J. Plant Biol. (2014) 57:357-365
DOI 10.1007/s12374-013-0481-z

ORIGINAL ARTICLE

# Overexpressing a Novel RING-H2 Finger Protein Gene, *OsRHP1*, Enhances Drought and Salt Tolerance in Rice (*Oryza sativa* L.)

**De-Er Zeng[1,2], Pei Hou[1], Fangming Xiao[3] and Yongsheng Liu[1,4]***

[1]*Ministry of Education Key Laboratory for Bio-resource and Eco-environment, College of Life Science, State Key Laboratory of Hydraulics and Mountain River Engineering, Sichuan University, Chengdu 610064, PR China*
[2]*College of Life Sciences, AnQing Normal University, AnQing 246011, PR China*
[3]*Department of Plant, Soil and Entomological Sciences, University of Idaho, Moscow, Idaho 83844-2339, USA*
[4]*College of Agricultural and Life Sciences, Chongqing University, Chongqing 400030, China*

Received: November 14, 2013 / Accepted: September 4, 2014


**Abstract** RING (really interesting new gene) zinc-finger proteins have important regulatory roles in the development of a variety of organisms. In the present report, a full length cDNA encoding a novel RING-H2 finger protein from rice, designated as OsRHP1, was isolated and characterized. OsRHP1 encodes a small protein (167 amino acids) with two N-terminal trans-membrane domains and a canonical RING-H2 zinc-finger motif located at the C-terminus. Ten putative homologs of OsRHP1 harboring one canonical RING-H2 finger domain exhibit in other plant species. In our work, OsRHP1 was expressed ubiquitously in various tissues, but its transcript is accumulated more in mature leaf. Compared with wild-type (WT) rice plants, transgenic plants overexpressing OsRHP1 exhibited more tolerance to drought and salt stress. Meanwhile, the transgenic T2 lines showed a significant increase of endogenous ABA content in normal condition. When subjected to drought and salt stress, much stronger accumulation of ABA was detected compared with WT. Furthermore, a significant increase of transcriptional expression, of five ABA biosynthesis or ABA-mediated response genes, *OsNCED*, *OsZEP*, *OsAAO*, *OsABI5*, *OsABF2*, *OsLEA3-1* were observed in the transgenic plants under drought and salt stress condition. These results demonstrate that overexpression of OsRHP1 substantially enhances drought and salt tolerance through increased ABA level and enhanced ABA-mediated stress response.

**Keywords:** Abscisic acid, Drought stress, Rice, RING-H2 finger; Salt stress

*Corresponding author; Yongsheng Liu
Tel : +86-28-85460570
E-mail : yongshengliu1122@163.com

## Introduction

Abiotic stresses, such as drought and salinity, are major limiting factors for crops to reach their yield potential. Crop plants with enhanced resistance to drought and salt stress can broaden the spectrum of growth conditions, thereby increasing yield stability and productivity (Hou et al. 2009). Nowadays, hundreds of genes have been cloned, identified and used as candidate genes in genetic engineering for the goal of enhancing drought and salt stress tolerance. Among these genes, RING (really interesting new gene) finger genes are represented in a variety of organisms and play important roles in the developmental process. The RING finger motif was initially defined as a novel zinc-finger domain, involved in protein–protein interaction and necessary for E3 ligase activity in protein ubiquitination (Freemont et al. 1993; Park et al. 2010). There are two distinct variants, namely RING-H2 (C3H2C3) and RING-HC (C3HCC3) domain, depending on which amino acid (Cys or His) occupied the fifth position of the motif (Lim et al. 2010).

The data indicate that some RING finger proteins are associated with plant growth and development as well as with plant-environment interactions (Schwechheimer et al. 2009). *COP1* was the first RING finger gene identified from Arabidopsis, involved in the repression of photomorphogenesis (Yi and Deng 2005), BIG BROTHER, a RING finger E3 ligase, controls organ size by restricting the duration of cell proliferative growth (Disch et al. 2006). In rice, EL5 is a RING-H2 type ubiquitin ligase associated with root development (Koiwai et al. 2007). TaRZF70, containing four RING-H2 domains, responses to drought in wheat (*Triticum aestivum* L.) plants. (Kam et al. 2007); OsBIRF1, one RING-H2 finger protein, regulates the growth and defense

responses against abiotic and biotic stress in rice (Liu et al. 2008) and PtaRHE1, a poplar RING-H2 protein with E3 ligase activity, alters plant development and induces defence-related responses (Bopopi et al. 2010). Some RING finger proteins are involved in the regulation of hormone signaling pathways in *Arabidopsis*. SINAT5, promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals (Xie et al. 2002), and BRH1, encodes a C-terminal RING-H2 finger protein, down-regulated by brassinosteroids and can be induced by the pathogen elicitor chitin (Molnar et al. 2002).

Recently, it has been demonstrated that some of the RING-H2 finger proteins play important roles in regulation of abiotic stress tolerance, suggesting a linkage between RING-H2 finger protein and ABA signaling in plants. *Arabidopsis* RING finger protein RHA2a is a novel, positive regulator of ABA signaling during germination and early seedling development (Bu et al. 2009). AIP2 is another RING finger protein, which involves in ABA signaling, interacting with ABI3 and ubiquitinating the target molecule for degradation (Zhang et al. 2005). The Arabidopsis C3H2C3-Type RING finger protein AtAIRP1 is a positive regulator of an ABA-Dependent response to drought stress (Ryu et al. 2010). SDIR1, a RING finger E3 ligase, appears likely to act as a positive regulator upstream of both ABI5 and AREB/ABF family TFs, promoting ABA-mediated drought tolerance (Zhang et al. 2007). ZmRFP1, the putative ortholog of SDIR1, encodes a RING-H2 E3 ubiquitin ligase and responds to drought stress in an ABA-dependent manner in maize (Xia et al. 2011). XERICO, encoding a small protein with an N-terminal trans-membrane domain and a canonical RING-H2 finger motif located at the C-terminus, positively regulates drought tolerance through increased abscisic acid (ABA) biosynthesis (Ko et al. 2006). *ZmXERICO*, putative homolog of *XERICO*, played an important role in ABA and light signal pathways, involved in plant defense system against environmental stresses in maize (Gao et al. 2012).

ABA plays a pivotal role in a variety of developmental processes and adaptive stress responses to environmental stimuli in plants. Endogenous level of ABA increases under drought and salt stress condition, and through its complex signaling cascade, modulates downstream ABA-responsive genes (Hadiarto et al. 2011). These ABA-mediated genes include those that encode proteins associated with stress response and tolerance, including late embryogenesis abundant (LEA) proteins; a number of regulatory proteins, such as TFs, protein kinases, and phosphatases; a variety of transporters; and enzymes involved in osmoprotectant synthesis, phospholipid signaling, fatty acid metabolism, cellular metabolism, carbohydrate metabolism, and secondary metabolism (Fujita et al. 2011).

In our work, we isolated the full-length cDNA encoding a novel RING-H2 finger protein from rice, designated as *OsRHP1*. Then we characterized *OsRHP1* gene using transgenic approach. A significant enhanced tolerance to drought and salt stress was observed in the transgenic lines overexpressing *OsRHP1*. To further understand drought and salt tolerance mechanism in rice, ABA accumulation level was detected under different conditions. Meanwhile, transcriptional expression of six ABA biosynthesis or ABA-mediated responsive genes was tested.

## Results

### Isolation and Sequence Analysis of *OsRHP1*

Based on the amino acid sequence of XERICO from Arabidopsis, a sequence similarity search of XERICO against the genome databases (http://www.ncbi.nlm.nih.gov/BLAST/) resulted in one putative RING-H2 finger protein homolog in rice, named OsRHP1. Through protein sequence analysis against SMART (http://smart.embl-heidelberg.de/), we predicted that *OsRHP1* encoded a small protein (167 amino acids) with two N-terminal transmembrane (TM) domain, a RING-H2 zinc-finger motif located at the C-terminus.

The structure of OsRHP1 is showed in Fig. 1A. From Blast search results against the NCBI databases ten tentative homologous proteins from other species were retrieved. Alignment of protein sequences showed OsRHP1 shared 62.13%, 64.29%, 62.36%, 48.50%, identity with homologs from monocotyledons *H. vulgare* (BAK03428), *B. distachyon* (accession no. XP-003578295), *Sorghum bicolor* (accession no. XP_002444825), *Z. mays* (accession no. NP-001147769), respectively. It also shared 39.52%, 30.54%, 31.55%, 34.32%, 32.35%, 33.73% identity with dicotyledons *Glycine max* (accession no. NP_001240255), *E. halophilum* (accession no.AAM19707), *B. napus* (accession no.AEQ19306), *C. rubella* (accession no. EOA31606), *A. thaliana* (accession no. NP_178507), *A. lyratal* (accession no. XP_002885748), respectively (Fig. 2B). The RING-H2 domain in its C-terminal region of OsRHP1 is 70% to 100% identical to the corresponding regions of other RING-H2 proteins. The phylogenetic tree based on the protein sequences agrees well with the evolutionary relation among these species (Fig. 1C). To further characterize the expression pattern of *OsRHP1* further, semi-quantitative RT-PCR and real-time PCR were performed synchronously. The data revealed *OsRHP1* was expressed in all the tissues or organs investigated, although the expression level is much higher in leaves than in other tissues or organs (Fig. 2D, 2E). Furthermore, the semi-quantitative RT-PCR result displayed expression of *OsRHP1* was enhanced in various degrees under different abiotic stress condition (Fig. S1).

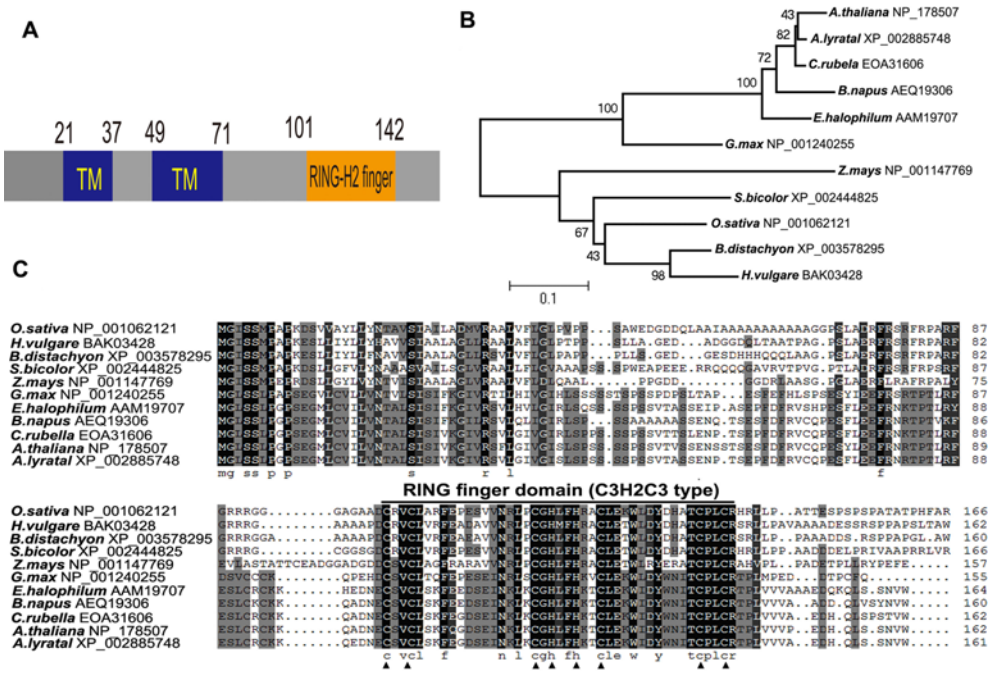


**Fig. 1.** Structure of *OsRHP1* protein and bioinformatics analysis. (A) Structure of OsRHP1 protein showing two transmembrance domain (TM), serine rich domain and conserved RING-H2 domain. (B) Multiple alignment of amino acid sequences of OsRHP1 and other plant homologs by MEGA 5.1. The identical residues are shaded in black with white letters. Residues with at least 75% conservation are shaded in deep gray with black letters. Residues with at least 50% conservation are shaded in gray with black letters. Cysteine and histidine are emphasized by small black triangles. (C) Phylogenetic analysis of OsRHP1 amino acid sequence and homologous sequences in other species by neighbor-joining method by MEGA5.1.

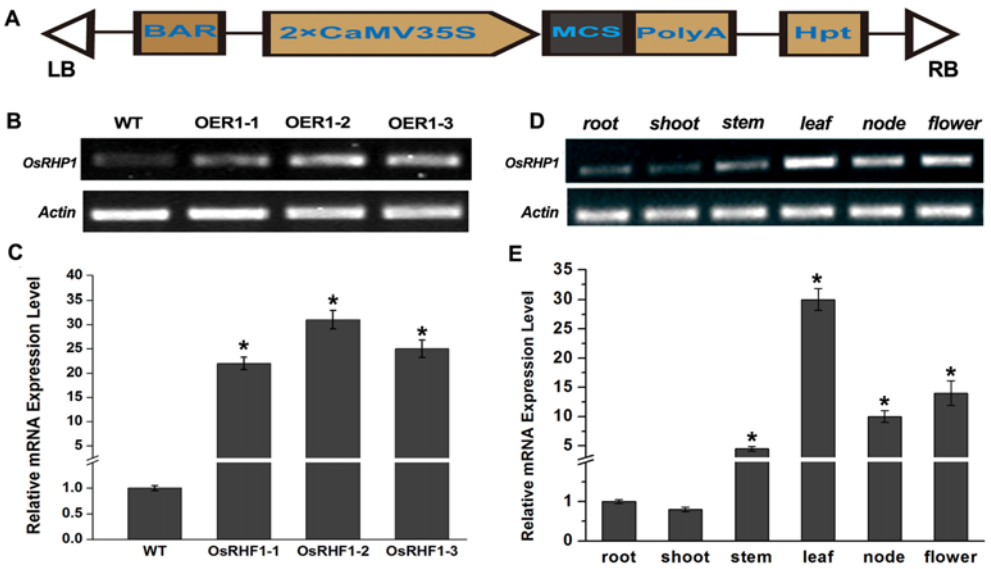


**Fig. 2.** Semi-quantitative and quantitative real-time RT-PCR analyses of *OsRHP1.* Schematic diagram of part of the T-DNA region of the transforming construct CMV *35S-OsRHP1*. B,C Semi-quantitative RT-PCR and real-time analysis of *OsRHP1* mRNA levels in WT and three independent T2 lines. D, E Semi-quantitative RT-PCR and real-time analysis of *OsRHP1* expression in different tissues. Error bars and mean ±SE are from three independent experiments.

## The Expression Level of *OsRHP1* Enhanced in Transgenic rice

We generated transgenic rice lines overexpressing *OsRHP1* driven by the CaMV *35S* promoter. Through PCR amplification of hygromycin resistance marker, nine independent transgenic lines of overexpressing *OsRHP1* were obtained. Three representative independent T2 transgenic lines (OER1-1,

OER1-1, OER1-3) were selected for further analysis. To confirm the expression level of *OsRHP1* in OER1 lines, total RNA was extracted from the leaves of WT and OER1 lines. The semi-quantitative RT-PCR and real-time PCR were used to determine the transcript level of *OsRHP1*. As shown in Fig. 2B, 2C, compared to that of WT, the expression levels of *OsRHP1* in OER1 lines were distinctly increased with more than 22-fold, 31-fold, and 25-fold, respectively.

## Overexpression of *OsRHP1* Enhanced ABA Accumulation in Rice

Intriguingly, when WT and transgenic T2 seed stratified on MS media, germination and seedling growth of three transgenic lines were retarded compared with WT. To determine whether the growth arrest was partially attributable to endogenous ABA rise, we measured endogenous ABA content with or without drought and salt treatment by ELISA. Results from three independent experiments showed that levels of ABA in OER1 lines were more than 4.5-fold higher than in the WT plants when grown on soil for 2 weeks under normal condition (Fig. 3).When subjected to drought and salt treatment, endogenous ABA content of OER1 lines is up to 6 and 4.8 fold higher than that of WT, respectively (Fig. 3). It should be noted that overexpression of *OsRHP1* increased endogenous ABA accumulation to a higher degree under drought or salt stress condition than normal condition.

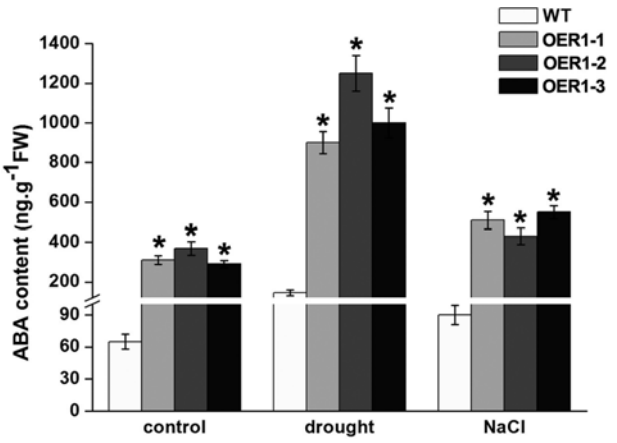


**Fig. 3.** Accumulation of ABA between WT and three Transgenic T2 lines (OER1-1, OER1-2, OER1-3).

## Overexpression of *OsRHP1* Enhances Tolerance to Drought and Salt Stress in Transgenic Rice

As ABA was significantly increased in the OER1 lines, we speculate their stress tolerance should be improved. To this end, the OER1 lines and WT were exposed to drought (withholding water for 10 to14 d) or high salinity (200 mM NaCl for 5 d), and then transferred to normal conditions to restore growth. After 6 d of growth recovery, the survival rates of seedlings were evaluated. Apparently, the OER1 lines exhibit enhanced drought and high salinity tolerances than WT under stress condition (Fig 4). Meanwhile, data showed that the OER1 lines had higher survival rates than that of WT. The average survival rate of the transgenic plants under drought stress was 39.9%, whereas that of the WT was only 17.5%. Under salt stress, the average survival rate of the

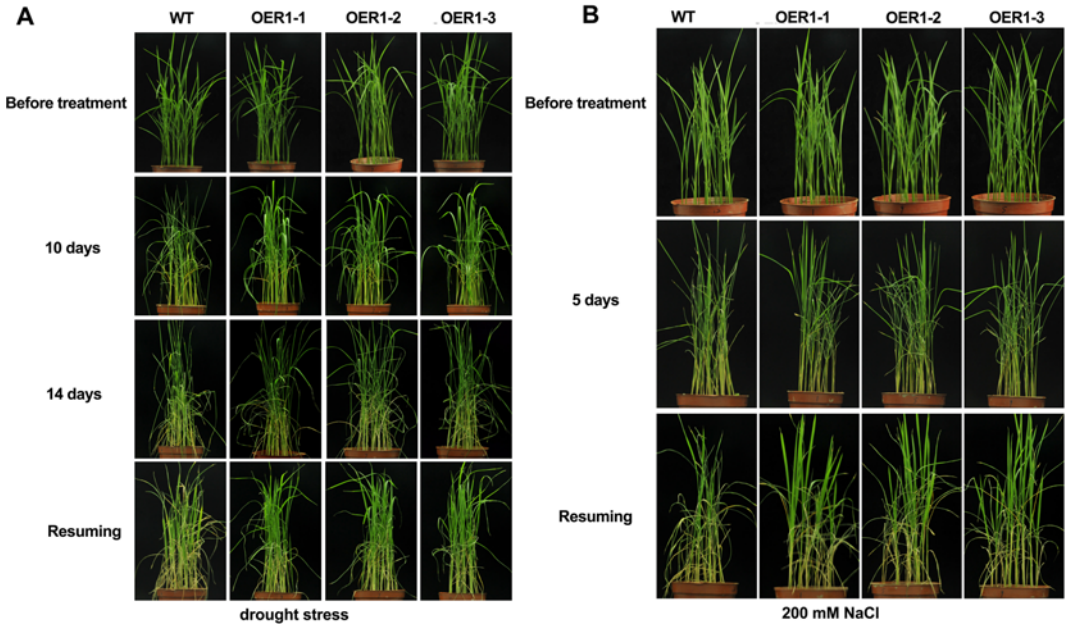


**Fig. 4.** Overexpression of *OsRHP1* in rice exibits more tolerance under drought and salt stress condition.

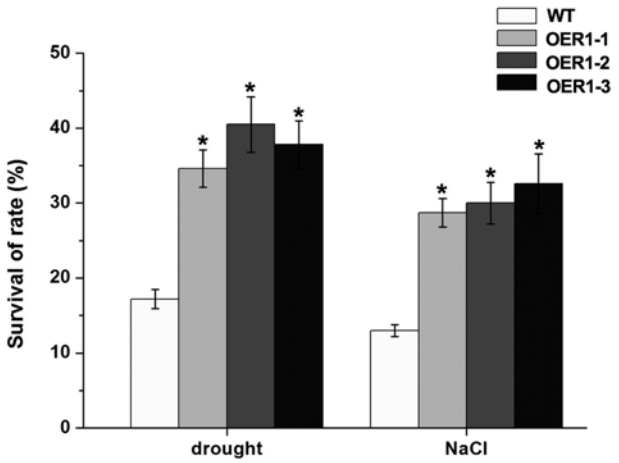


**Fig. 5.** Survival rate of the three transgenic T2 plants (OER1-1, OER1-2, OER1-3) after resuming watering. Each data point is the average of at least three replications (* t test, with $P < 0.05$).

transgenic plants was 30.8%, whereas that of the WT was only 13.2% (Fig. 5). These data clearly demonstrate that overexpression of *OsRHP1* confers enhanced drought and salt tolerance in rice. Impressively, we performed extreme drought experiment with OER1-2 line, withholding water for 22 d all along. The result displayed that the OER1-2 line still have some survivals about 7.8% percentage. However, WT plants were almost withered and dead for harsh drought stress (Fig. 6).

Expression Levels of ABA-biosynthesis Genes and Stress-responsive Genes

Given the above results that overexpression of *OsRHP1* substantially increased endogenous ABA content and enhanced drought and salt stress tolerance in transgenic rice, we speculate that *OsRHP1* plays a key regulatory role in ABA biosynthesis pathway, and likely participate in drought and salt stress response process in ABA-dependent manner. To confirm this hypothesis, transcript levels of 6 ABA-biosynthesis and stress-responsive genes (*OsNCED*, *OsZEP*, *OsAAO*, *OsABI5*, *OsABF2*, *OsLEA1-3*) were assayed in the OER1 and WT plants under normal and drought stress conditions. All these six genes were selected based on the literature. Moreover, these genes showed significant higher expression level in the OER1-2 line than in WT under normal condition. And under drought or salt stress conditions, all of these genes showed much higher expression level than normal condition to different degrees in OER1-2 line.

## Discussion

Previous studies demonstrate some RING-H2 finger proteins respond to drought or salt stress in an ABA-dependent way. Overexpression of *XERICO*, an Arabidopsis RING-H2 finger gene, confers drought tolerance through increased ABA biosynthesis. And *ZmXERICO*, a putative homolog of *XERICO*, might be involved in ABA signaling pathway against environmental stresses in maize (Ko et al. 2006; Gao et al. 2012).

In our work, we are fortunate to seek out a novel RING-H2 finger gene *OsRHP1*, putative homolog of *XERICO* and *ZmXERICO*. Intriguing, OsRHP1, containing two trans-membrane domains and a RING-H2 zinc finger motif shared high sequence homology with XERICO and ZmXERICO. Meanwhile, ten putative RING-H2 finger protein homologs from other species were excavated. Amino acid sequence alignment indicates that they share high sequence homology in RING-H2 finger motif. Phylogenetic analysis demonstrates the evolutionary conservation and divergence of different RING-H2 finger proteins (Fig. 1). Based on previous study and our comparative analysis, we have reason to hypothesize that *OsRHP1* probably plays a resemblant role like *XERICO* or *ZmXERICO*, and it might be implicated in ABA-mediated stress response pathway.

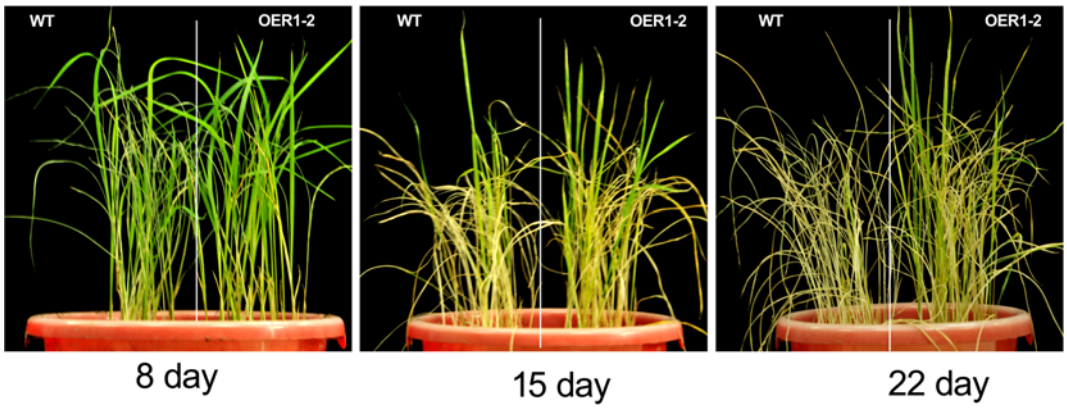


**Fig. 6.** Extreme experiment of WT and OER1-2 plants under drought stress.

To characterize *OsRHP1* and confirm the hypothesis, a series of assays were investigated. Our data showed that overexpression of *OsRHP1* substantially increases cellular ABA level in rice plants under normal and stress conditions (Fig. 3).

ABA accumulation is a common trait of transgenic plants that upregulate genes involved in ABA biosynthesis. Several genes involved in ABA biosynthesis have been cloned and characterized in Arabidopsis thaliana and other plant species (Xiong and Zhu 2003; Taylor et al. 2005). These genes include zeaxanthin epoxidase (ZEP), which catalyses the epoxidation of zeaxanthin to produce epoxycarotenoid, 9-cis-epoxycarotenoid dioxygenase (NCED), which catalyses the cleavage reaction of epoxycarotenoids to produce xanthoxin, and abscisic aldehyde oxidase (AAO), which catalyses the final step of ABA biosynthesis whereby ABA aldehyde is converted to ABA (Seo et al. 2001). Previous studies have demonstrated that overexpression of *ZEP* in transgenic Arabidopsis (Park et al. 2008), *NCED* in transgenic lines of tomato (Thompson et al. 2001), Arabidopsis (Iuchi et al. 2001), tobacco (Qin and Zeevaart 2002) and bentgrass (Aswath et al. 2005) could substantially enhance ABA level.

Thus, it is convincible that accumulation of ABA could be attribute to enhanced expression of ABA-biosynthesis genes

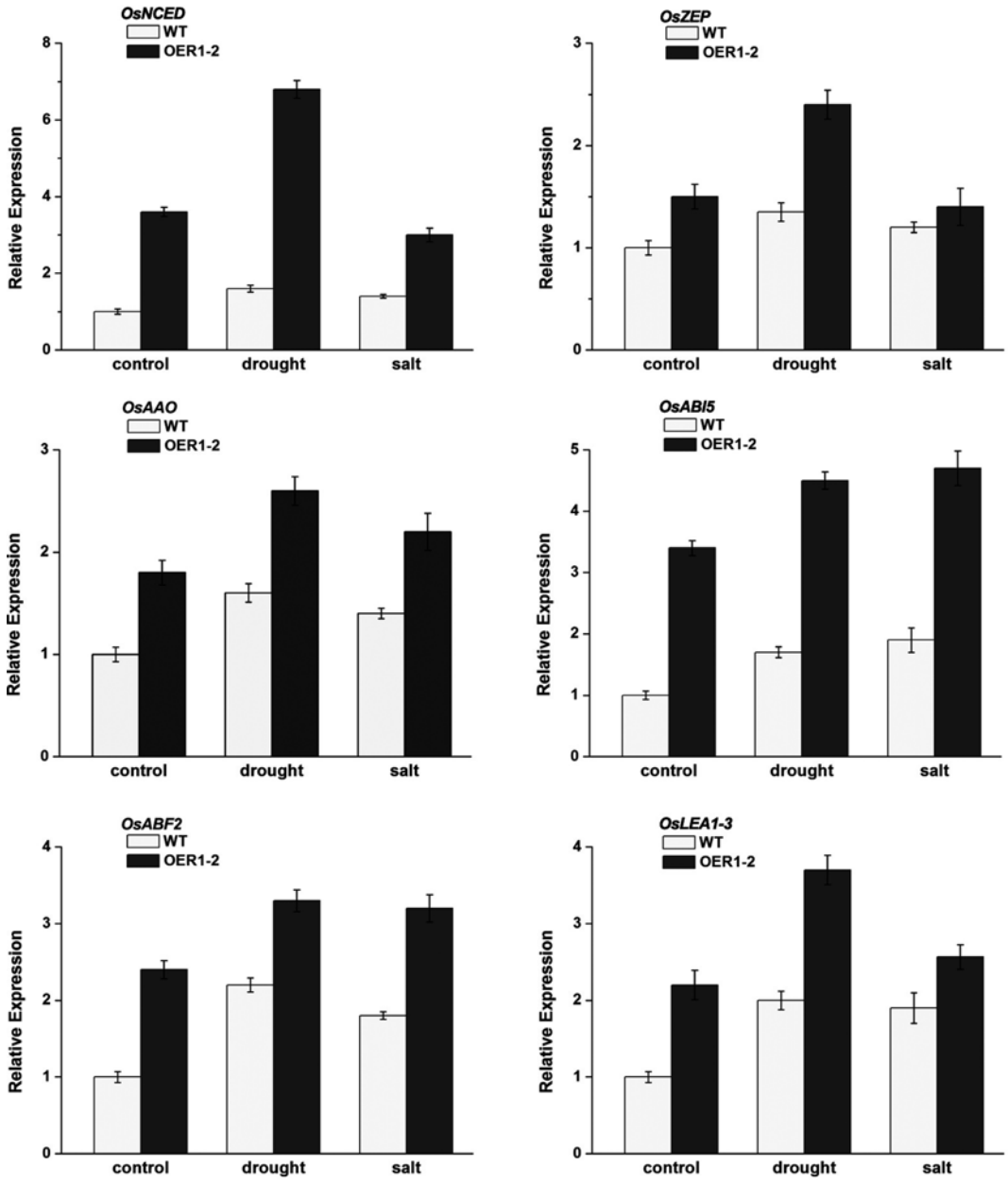


**Fig. 7.** Expression analysis of ABA-related genes in three transgenic T2 plants (OER1-1, OER1-2, OER1-3) and WT. Under drought for 16 h and 200 mM NaCl for 6 h. Error bars and mean ± SD are from three independent experiments.

such as *NCED*, *ZEP* and *AAO*. Our data demonstrates that overexpression of OsRHP1 substantially enhances the expression level of *OsNCED, OsZEP* and *OsAAO* under normal condition. However, under drought or salt stress condition, the expression level of *OsNCED*, *OsZEP* and *OsAAO* were enhanced more than normal condition in OER1 plants (Fig. 7).

Increased ABA levels in response to osmotic stresses, such as drought and high salinity, induce the expression of many genes that appear to play multifaceted roles in dehydration response and tolerance in both vegetative tissues and seeds (Finkelstein et al. 2002; Yamaguchi-Shinozaki and Shinozaki 2006). Expanding transcriptome data sets have uncovered a global picture of ABA-regulated genes in Arabidopsis plants. ABA-induced genes include those that encode proteins associated with stress response and tolerance, including late embryogenesis abundant (LEA) proteins; a number of regulatory proteins, such as TFs, protein kinases, and phosphatases; a variety of transporters; and enzymes involved in osmoprotectant synthesis, phospholipid signaling, fatty acid metabolism, cellular metabolism, carbohydrate metabolism, and secondary metabolism (Fujita et al. 2010). Thus, we examined transcript levels of two well-known ABA-responsive genes: *OsABF2*, and *OsABI5*. *OsABI5* is one important transcription factor modulating ABRE-dependent gene expression in response to osmotic stress, and ABA could increase *OsABI5* expression level by protecting *OsABI5* from ubiquitin-mediated degradation. And *OsABF2* is one transcription factor that regulates ABA-mediated ABRE-dependent gene expression under osmotic stress condition (Fujita et al. 2010; Shinozaki et al. 2006).

Our data showed that the transcript level of *OsABF2* and Os*ABI5*, were significantly elevated under stress condition than normal condition in the OER1 plants (Fig. 7). Token into consideration of the expression level of *OsNCED*, *OsZEP* and *OsAAO* were enhanced more than normal condition in OER1 plants under drought or salt stress condition as well (Fig. 7). It stands to reason that OsRHP1 is implicated in drought and salt stress signal pathway through modulating expression of ABA biosynthesis and ABA-responsive genes.

During the ABA-mediated stress response, many genes including late embryogenesis abundant proteins (LEA) genes, heat shock proteins, lipid desaturases, water channel regulators, ion transporters and other antioxidant enzymes, downstream of transcription factors, were up-regulated (Yamaguchi-Shinozaki and Shinozaki 2006). Among these genes, *LEA* is one canonical gene involved in plant stress resistance to drought, salt, and cold stress. Previous study showed overexpression of *OsLEA3-1* in rice significantly enhanced drought and salt resistance under field condition (Xiao et al. 2007). Our data showed that expression of *OsLEA1-3* was also remarkably enhanced under stress condition than normal condition in the OER1 plants (Fig. 7). These results might explain why overexpressing *OsRHP1* in rice plants conferred enhanced drought and salt tolerance (Fig. 4-6).

Although we have shown that overexpression of *OsRHP1* conferred enhanced drought and salt tolerance in rice (Oryza sativa L.), exhibited a significant increase in ABA content, and expression level of ABA biosynthesis genes and ABA-responsive genes under drought and salt stress. It is still not clear how OsRHP1 stimulates transcriptional regulation of the genes involved in ABA signaling pathway. Further questions remain to be addressed, including whether OsRHP1 could serve as an E3 ubiquitin ligase, and how OsRHP1 interacts with other ABA-related genes in rice. Further study about these questions will shed more light on regulatory mechanism of *OsRHP1*, and accelerate potential application in transgenic engineering for improving drought and salt stress tolerance in crop plants.

## Materials and Methods

### Plant Materials and Growth Conditions

Rice cultivar Nipponbare (*Oryza sativa* L. ssp. *japonica*) was used in this study. The wild type (WT) and transgenic rice plants were first planted in the artificial climate incubators (BINDER, Tuttlington, Germany) under standard conditions (28°C day, 20°C night; 12 h light, 12 h dark), and transplanted into the field 5 weeks later. WT and the transgenic progeny plants were grown side by side in farm's field at the Sichuan University.

### Isolation of *OsRHP1* cDNA

Total RNAs derived from rice seedling leaves were extracted using Trizol reagent following the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using the First Strand cDNA Synthesis kit (Toyobo, Osaka, Japan). *OsRHP1* cDNA fragment was amplified by high fidelity PCR using PrimeSTAR HS DNA polymerase (TaKaRa, Dalian, China). Briefly, upstream (5'-AAGCTT GAGATGGGCATCTCGAGCATG-3') and downstream (5'-GGATCC GCCCAGTGGCGGATTGAAG-3') primers were used to amplify a 539 bp DNA fragment.

### Construct Overexpressing *OsRHP1* and Rice Transformation

A 539-bp *Hind*III/*Bam*HI *OsRHP1* cDNA fragment with 3 bp of 5'-untranslated leader and 32 bp of 3'-untranslated trailer was cloned into the plant gene expression vector pHB, which contains a double CaMV *35S* promoter and the rbcS polyadenylation signal (Mao et al. 2005). The resulting recombinant was named as pHB-*OsRHP1* and introduced into *Agrobacterium tumefaciens* EHA105. Rice cultivar Nipponbare (*O. sativa* L. ssp. *japonica*) was used as plant material and transformed by the standard *Agrobacterium*-mediated method (Liang et al. 1997). Primary transformants ($T_0$) were selected on MS agar containing 50 mg $L^{-1}$ hygromycin.

### Semi-quantitative and Quantitative Real-Time RT-PCR

Primary rice transformants ($T_0$) were verified by PCR using *hygromycin phosphotransferase (hpt)* gene-specific primers *hpt*-f (5'-TAGGAG-

GGCGTGGATATGTC-3') and *hpt*-r (5'-TACACAGCCATCGGTCC-AGA-3'). For semi-quantitative RT-PCR, total RNAs were extracted using Trizol reagent following the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA) and treated with DNase I (TaKaRa, Dalian, China). About 1 μg of total RNA from each sample was used for first-strand cDNA synthesis (Toyobo, Osaka, Japan). Actin gene was employed as positive internal control. *Actin*-f1 (5'-AAGATCCTGACGGAGCGTGGTTAC-3'), *Actin*-r1 (5'-CTTCCTA-ATATCCACGTCGCACTTC-3'); *Actin*-f2 (5'-ACCTTCAACACC-CCTGCTAT-3'), *Actin*-r2 (5'-CACCATCACCAGAGTCCAAC-3') were used for the RT-PCR and real-time RT-PCR, respectively. *OsRHP1*-f2 (5'-GAGATGGGCATCTCGAGCATG-3'), *OsRHP1*-r2 (5'-GCCCA-GTGGCGGATTGAAG-3'); *OsRHP1*-f3 (5'-GCCACCCCCAAAT-CTGAAAC-3'), *OsRHP1*-r3 (5'-TACGCCACCACGCTGTCCTT-3') were used for the RT-PCR and real-time RT-PCR, respectively. To test the expression level of ABA-related genes, total RNA was extracted from 12-d fresh leaf. Drought and salt stress were applied for 12 h and 6 h, respectively (Zhang et al. 2011). The primers for real-time PCR analysis of ABA synthesis and ABA-regulated genes were as follows (in parentheses): *OsNCED* (5'-CGTCCCGG-ACTGCTTCTGCT-3') and (5'-AGGCGGTCGTCGGACTCGTT-3'), *OsZEP* (5'-TATGACCGACCACCTACTTT-3') and (5'-GGTAACC-ATCCTCAATAGCC-3'), *OsAOO* (5'-TCTTCATAGAGTCTCCC-ACA-3') and (5'-GTAACTTTGCACCTCATACC-3'), *OsABI5* (5'-AGCGGTGAACCAGTTTGATT-3') and (5'-ATCTGCCTGTTTCCT-CTCCA-3'), *OsABF2* (5'-GATTTTGCCTCCGACGAACA-3') and (5'-GAGCCATCACCATTCACCAA-3'), *OsLEA3-1* (5'-AATGATTT-CCCTTTGGGTC-3') and (5'-CATCAGTACACATCACCCA-3'). All real-time PCR reactions were performed in triplicate on the iCycler iQ™ Real-Time PCR and Detection System (Bio-Rad Laboratories, Tokyo, Japan). Relative expression software tool (REST) was used to analyze the results.

Stress Tolerance Experiments

For drought and salt stress treatment on soil-grown plants, homozygous $T_2$ transgenic plants were selected by germinating seeds on MS medium containing 50 mg/L hygromycin. After germination, all seedlings showing positive transgene were transplanted into soil pots (vermiculite:soil = 2:1) in a growth chamber under a 16 h light/8 h dark cycle at 25°C. After 3-week growth with normal water supply, drought stress was imposed by withholding water for 10 d and 14 d, salt stress was carried out by water solution containing 200 mM NaCl for 5 d.

Determination of ABA Content

Drought and salt stresses were applied to transgenic plants as described previously (Ouyang et al. 2010). ABA content of samples was determined with a Phytodetek ABA test Kit (Agdia, USA) by an enzyme-linked immunosorbent assay (ELISA). For ABA extraction, 1 g of flesh leaf was ground in a mortar and homogenized using extraction solution (80% methanol, v/v). Extracts were centrifuged at 12,000 *g* for 20 min. The supernatant liquid was eluted through a Sep-PakC18 cartridge (Waters, Milford, MA, USA) to remove polar compounds and stored at -20°C for enzyme linked immunosorbent assay (ELISA). The ELISA procedures were conducted according to the instructions provided by the manufacturer. ABA was determined by the SpectraMax M2 system (molecular devices, USA). SoftMax Pro v5.0.1 was used to analyze the results.

## Acknowledgments

This work was Supported by the 973 Program from Chinese Ministry of Science and Technology (2011CB100401), the Key Project from Chongqing Local Government (2010AA1019) and the National Science Fund of China for Distinguished Young Scientists (30825030).

## Author's Contributions

YL, FX and DZ designed the experimental plan. DZ performed the experiments. DZ, YL, FX and PH analyzed the data. DZ drafted the initial manuscript. DZ, FX and YL wrote the paper. All the authors agreed on the contents of the paper and post no conflicting interest.

## Supporting Information

Fig. S1. semi-quantitative RT-PCR analyses of *OsRHP1* under different abiotic stress conditions.

## References

Aswath CR, Kim SH, Mo SY, Kim DH (2005) Transgenic plants of creeping bent grass harboring the stress inducible gene, 9-cis-epoxycarotenoid dioxygenase, are highly tolerant to drought and NaCl stress. Plant Growth Regul 47:129–139

Bopopi JM, Vandeputte OM, Himanen K, Mol A, Vaessen Q, El Jaziri M, Baucher M (2010) Ectopic expression of *PtaRHE*1, encoding a poplar RING-H2 protein with E3 ligase activity, alters plant development and induces defence-related responses. J Exp Bot 61:297–310

Bu QY, Li HM, Zhao Q, Jiang H, Zhai Z, Zhang J, Wu X, Sun J, Xie Q, Wang D, Li C (2009) The Arabidopsis RING Finger E3 Ligase *RHA2*a is a novel positive regulator of abscisic acid signaling during seed germination and early seedling development. Plant Physiol 150:463–481

Disch S, Anastasiou E, Sharma VK, Laux T, Fletcher JC, Lenhard M (2006) The E3 ubiquitin ligase BIG BROTHER controls Arabidopsis organ size in a dosage-dependent manner. Current Biol 16:272–279

Finkelstein RR, Gampala SSL, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell 14(suppl 1):S15–S45

Freemont PS (1993) The RING finger. A novel protein sequence motif related to the zinc finger. Ann NY Acad Sci 684:174–192

Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants. J Plant Res. 124:509–525

Gao Y, Li H, Deng D, Chen S, Jiang W, Chen J (2012) Characterization and expression analysis of the maize RING-H2 finger protein gene *ZmXERICO* responsive to plant hormones and abiotic stresses. Acta Physiologiae Plantarum 34:1529–1535

Hadiarto T, Tran LSP (2011) Progress studies of drought-responsive genes in rice. Plant Cell Reports 30:297–310

Hou X, Xie K, Yao J, Qi Z, Xiong L (2009) A homolog of human ski-interacting protein in rice positively regulates cell viability and stress tolerance. Proc Natl Acad Sci USA 106:6410–6415

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. Plant J 27:325–333

Kam J, Gresshoff P, Shorter R, Xue G-P (2007) Expression analysis of RING zinc finger genes from Triticum aestivum and identification of *TaRZF*70 that contains four RING-H2 domains and differentially responds to water deficit between leaf and root. Plant Sci

173:650–659
Ko JH, Yang SH, Han KH (2006) Upregulation of an Arabidopsis RING-H2 gene, *XERICO*, confers drought tolerance through increased abscisic acid biosynthesis. Plant J 47:343–355
Koiwai H, Tagiri A, Katoh S, Katoh E, Ichikawa H, Minami E, Nishizawa Y (2007) RING-H2 type ubiquitin ligase EL5 is involved in root development through the maintenance of cell viability in rice. Plant J 51:92–104
Li H, Jiang H, Bu Q, Zhao Q, Sun J, Xie Q, Li C (2011) The Arabidopsis RING finger E3 ligase RHA2b acts additively with RHA2a in regulating abscisic acid signaling and drought response. Plant Physiol 156:550–563
Liang Z, Ma D, Tang L, Hong Y, Luo A, Zhou J, Dai X (1997) Expression of the spinach betaine aldehyde dehydrogenase (BADH) gene in transgenic tobacco plants. Chin J Biotechnol 13:153
Lim SD, Yim WC, Moon JC, Kim DS, Lee BM, Jang CS (2010) A gene family encoding RING finger proteins in rice: their expansion, expression diversity, and co-expressed genes. Plant Mol Biol 72:369–380
Liu H, Zhang H, Yang Y, Li G, Wang X, Basnayake BMVS, Li D, Song F (2008) Functional analysis reveals pleiotropic effects of rice RING-H2 finger protein gene *OsBIRF*1 on regulation of growth and defense responses against abiotic and biotic stresses. Plant Mol Biol 68:17–30
Mao J, Zhang YC, Sang Y, Li QH, Yang HQ (2005) A role for Arabidopsis cryptochromes and *COP*1 in the regulation of stomatal opening. Proc Natl Acad Sci USA 102:12270–12275
Molnar G, Bancoş S, Nagy F, Szekeres M (2002) Characterisation of *BRH*1, a brassinosteroid-responsive RING-H2 gene from *Arabidopsis thaliana*. Planta 215:127–133
Ouyang SQ, Liu YF, Liu P, Lei G, He SJ, Ma B, Zhang WK, Zhang JS, Chen SY (2010) Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (*Oryza sativa*) plants. Plant J 62:316–329
Park HY, Seok HY, Park BK, Kim SH, Goh CH, Lee B, Lee CH, Moon YH (2008) Overexpression of Arabidopsis *ZEP* enhances tolerance to osmotic stress. Biochem Biophys Res Commun. 375:80–85
Park GG, Park JJ, Yoon JM, Yu SN, An GH (2010) A RING finger E3 ligase gene, *Oryza sativa* delayed seed germination 1 (*OsDSG*1), controls seed germination and stress responses in rice. Plant Mol Biol 74:467–478
Qin X, Zeevaart JAD (2002) Overexpression of a 9-cis-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. Plant Physiol 128:544–551
Ryu MY, Cho SK, Kim WT (2010) The Arabidopsis C3H2C3-type RING E3 ubiquitin ligase AtAIRP1 is a positive regulator of an abscisic acid-dependent response to drought stress. Plant Physiol 154:1983–1997
Seo M, Koiwai H, Akaba S, Komano T, Oritani T, Kamiya Y, Koshiba T (2001) Abscisic aldehyde oxidase in leaves of *Arabidopsis thaliana*. Plant J 23:481–488
Taylor IB, Sonneveld T, Bugg TDH, Thompson AJ (2005) Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. J Plant Growth Regul 24:253–273
Thompson AJ, Jackson AC, Symonds RC, Mulholland BJ, Dadswell, AR, Blake PS, Burbidge A, Taylor IB (2001) Ectopic expression of a tomato 9-cis-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. Plant J 23:363–374
Xia Z, Liu Q, Wu J, Ding J (2011) ZmRFP1, the putative ortholog of SDIR1, encodes a RING-H2 E3 ubiquitin ligase and responds to drought stress in an ABA-dependent manner in maize. Gene 495:146–153
Xiao B, Huang Y, Tang N, Xiong L (2007) Over-expression of a *LEA* gene in rice improves drought resistance under the Weld conditions. Theor Appl Genet 115:35–46
Xie Q, Guo HS, Dallman G, Fang S, Weissman AM, Chua NH (2002) SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. Nature 419:167–170
Xiong L, Zhu JK (2003) Regulation of abscisic acid biosynthesis. Plant Physiol 133:29–36
Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803
Yi C, Deng XW (2005) COP1–from plant photomorphogenesis to mammalian tumorigenesis. Trends Cell Biol 15:618–625
Zhang X, Garreton V, Chua NH (2005) The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. Genes Dev 19:1532–1543
Zhang X, Zhen J, Li Z, Kang D, Yang Y, Kong J, Hua J (2011) Expression profile of early responsive genes under salt stress in upland cotton (*Gossypium hirsutum* L.). Plant Mol Biol Rep 29:626–637
Zhang Y, Yang C, Li Y, Zheng N, Chen H, Zhao Q, Gao T, Guo H, Xie Q (2007) SDIR1 is a RING finger E3 ligase that positively regulates stress-responsive abscisic acid signaling in Arabidopsis. Plant Cell 19:1912–1929