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Molecular cloning and expression of 12-oxophytodienoic acid reductase gene from barley

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Abstract

The 12-oxophytodienoic acid reductases (OPRs) are flavin mononucleotide-dependent oxidoreductases that catalyse the reduction of double bonds in α,β-unsaturated aldehyde and ketones and some of them are part of the octadecanoid pathway leading to jasmonic acid biosynthesis. In this paper, a member of the OPR gene family (*HvOPR1*) was isolated from barley, the full length cDNA of *HvOPR1* was 1298 bp containing a 1089 bp ORF encoding 362 amino acids. Bioinformatic analyses revealed that the deduced HvOPR1 has considerable homology with other plant OPRs. Phylogenetic analysis showed that HvOPR1 codes for OPR of subgroup I, containing enzymes which are not required for jasmonic acid biosynthesis. Expression of *HvOPR1* during development and in response to abiotic stresses and hormonal treatments was analyzed by semi-quantitative reverse-transcription PCR. *HvOPR1* expression was differentially regulated during primary leaf development and up-regulated in response to drought, hydrogen peroxide and wounding. Moreover, the accumulation of *HvOPR1* mRNA was increased in response to jasmonic acid, salicylic acid and abscisic acid. The results suggested that *HvOPR1* might play an important role during development and in response to abiotic stresses and signaling molecules.

Keywords: Abiotic stress, Barley, Jasmonic acid, Oxophytodienoic acid reductase, Phytohormone.
Abbreviations: ABA-abscisic acid; JA-jasmonic acid; SA-salicylic acid; OPR-oxophytodienoic acid reductase; H_2O_2-hydrogen peroxide; DAS-days after sowing.

Introduction

12-Oxophytodienoate reductases (OPRs) are a small group of flavin-dependent oxidoreductases in plants related to the yeast old yellow enzyme (OYE) (Williams and Bruce, 2002) with the general activity of reducing double bonds adjacent to an oxo group in α,β-unsaturated carbonyls (Schaller and Weiler, 1997; Strassner et al., 1999). The OPR names are derived from OPR3 in tomato and *Arabidopsis thaliana*, which catalyzes the reduction of the cyclopentenone (9S,13S)-12-oxophytodienoate [(9S,13S)-OPDA] to the corresponding cyclopentanone in the biosynthesis of jasmonic acid (JA) (Vick and Zimmerman, 1984). JA and other octadecanoids constitute a family of plant hormones, collectively called jasmonates, which serve multiple roles as signalling molecules in plant resistance responses against herbivores and pathogens (Turner et al., 2002; Schilmiller and Howe, 2005), as well as growth regulator in numerous developmental processes including male and female reproductive development, fruit ripening, tuber formation, tendril coiling, and seed germination (Creelman and Mullet, 1997; Liechti and Farmer, 2002).

In plants, OPRs are usually encoded by a multigene family. The first member of the OPR family in higher plants was identified from *Arabidopsis thaliana*, and named AtOPR1 (Schaller and Weiler, 1997). Subsequently, other OPR genes were identified in the tomato (Strassner et al., 1999; 2002), pea (Matsui et al., 2004), rice (Agrawal et al., 2003; Sobajima et al., 2003) and maize (Zhang et al., 2005) genomes. All plant OPRs are classified into two subgroups depending on their substrate specificity (Schaller et al., 1998; Strassner et al., 1999). Members of the OPRI group have a rather broad substrate specificity and catalyze the reduction of double bonds in α,β-unsaturated aldehydes or ketones (Kohli and Massey, 1998; Uchida, 2003). Moreover, they preferentially catalyze the reduction of cis-(-) OPDA over cis-(+) OPDA, the only natural precursor of JA, and therefore, are not the enzymes of the JA biosynthetic pathway. AtOPR1, AtOPR2, pea PsOPR1 through PsOPR6 and rice OsOPR1 are OPRI enzymes (Schaller et al., 1998; Sobajima et al., 2003; Matsui et al., 2004). Members of the OPRII group are required for JA biosynthesis because they effectively catalyze the reduction of cis-(+) OPDA which is the only precursor of biologically active JA (Schaller et al., 1998). Based on their substrate specificity, AtOPR3 of *Arabidopsis* and LeOPR3 of tomato, are classified into subgroup OPRII (Stintzi and Browse, 2000; Strassner et al., 2002). The biological significance of plants with multiple OPRs is not clearly understood. To date, the physiological role of only one plant OPR isoform, *Arabidopsis* OPR3, has been established. Genetic studies have confirmed the biochemical data regarding the requirement of AtOPR3 for the JA biosynthesis, *AtOPR3* loss of function mutant is jasmonate deficient, male sterile, and impaired in jasmonate dependent gene expression (Sanders et al., 2000; Stintzi and Browse, 2000; Stintzi et al., 2001). Individual *OPR* family

members exhibit distinct tissue specific, developmental, stress and hormonal patterns of gene expression. For example, *AtOPR3* is transcribed more actively in flowers or anthers than in roots and leaves (Stintzi and Browse 2000). Conversely, *AtOPR1/2* is transcribed more actively in roots and leaves and *AtOPRs* expression is rapidly and transiently up-regulated in response to wounding (Biesgen and Weiler, 1999). Likewise, the expression of β-glucuronidase (GUS) under the control of the *AtOPR1* and *AtOPR2* promoters is up-regulated after stimulation by touch, wounding, and ultraviolet (UV) irradiation (Biesgen and Weiler, 1999). In addition, mutations in *AtOPR3*, which encodes the enzymes belonging to group II, were shown to be deficient in the biosynthesis of JA and the males were sterile (Stintzi and Browse, 2000; Sanders et al., 2000). *OsOPR1*, the first rice *OPR* gene, was characterized at the biochemical and molecular levels (Agrawal et al., 2003). It is rapidly and transiently up-regulated in response to a variety of environmental cues including JA, SA, ethylene and H_2O_2 (Agrawal et al., 2003). Similar results were found for the expression of *OsOPR7* (Tani et al., 2008). Moreover, over-expression *OsOPR7* (OPRII) was able to compensate for the phenotype of *AtOPR3* mutants, whereas *OsOPR1* (OPRII) was unable to compensate for the same phenotype (Tani et al., 2008). Furthermore, maize *ZmOPR1* and *ZmOPR2* are highly induced by SA and pathogen infection, whereas *ZmOPR7* and *ZmOPR8* appear to be wound- and JA-responsive (Zhang et al., 2005). With the exception of rice and maize, little is known about OPR gene family in cereals. Therefore, the aims of this research are to isolate and characterize *HvOPR1* gene from barley and, in particular, to examine its expression patterns during development and after abiotic stresses and hormonal treatment.

Results and Discussion

Cloning and sequence analysis of HvOPR1

Based on screening of the barley ESTs in the TriFLDB database (Mochida et al., 2009), a cDNA fragment with high homology to several plant OPRs was identified. The full length cDNA was cloned by RT-PCR and designated as *HvOPR1* (GenBank accession number JN797728). The full length of *HvOPR1* is 1298 bp and has a start and stop codon, indicating that the gene is complete. The sequence consists of a complete open reading frame (ORF) of 1089 bp with a 5'-untranslated region of 84 bp, and a 3'-untranslated region of 124 bp, respectively. The gene encodes a protein of 362 amino acids with a predicted molecular mass of 40.3 kDa and a theoretical isoelectric point of 5.97 (Fig 1). Analysis of this polypeptide sequence indicated a high probability of being a cytoplasm targeted protein (data not shown). However, further experimental proof is necessary to define the exact subcellular localization site of HvOPR1. Protein-protein BLAST and multiple alignment analysis showed that the deduced HvOPR1 amino acid sequence had considerable homology with OPR sequences from other plant species, such as Arabidopsis OPR1 (69%), rice OPR1 (73%), maize OPR4 (78%), foxtail millet (75%), suggesting that *HvOPR1* belonged to the OPR family. Analysis of conserved domain architecture revealed the existence of a conserved NADH: flavin oxidoreductase/NADH oxidase domain in the *HvOPR1* sequence (Fig 2), suggesting that HvOPR1 belongs to the flavin oxireductase enzyme family. To determine the phylogenetic relationship of HvOPR1 with OPRs from other plant species, the sequences of various OPRs were aligned, and a phylogenetic tree was constructed using the neighbor-joining method. As shown in Fig 3, phylogenetic analysis clearly separated two subgroups of OPR proteins (designated subgroup I and II), and within each subgroup, proteins from dicots and monocots clustered separately with strong bootstrap support. OPR subgroup I and II contains protein from almost all plant species included in the analysis. Thus the OPR subgroups are likely the products of ancient duplication event that occurred in a common ancestor prior to speciations (Zhang et al., 2005). HvOPR1 clustered with proteins in OPR subgroup I (OPRI), which are not involved in the biosynthesis of JA (Schaller et al., 1998; Sobajima et al., 2003). Therefore, the physiological function might not be involved in JA biosynthesis.

Expression patterns of HvOPR1

Expression pattern analysis can help to reveal the possible biological functions of target genes. To investigate the expression pattern of *HvOPR1* gene, semi-quantitative RT-PCR was performed. *HvOPR1* expression was examined during primary leaf development. As shown in Fig 4, *HvOPR1* was detected strongly after 10 and 20 DAS, whereas its expression declined to almost undetectable levels after 12 to 18 DAS. These results indicated that the expression of *HvOPR1* is developmentally regulated during primary leaf development. It has been previously reported that the *Arabidopsis* genes included in OPRI subgroup (*AtOPR1* and *AtOPR2*) are expressed in different developmental stages with distinct transcript profiles (Biesgen and Weiler, 1999; Li et al., 2011). Furthermore, similar results were observed for OPR family members in maize and rice (Zhang et al., 2005; Li et al., 2011). The expression patterns of *HvOPR1* were further examined under abiotic stress conditions (Fig 5). The barley seedlings were exposed to drought, cold (4 ℃), H_2O_2 and wounding. *HvOPR1* expression responds differentially to drought, H_2O_2, and wounding. However, no obvious change of the *HvOPR1* expression was observed under low temperature condition (4 ℃). In contrast to this finding, Biesgen and Weiler (1999) reported that cold treatment increased the mRNA level of *OPR1* in *Arabidopsis*. *HvOPR1* was up-regulated slightly and gradually in drought-stressed leaves. Drought stress also was shown to cause a transient up-regulation of *OsOPR1* transcript (Agrawal et al., 2003). Foxtail millet *SiOPR1* was up-regulated by PEG stress only in the roots and its expression level was continuously increased with the water loss (Zhang et al., 2007). Both H_2O_2 and wounding had positive effects on the expression of *HvOPR1*, where in both treatments the maximum levels of transcripts were obtained at 6 h. H_2O_2 is a major kind of ROS produced by plants under normal conditions during the metabolic processes and acting as a key regulator in a broad range of physiological processes (Foreman et al., 2003). Furthermore, H_2O_2 plays an important role in plants under various biotic and abiotic stresses (Dat et al., 2000), and when accumulated at high concentrations. The H_2O_2 can lead to oxidative stress in plants and trigger the programmed cell death (Mittler et al., 2004). The accumulation of *HvOPR1* transcripts when barley seedlings were sprayed with H_2O_2 showed that H_2O_2 or an H_2O_2-derived signal is involved in HvOPR1 function, and therefore suggesting a possible role of HvOPR1 in oxidative stress responses. The OPRs belong to OPRI subgroup are proposed to reduce the double bond of α,β-unsaturated carbonyl compounds which are produced in response to stress-related lipid oxygenation as a result of oxidative damage (Tani et al.,

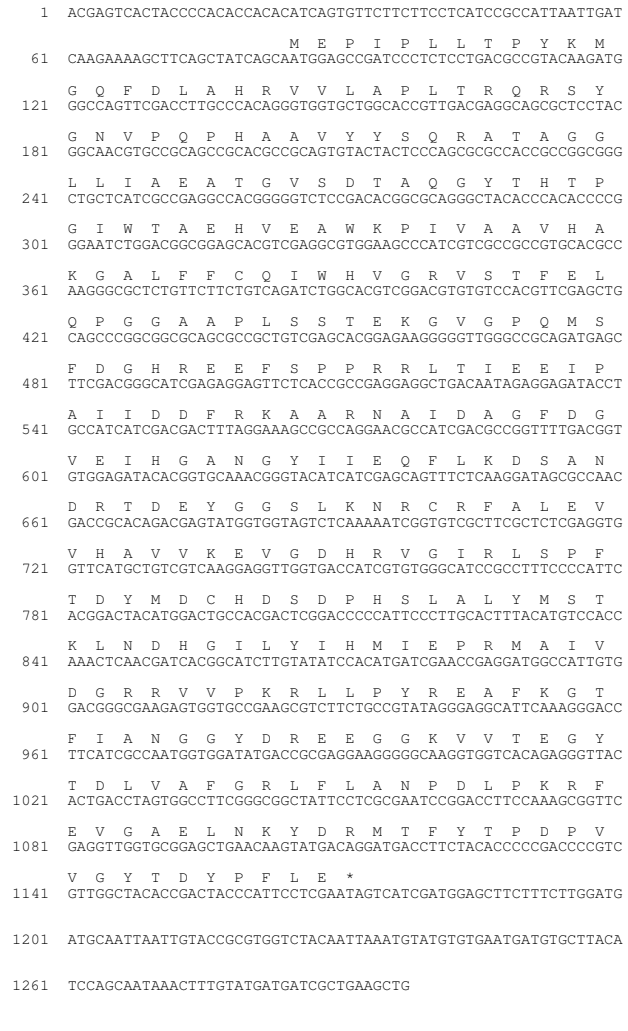

Fig 1. Nucleotide and deduced amino acid sequence of HvOPR1. The deduced amino acid sequence for the open reading frame is designated as a single-letter code above the nucleotide sequence. Numbers to the right refer to nucleotides. The translational termination codon is marked by an asterisk.

2008; Beynon et al., 2009). *HvOPR1* is wound-inducible; therefore, HvOPR1 may have a potential role in wound-associated defenses, perhaps including those triggered by insects invasion. Wounding is one of the severest stresses to plants caused by herbivory, parasitism or by mechanical injury. Wounding is also known to inhibit plant growth (Aldea et al., 2005) and creates potential points of entry for pathogenic microorganisms (Paul et al., 2000). This result is in agreement with the wound-inducible expression of the *HvOPR1* closest homologue in *Arabidopsis* and rice, *AtOPR1* and *AtOPR2* and *OsOPR1* (Biesgen and Weiler 1999; Agrawal et al., 2003; Sobajima et al., 2003). In contrast to these findings, wounding only stimulated the expression of tomato *LeOPR3* (OPRII) in local leaves, but did not affect the expression of *LeOPR1* and *LeOPR2* (both OPRI) (Strassner et al., 2002). Phytohormones are implicated in complex signaling networks involved in regulating a variety of developmental processes, as well as plant responses to a wide range of biotic and abiotic stresses (Rajendra and Jones, 2009; Robert-Seilaniantz et al., 2007). To investigate the effect of phytohormones on *HvOPR1* expression, three phytohormones, JA, SA, and ABA were used to treat 14 day-old seedlings, and the expression patterns of *HvOPR1* at transcription level were examined. The results showed that JA, SA, and ABA could enhance the expression levels of *HvOPR1* to different extent. Both JA and SA induced the *HvOPR1* expression quickly and reached the maximum level at 3 h. *HvOPR1* is induced by JA treatment. JA is a signaling molecule that functions in the control of various metabolic and developmental processes in plant, as well as in mediating plant responses to wounding, herbivore and pathogen attack (Turner et al., 2002; Wasternack, 2007). Being induced by JA, might explain the up-regulation of *HvOPR1* by wounding. Upon wounding, cellular mechanism quickly convey the signal to the plastid to activate the immediate accumulation of JA (Kallenbach et al., 2010). This wound activation of the JA pathway is potentially complex due to the interaction of the JA pathway with ethylene, SA and ABA pathway (Ribot et al., 2008). *HvOPR1* is also SA-inducible, which therefore makes *HvOPR1* prime candidate for involvement in SA-inducible disease defense responses. SA has been suggested to function as a natural transduction signal mediator to activate hypersensitive response and systemic acquired resistance inside plants (Klessig and Malamy, 1994). SA also serves as the central signaling compound for activation of numerous defense-related genes including a set of pathogenic related genes. SA treatment also induced maize *ZmOPR1* and *ZmOPR2*. This is possibly due to the presence of typical binding elements for the TGA transcription factor in their promoter (Zhang et al., 2005). After ABA treatment, the expression of *HvOPR1* increased slightly at 3 and 6 h. This induced level of *HvOPR1* transcript by ABA was obviously lower than those seen with JA and SA. In contrast to this result, Zhang et al. (2005) reported that maize *ZmOPR1* and *ZmOPR2* (both OPRI) mRNA level did not increase in response to exogenous ABA application. In this paper, the full length cDNA of *HvOPR1* was isolated from *H. vulgare*. An expression analysis was performed by semi-quantitative RT-PCR, and the results showed that *HvOPR1* was regulated during primary leaf development. Our results also revealed the up-regulation of *HvOPR1* in response to drought, H_2O_2, wounding and hormonal treatments (JA, SA and ABA). The cloning and characterization of *HvOPR1* will be helpful to understand more about the function of OPRI in plants.

Materials and methods

Plant culture and treatments

Barley (*Hordeum vulgare*, cv. Acsad 176) plants were grown in peat moss-filled pots in a greenhouse at 25±2 ºC with a 14 h light and 10 h dark cycle. To investigate the expression pattern of *HvOPR1* during primary leaves development, primary leaves were harvested at 2-day intervals between 4 and 24 days after sowing (DAS). Several treatments were applied to 2-week-old seedlings for 6 h. For drought stress, primary leaves were cut off and left on the lab bench at room temperature (22 ± 2 ºC) and under light. Cold stress was induced by transferring seedlings to a growth chamber set to 4 ºC. Wounding experiment was carried out in intact plants by wounding each primary leaves four times with a pair of scissors. Intact unwounded leaves were used as a control. Different set of seedlings were prayed under greenhouse

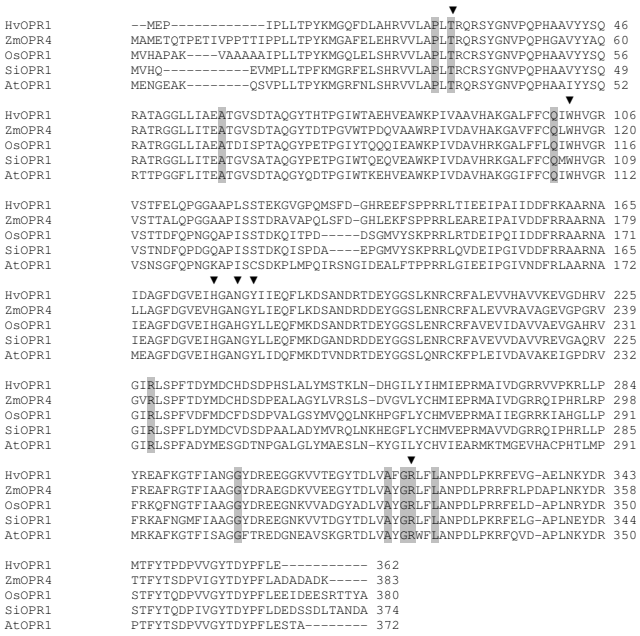

Fig 2. Amino acid sequence alignment of HvOPR1 and other plant OPRs using the ClustalW program. Gray background (box) indicates the conserved FMN binding sites. Amino-acid residues involved in substrate binding are represented with triangles. Numbers to the right indicates the amino acid positions.

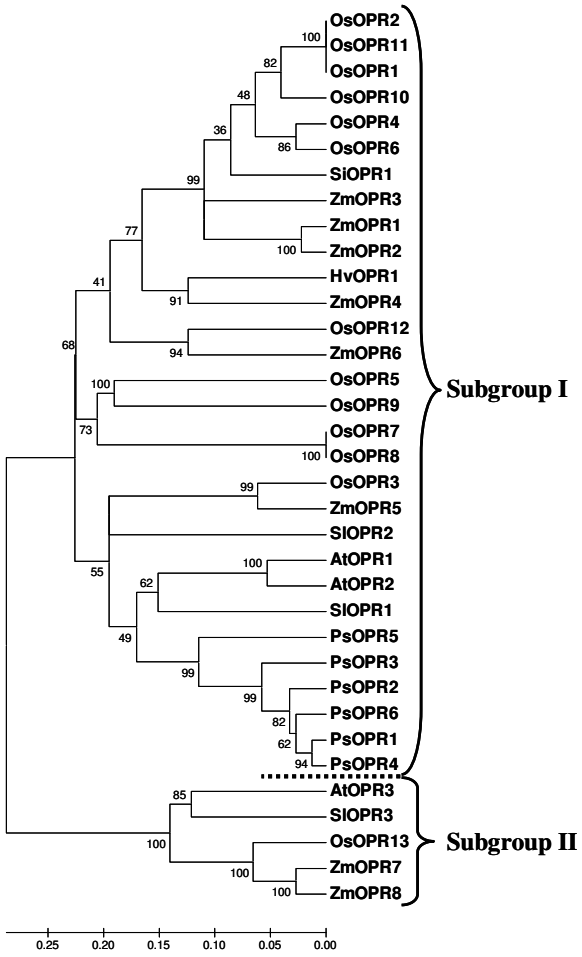

Fig 3. Phylogenetic analysis of OPR family member proteins. The neighbor-joining tree was constructed with MEGA program. The Bootstrap values on the nodes indicate the number of times that each group occurred with 1000 replicates. The species and GenBank accession numbers are indicated in Materials and Methods.

conditions with hydrogen peroxide (H_2O_2) (10 mM), jasmonic acid (JA) (100 µM), salicylic acid (SA) (200 µM), or abscisic acid (ABA) (100 µM). In all treatments, leaf samples were collected after 0, 3, and 6 h of treatment. For both control and treatments, eight indistinguishable leaves were harvested and pooled together for RNA extraction. The harvested leaf tissues were quickly frozen in liquid nitrogen and stored at -70 ℃ for further analysis.

RNA extraction and cDNA synthesis

Total RNA was extracted from the leaf samples with the Trizol Reagent (Promega, USA) and treated with DNase I (Promega, USA) according to user's manual. RNA concentration and purity were estimated based on absorbance at 260 and 280 nm. Five microgram of RNA was used to reverse transcribe the first strand cDNA using GoScript™ Reverse Transcription System (Promega, USA) according to the manufacturer's protocols with oligo $(dT)_{18}$ as a primer in a reaction volume of 20 µl.

Isolation of the HvOPR1 cDNA

One full length expressed sequence tag (EST) sequence (AK249171) of barley, which shares high similarity with rice *OPR1*, maize *OPR1* and *OPR4* and *Arabidopsis OPR1*, was selected from TriFLDB database (Mochida et al., 2009). On the basis of this sequence, a pair of specific primers (5'-ACGAGTCACTACCCCACACCACA-3') (sense) and (5'-GCAGCTTCAGCGATCATCATACAAAG-3') (antisense) were designed for amplification of the complete putative ORF using cDNA generated from leaves of 3 h JA-treated seedlings as template. A total of 25 µl of the RT-PCR reaction mixture containing 20 ng template cDNA, 2.5 µl 10X PCR buffer, 2.5 µl dNTPs mixture (2.5 mM each), 0.1 µl of each primer (10 µM), 0.1µl proof reading *Pfu* DNA polymerase (Promega, USA), and distilled H_2O was added to make up the final volume of 25 µl. The PCR condition was as follows: 1 min at 94 ℃, then 30 cycles of 40s at 94 ℃, 40s at 55 ℃ and 1.5 min at 72 ℃, and a final extension of 10 min at 72 ℃. PCR products were separated on 1% agarose gels and the single specific PCR product band was purified and cloned into the pGEM-T easy vector (Promega, USA) for sequencing.

Bioinformatics analysis

The amino acids sequence of HvOPR1 was deduced and analyzed with ProtParam tool (http://cn.expasy.org/tools/protparam.html), and the prediction of subcellular localization was performed using the online tool ProtComp 9.0 (http://linux1.softberry.com/berry.phtml). Nucleotide

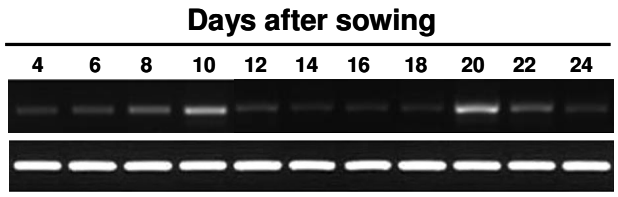

Fig 4. Expression pattern of *HvOPR1* during primary-leaf development as detected by RT-PCR analysis. Total RNA was extracted at marked days after sowing. The β-actin gene was used as the internal control for normalization of RNA loading (lower panel).

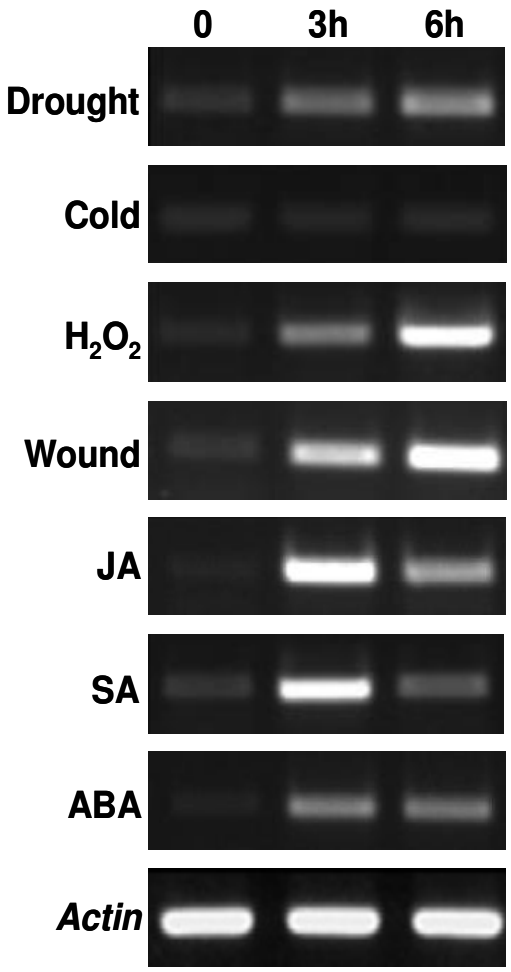

Fig 5. Expression pattern of *HvOPR1* in response to different stress stimuli and hormonal treatments as detected by RT-PCR analysis. Total RNA was extracted from two-week-old seedlings at marked time under drought, cold, H_2O_2, wounding, JA, SA and ABA treatments. The β-actin gene was used as the internal control for normalization of RNA loading (lower panel).

sequence and protein similarity analyses were carried out at the NCBI server using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/), and protein domains were identified by searching the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al., 2009). ClustalW (Chenna et al., 2003) was used to perform multiple alignment analysis. Phylogenetic analysis of OPR proteins was carried out by neighbor-joining algorithm implemented in MEGA 4.1 program (Tamura et al. 2007), after bootstrap re-sampling analysis with 1000 replicates to assess branch support. Sequences from the following list (GenBank accession numbers in parentheses) were used for the phylogenetic analyses: *Hordeum vulgare* OPR1 (JN797728); *Arabidopsis thaliana* OPR1 (AAC78440), OPR2 (AAC78441) and OPR3 (AAG15379); *Zea mays* OPR1 (AY921638), OPR2 (AY921639), OPR3 (AY921640), OPR4 (AY921641), OPR5 (AY921642), OPR6 (AY921643), OPR7 (AY921644) and OPR8 (AY921645); *Oryza sativa* OPR1 (AJ557138), OPR2 (AJ557139), OPR3 (AK102440), OPR4 (AK108079), OPR5 (AK106409), OPR6 (AK061212), OPR7 (AK059887), OPR8 (AK069898), OPR9 (AK067218), OPR10 (AK105590), OPR11 (AK103067), OPR12 (AK100034) and OPR13 (AK104843); *Solanum lycopersicum* OPR1 (AJ242551), OPR2 (AJ278331) and OPR3 (AJ278332); *Seteria italica* OPR1 (DQ393721); *Pisum sativum* OPR1 (BAD06518), OPR2 (BAB40340), OPR3 (BAD06519), OPR4 (BAD06521), OPR5 (BAD06520) and OPR6 (BAD06522).

Gene expression analysis

The first strand cDNA generated for all the samples was used for semi-quantitative RT-PCR using the aforementioned primers to monitor the transcript levels. To verify equivalent loading of cDNA, a barley β-actin gene (GenBank accession No. AAN59955) fragment was amplified using sense primer 5'-CATAGTTGGTCGCCCTCGGCAC-3' and antisense primer 5'-GCTGGAAGAGGACCTCAGGGCA-3' under conditions similar to those described for *HvOPR1*, except for the extension time of 1 min. All RT-PCR expression assays were performed and analyzed three times in independent experiments.

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