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Overexpression of *BdRHP1* improves drought tolerance and reduces yield loss in rice

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Abstract

Drought is a major factor decreasing the growth, development, and productivity of rice in about one-third of the world area. The characterization of genes imparting tolerance to drought in rice, is an attractive strategy for genetic engineering to improve drought tolerance. It is demonstrated that ectopic overexpression of *Brachypodium distachyon* RING-H₂ finger gene (designated as *BdRHP1*) enhances drought tolerance in rice at both the vegetative and reproductive stages. When subjected to drought, positive transgenic lines showed delayed wilting, and improved recovery after rewatering. However, the transgenic plants exhibited more significant germination delay and shoot and root growth arrest than WT under 5 μ M abscisic acid (ABA) treatment. When they were subjected to drought at the reproductive stage, the transgenic plants lost water more slowly compared to WT and they had higher leaf relative water content. After 28 d of slow progressive soil drying, transgenic plants recovered better after rewatering and flowered earlier than WT plants. The yield of water-stressed transgenic plants was higher than that of WT plants. Together, the data suggest that *BdRHP1* has a specific function in positive modulation of improving drought tolerance in rice.

Additional key words: abscisic acid, *Brachypodium distachyon*, *Oryza sativa*, relative water content, RING finger.

Introduction

Rice (*Oryza sativa* L.) is an important cereal crop and provides staple food for more than half of the world's population (Hadiarto and Tran 2011). Water shortage is one of the limiting factors for rice yield affecting the majority of the farmed regions around the world. Recent years, tremendous efforts have been devoted to enhancing drought tolerance and alleviating yield loss caused by drought stress.

It is documented that plants respond to drought *via* alteration of gene expression, accumulation of abscisic acid (ABA) or osmolytes, stomatal closure, decrease in photosynthetic rate and growth, activation of respiration, *etc.* (Baldoni *et al.* 2015, Fujita *et al.* 2011, Haak *et al.* 2017, Joshi *et al.* 2016). Recently, several hundred genes

induced or repressed under drought condition have been identified as candidate genes for genetic engineering (Abou-Elwafa and Song 2017, Fleury *et al.* 2010, Jiang *et al.* 2012, Serba and Yadav 2016). In addition to introducing genes encoding metabolites or osmo-protectants into plants to avoid cellular injury, some genes involved in signalling and regulatory pathways offer new ways for enhancing drought tolerance in crops (Singh *et al.* 2015).

In eukaryotes, RING (really interesting new gene) zinc-finger proteins, most of which possess E3 ligase activity, are a large gene family (Jung *et al.* 2012). The data indicates these RING finger proteins are associated with plant growth and development *via* plant-environment

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Abbreviations: ABA - abscisic acid; *BdRHP1* - *Brachypodium distachyon* RING-H₂ finger gene; CaMV - cauliflower mosaic virus; GA - gibberellin; LEA - late embryogenesis abundant protein; PCR - polymerase chain reaction; RING - really interesting new gene; RT-PCR - reverse transcription PCR; RWC - relative water content; TM - transmembrane; WS - water-stressed; WT - wild type; WUE - water use efficiency; WW - well watered.

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interactions (Schwechheimer *et al.* 2009). Particularly, RING-H₂ zinc finger genes have been shown to play important roles involved in seed dormancy, germination, plant seedling growth, and drought stress response dependent on ABA-mediated pathway. *XERICO* confers enhanced drought tolerance through up-regulation of ABA biosynthesis (Ko *et al.* 2006). RING-H₂ finger E3 ligase (RHA2a) is a novel positive regulator of ABA signalling during seed germination and early seedling development (Bu *et al.* 2009), and RHA2b acts additively with RHA2a in regulating ABA signalling in drought response (Li *et al.* 2011). *Arabidopsis* ABA-insensitive RING protein 1 (AtAIRP1) is a positive regulator of an ABA-dependent response to drought stress (Ryu *et al.* 2010). Salt- and drought-induced RING-finger protein 1 (SDIR) positively regulates drought and salt stress-responsive ABA signalling pathway in *Arabidopsis* (Zhang *et al.* 2007). *OsSDIR1* overexpression greatly improves drought tolerance in transgenic rice (Gao *et al.* 2011). *Zea mays* RING-finger protein 1 (*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.* 2012). The pepper RING-H₂ finger E3 ligases, CaDIR1 and CaREL1, also regulate the drought

stress response via ABA-mediated signalling (Joo *et al.* 2017). These genes offer a new way for genetic engineering and facilitate our understanding of drought stress response pathway.

The grass family *Poaceae* includes the most important crop plants and comprises several subfamilies such as *Pooideae* (wheat, barley, and oats), *Ehrhartoideae* (rice), and *Panicoideae* (maize, sorghum, and millets). The wild grass *Brachypodium distachyon* belongs to the *Brachypodieae* tribe, a sister tribe to the *Triticeae* in the *Pooideae* subfamily (Mochida and Shinozaki 2013). Due to its simple diploid genome, close phylogenetic links with other temperate grass species, relative ease of genetic transformation, short life cycle, simple growth requirements, and especially keeping maximal genetic variability, *Brachypodium* may be well placed as a model cereal for functional genomic studies in order rapidly to decipher gene functions (Girin *et al.* 2014).

The aim of this work, was to clone and characterized the full-length cDNA encoding RING-H₂ protein from *Brachypodium distachyon*, designated as *BdRHP1*. Then, to obtain transgenic rice plants overexpressing *BdRHP1*, and decipher its function in drought stress responses.

Materials and methods

Plants and growth conditions: Rice (*Oryza sativa* L.) cultivar Nipponbare was used in our study. The wild type (WT) and transgenic rice plants were firstly planted in the climate chamber (BINDER, Tuttlington, Germany) under standard conditions (12-h photoperiod, day/night temperatures 28/20 °C, air humidities 70 %, and irradiance 675 $\mu\text{mol m}^{-2} \text{s}^{-1}$), and transplanted into the field 3 weeks later. WT and the transgenic progeny plants were grown side by side in farm's field around the Anqing Normal University.

***BdRHP1* constructs and transformation:** A 493-bp fragment, contained *Brachypodium distachyon* RING-H₂ protein 1 (*BdRHP1*) coding sequence with restriction endonuclease sites *Bam*HI/*Sac* I was cloned into the plant gene expression vector pHB, which possesses a double *CaMV* 35S promoter and the *rbcS* polyadenylation signal (Mao *et al.* 2005). The resulting construct was introduced into *Agrobacterium tumefaciens* strain EHA105 and rice plants were transformed by the standard *Agrobacterium*-mediated method (Liang *et al.* 1997). Eight independent transgenic lines were generated and grown to T₂ generations. The used primers are given in Table 1 Suppl.

Analysis of abscisic acid sensitivity and water loss curves: Around 20 seeds of WT and two of T₂ generation independent lines were surface-sterilized in 2 % (m/v) NaClO for 15 min and then washed thoroughly with distilled water. Seeds were placed in Petri dishes with

15 cm³ of autoclaved water containing 0 or 5 μM ABA. Germination percentage was calculated on day 3, 5, and 7 after sowing. After 7 d, root and shoot lengths were also measured.

Three independent transgenic seeds along with wild type Nipponbare were sown in polyvinyl pots filled with 2 kg of air-dried commercial potting mixture (*Pioneer Horticultural Company*, Anqing, China) saturated with water. For water loss analysis, ten fully expanded leaves (4-leaf stage) from WT and three homozygous transgenic lines were detached and weighed immediately. Detached leaves were placed on filter paper in the growth chamber and weighed at designated time intervals. The rate of water loss was calculated on the basis of the initial fresh mass of the leaves.

Drought treatments: The T₂ seeds of independent transgenic lines with 3 replications per line along with WT were sowed in pots with the same amount of soil. The experiment was conducted in a growth chamber. At 10 d after sowing, the pots were saturated with water and the individual pots along with plants were weighed. Water was withheld from 11 d after sowing until WT plants showed complete wilting. Individual pot weight was taken every day to assess the water loss. At the end of drought period, plants were watered and recovery was recorded 5 d later (Manavalan *et al.* 2012).

Drought stress (WS) was also imposed at the early booting stage by withholding water for 28 d. Control plants

were regularly irrigated (WW plants). Development of stress in the WS plants was routinely monitored by visual symptoms of leaf rolling and leaf drying and by leaf relative water content (RWC). The plant traits and yield components of rice namely, panicle length (cm), panicle number, grains per panicle, spikelet sterility (%), grain yield per plant (g) at maturity were measured as described

by (Yoshida *et al.* 1976).

Statistical analysis: Data was analysed using *SAS* v. 9.2 software (*SAS Institute*, Cary, NC, USA). by a three factor *ANOVA* model (event, plant type, and treatment). Means were tested for significance according to least significant differences.

Results

Based on comparative genomics analysis we acquire a novel RING-H₂ finger homolog of *Oryza sativa* *OsRHP1* in *Brachypodium distachyon* designated as *BdRHP1*. *BLAST* search exhibited thirteen homologous proteins from other species. In general, most of the plant species have the single conserved RING-H₂ finger protein. Sequence similarity analysis revealed that *BdRHP1* (XP-003572327) enjoyed 89.42, 89.40, 62.23, 58.75, and 56.89 % homology with *O. sativa* var. *japonica* (XP-015648819), *O. sativa* var. *indica* (OS_08G36800), *Hordeum vulgare* (BAK-03428), *Sorghum bicolor* (XP_002444825) and *Zea mays* (NP-001147769), respectively (Fig. 1). Alignment of peptide sequences of plant homologs displayed 56.69 % uniform identity. In addition, the RING-H₂ domain of *BdRHP1* is 70 to 100 % identical to the corresponding regions of other RING-H₂ protein homologs. Other species like *Solanum tuberosum*, *Solanum lycopersicum*, *Nicotiana tabacum*, *Glycine max*, *Brassica napus*, and *Arabidopsis thaliana* also have only one gene for the RING-H₂ finger protein. Multiple

sequence alignments of divergent protein sequences were done using *ClustalW.2* and through the next generation biology workbench (<http://www.ngbw.org>). The most highly conserved regions of proteins were searched by using *SMART* tool (<http://smart.embl-heidelberg.de/>), and it showed that the RING-H₂ finger protein motif was highly conserved among the species (Fig. 1 Suppl.). Phylogenetic trees based around the neighbour-joining method were constructed by *MEGA* (Saitou *et al.* 1987). It was indicated that the RING-H₂ finger genes from monocots and dicots form distinct groups, and the clustering of families is seen (Fig. 2). In addition, the analysis revealed that *BdRHP1* encoded a small protein (158 amino acids) with one N-terminal transmembrane (TM) domain, a RING-H₂ zinc-finger motif located at the C-terminus. The tree revealed that the RING-H₂ finger genes of monocots are closely related.

Transgenic plants overexpressing *BdRHP1* were generated. A 493-bp fragment with *BamH* I/*Sac* I endonuclease sites was cloned into the plant gene

Fig. 1. Alignment of *BdRHP1* with other homologous proteins. Black colour indicates identical, grey colour indicates 75 % consistency, arrows indicates cysteins locus.

expression vector pHB, which contains a double *CaMV* 35S promoter and the *rbcS* polyadenylation signal (Fig. 3A). CDS encoded a small protein (158 amino acids) with one N-terminal TM domain, a RING-H₂ zinc-finger motif located at the C-terminus. Primary transgenic (T₀) lines were verified by PCR amplification using primers of *BdRHP1* and the hygromycin phosphotransferase gene. The T₁ transgenic seeds from eight independent positive

plants and four independent plants (designated as Bd-3, Bd-5, Bd-7, and Bd-8) were used in the drought treatment at the vegetative and reproductive stages, respectively. Three independent T₂ homozygous lines (Bd-5 Bd-7, and Bd-8, respectively) were picked out for semi-quantitative PCR and real-time quantitative PCR analyses. Expression of *BdRHP1* mRNA was detected in transgenic lines but not in WT (Fig. 3B,C).

Fig. 2. Phylogenetic relationship between BdRHP1 and homologs in other species determined by neighbour-joining method (MEGA7).

The T₂ seeds of transgenic plants overexpressing *BdRHP1* were subjected to ABA sensitivity assay. Under 5 μ M ABA, the transgenic plants displayed more remarkable seed germination delay than WT. Also the shoot and root growth of transgenic plants was more inhibited by ABA than that of WT plants (Fig. 2 Suppl.).

When subjected to drought in vegetative stage by withholding water, the transgenic plants did not exhibit any morphological difference from WT plant during the first 13 d after sowing. Symptoms of leaf wilting appeared in WT plants after 15 d of withholding water, but the same symptom appeared in the transgenic plants after 17 d. After 22 d some of WT plants died or showed severe leaf drying, whereas the transgenic plants exhibited some vitality (Fig. 3 Suppl.). Further, we measured changes of the fresh mass of detached leaves over different periods as an indicator of transpirational water loss. These data indicated that transgenic lines have more water retention ability than WT (Fig. 4 Suppl.).

All transgenic plants showed similar growth and tiller production as WT plants. Drought experiment was carried out 7 d before booting stage refer to the modified method described previously (Yue *et al.* 2006), in which water was withhold for 28 d. After 28 d the transgenic plants maintained greater flag leaf and higher relative water content than WT plants. In addition, the flag leaves of WT plants wilted much faster and had more rolled flag leaves (data not shown). Three days after re-watering, 49 - 55 % of the transgenic plants recovered, but only 19 - 23 % of WT plants (Fig. 4A). Furthermore, the plants from 3 independent transgenic lines exhibited slower water loss compared with WT plants and so maintained higher water content (Fig. 4B). In addition, the transgenic plants retained more green leaves than WT (data not shown). After re-watering for 5 d, transgenic plants showed 85 % recovery and even started flowering, whereas WT plants recovered only to 75 % and showed delay in flowering (Fig. 5 Suppl.). At maturity, there was a significant

difference between the transgenic lines and WT in filled grain mass. The grain mass of transgenic plants was 10.8 g plant⁻¹ under the WW treatment and 5.3 g plant⁻¹ under the WS treatment. However, the grain mass of WT plants was similar (10.2 g plant⁻¹) under the WW treatment but only 3.2 g plant⁻¹ under the WS treatment (Fig. 5C). Interestingly, the smaller yield reduction was discovered in transgenic lines, whereas WT showed a higher yield reduction (Fig. 5B).

Production parameters including panicle number, panicle length, spikelet sterility and number of grains per

panicle were presented (Fig. 6). There was a significant difference between transgenic plants and WT in spikelet sterility which ranged from 30.1 - 32.8 % in transgenic lines under drought condition, whereas in WT it was 56.6 %. There was also significant difference in number of grains per panicle and panicle length under WS condition. Meanwhile no significant difference between the WT and transgenic plants was noticed for panicle number. These results apparently indicated that overexpression of *BdRHP1* in rice can improve drought resistance during the reproductive stage.

Fig. 3 A - Schematic diagram of part of the T-DNA region of the transforming construct. B - Semi-quantitative RT-PCR analysis of *BdRHP1* mRNA in WT and three independent T₂ lines. C - Real-time PCR analysis of *BdRHP1* mRNA in WT and three independent T₂ lines. Means $\pm$ SEs are from three independent experiments.

Fig. 4 A - Survival rate of transgenic and WT plants after drought and re-watering for 3 d. B - Relative water content of transgenic and WT plants. Means $\pm$ SEs from three independent experiments (*, ** indicate significant differences at $P < 0.05$ or $P < 0.01$, respectively, according to *t*-test).

Discussion

RING-H₂ finger proteins are widely represented in plants and play important roles in the regulation of many developmental processes as well as in plant-environment interactions (Liu *et al.* 2008, Lim *et al.* 2010, Ryu *et al.* 2010, Gao *et al.* 2011). Previous studies have indicated the

RING-H₂ finger proteins of many species play a vital role in drought tolerance and in ABA-dependent drought signalling pathway (Ko *et al.* 2006, Liu *et al.* 2008, Mukoko Bopopi *et al.* 2010, Zeng *et al.* 2014). They may be conserved in plants, which is supported by the facts that

only one transcript of RING-H₂ finger homolog was detected in *Brachypodium distachyon* and other plant species.

Since *Brachypodium* may be considered as a model cereal for functional genomic studies in order rapidly to decipher gene functions (Girin *et al.* 2014), we isolated *BdRHP1* and transformed it in rice. The overexpression of

BdRHP1 in rice displayed hypersensitivity to ABA during seed germination and early seedling growth (Fig. 2 Suppl.). Our data also showed that overexpression of *BdRHP1* substantially increased cellular ABA content (data not shown) and enhanced drought stress tolerance in rice plants.

Fig. 5 *A* - Effect of drought stress on morphology of spikes from rice transgenic and WT plants. *B* - Percentage reduction of grain yield over control (well-watered). *C* - grain yield (filled grain mass per plant) of WT and different transgenic lines. Means $\pm$ SEs from three independent experiments (* - significant differences at $P < 0.05$ according to *t*-test).

It was documented that ABA affects some stress adaptation response during germination and early seedling growth, such as suppression of germination, shoot and root growth, transpiration rate, and wilting (Holdsworth *et al.* 2008, Fujita *et al.* 2009, Sreenivasulu *et al.* 2012, Nakashima and Yamaguchi-Shinozaki 2013, Chater *et al.* 2014, Lim *et al.* 2015). Interestingly, gibberellins (GAs) are phytohormones promoting germination and early growth and development (Dai and Xue 2010, Sun *et al.* 2010a,b, Daviere and Achard 2013, Kwon and Paek 2016). Hence, accumulation of ABA will antagonize GAs effects (Jiang and Fu 2007, Zentella *et al.* 2007, Daviere and Achard 2013). We found that rice plants overexpressing *BdRHP1* were hypersensitive to exogenous ABA as concert its effects on germination and growth during the early seedling stage.

Sessile plants have involved arrays of distinct

mechanisms to adapt to abiotic stresses (Fujita *et al.* 2011, Lata and Prasad 2011, Sah *et al.* 2016). Increased ABA content in response to drought induce expression of many genes that play multifaceted roles in dehydration response and tolerance in both vegetative and reproductive stages (Yamaguchi-Shinozaki *et al.* 2006, Zhu *et al.* 2016, Haak *et al.* 2017). Late embryogenesis abundant proteins (LEAs), heat shock proteins, lipid desaturases, water channel regulators, ion transporters, and antioxidant enzymes are important for plant tolerance (Nakashima and Yamaguchi-Shinozaki 2013). Besides this, ABA also improves the water-use efficiency (WUE) of plants under drought stress. One of the drought tolerance mechanisms in rice is achieved by delayed leaf rolling (Price Cairns *et al.* 2002). The maintenance of higher leaf RWC guaranteed slight leaf wilting or rolling symptoms, and higher rate of survival after resuming water (Fig. 4). Rapid

transpiration in the WT might have led to a quick loss of pressure potential and triggered early leaf rolling and drying in plants (Chandra Babu *et al.* 2004, Rivero *et al.* 2007, Zhang *et al.* 2010).

In rice, yield loss depends on both the severity and duration of drought (Farooq *et al.* 2009). Therefore, the identification of genes that impact yield improvement at the reproductive stage is imperative. In our work, water deficit was imposed at the critical stage of reproductive

development. Drought resulted in approximately two-third of grain yield loss in WT plants, whereas about a half of yield loss was noticed in transgenic plants (Fig. 5). This was associated with an increased number of filled grains and reduced spikelet sterility as coincided with other studies (Xiao *et al.* 2009, Jeong *et al.* 2010). However, no differences were observed between WT and transgenic plants under WW conditions.

Fig. 6 A - Panicle length in transgenic plants and WT. B - Spikelet sterility of transgenic plants and WT. C - Panicle number per transgenic or WT plant. D - Grain number per panicle in transgenic and WT plants. Means $\pm$ SEs from three independent experiments (* - significant differences at $P < 0.05$ according to t -test).

We used overexpression of *BdRHP1* in rice to decipher its function under drought. Transgenic plants exhibited less adverse effects of drought on plant architecture, growth, and development. When they were subjected to

drought during reproductive stage, less yield loss was noticed. Hence, *BdRHP1* is a candidate for rice genetic engineering for drought tolerance.

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