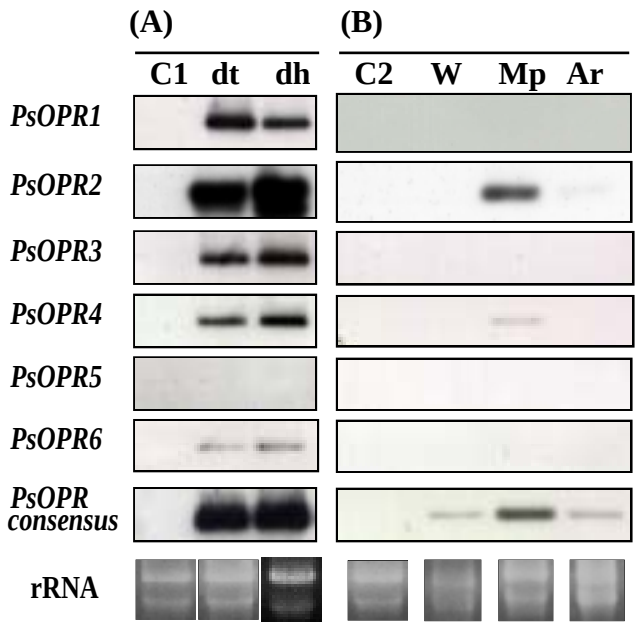

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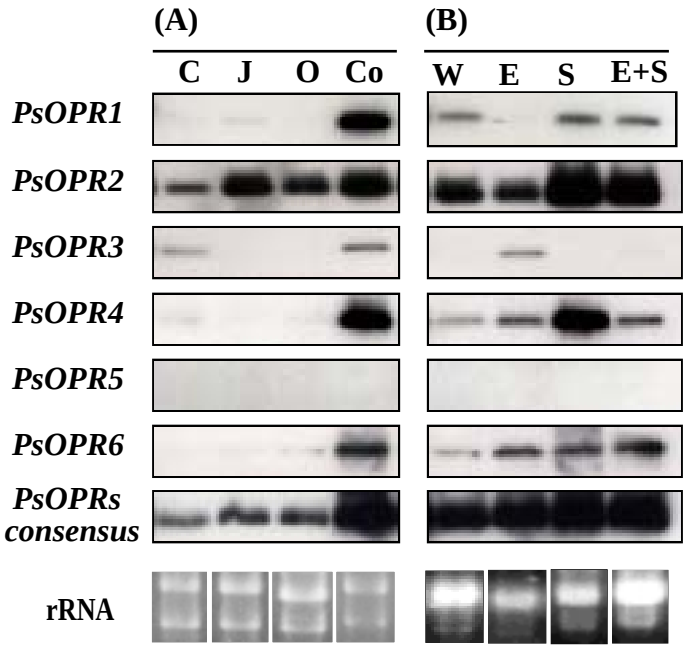
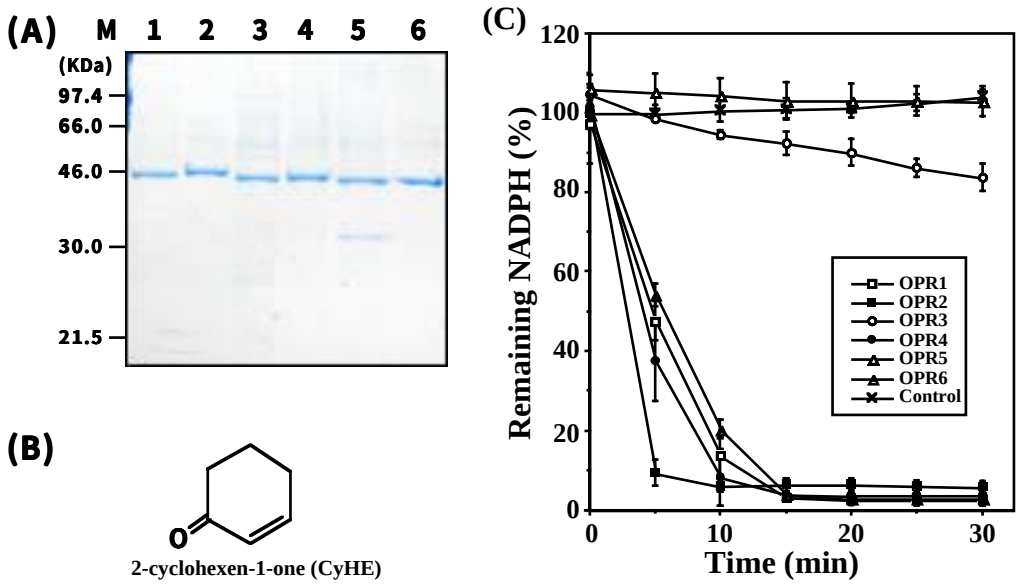
Fig.5

up



Matsui et al
Fig. 6

up



Matsui et al.
Fig. 7

up