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Cloning and expression analysis of a dirigent protein gene from the resurrection plant *Boea hygrometrica*

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Abstract

Resurrection plants are tolerant to extreme dehydration and are useful model systems to study genes that play a role in drought tolerance. A gene fragment encoding a dirigent protein that is predicted to function in lignin biosynthesis was identified from leaves of the resurrection plant *Boea hygrometrica* via cDNA microarray screening. A cDNA, designated *BhDIR1*, containing the complete predicted open reading frame, was obtained by 5′-RACE. *BhDIR1* transcripts were found to be accumulated in response to changes in plant dehydration status, exogenously applied phytohormones and signaling molecules, and temperature stresses. *BhDIR1* encodes a protein of 199 amino acids, which shows 20–40% similarity to dirigent proteins reported from other plants. BhDIR1 is predicted to contain a cleavable signal peptide at the N-terminal, and its plasma membrane/cell wall localization was confirmed using a GFP fusion protein assay. Consistent with this discovery, the acid-soluble lignin content decreased in dehydrated *B. hygrometrica* leaves. Taken together, our results indicate a protective role for a dirigent protein in response to drought stress by changing the physical characters of lignin which in turn is predicted to affect the mechanical strength and flexibility of the plant cell wall.

Keywords: Resurrection plant; Dirigent protein; Drought; Lignin; Cell wall

1. Introduction

Drought is an environmental stress frequently encountered by plants. Plants have evolved various strategies to cope with drought stress. Unlike most plants that can resist only mild water deficit, a group of so-called "resurrection plants" can tolerate severe water loss in a dormancy-like state for an indefinite period and are able to recover when water is available. The mechanisms underlying such extraordinary drought resistance have been investigated. Studies have shown that desiccation tolerance is primarily protoplasmic. High levels of antioxidant enzymes, osmolytes and protective macromolecules are commonly accumulated during desiccation, which is believed help to avoid reactive oxygen species (ROS) burst, to protect membranes and proteins, and to form a glassy state to reduce the metabolic rate during drought [1]. More recent studies suggest that cell wall flexibility and strength may play an important role in dehydration tolerance to ensure recovery after rewatering. In the resurrection plants *Craterostigma wilmsii*, *Myrothamnus flabellifolius* and *Sporobolus stapfianus*, mesophyll cells were found to shrink after dehydration, and the cell walls were subject to a high degree of folding [2,3]. Cell wall folding may contribute to reduce the extent of plasmolysis, to minimize the damage on the plasma membrane and to maintain the integrity of cell structures during dehydration. As major components of the cell wall and important determinants of cell wall elasticity besides carbohydrates, cell wall structural proteins such

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as expansins and glycine-rich proteins have been shown to accumulate upon dehydration [4,5].

Previously, we have reported studies on a Chinese resurrection angiosperm, *Boea hygrometrica* (Bunge) R. Br., using physiological, genomic and proteomic approaches to understand the dehydration tolerant basis [6–10]. It was observed that walls of *B. hygrometrica* leaf cells folded inwards soon after drought occurred [11]. Efforts to identify drought-induced genes from the cDNA library from *B. hygrometrica* leaves dehydrated for 2 h have revealed a gene fragment encoding a peptide highly homologous to dirigent proteins that may function in lignin synthesis [12]. In order to gain insight into the role of cell wall-related proteins in wall folding and drought-tolerance mechanisms, in this study, the full-length dirigent gene was cloned and its expression was analyzed.

2. Materials and methods

2.1. Materials

Boea hygrometrica plants were collected in Beijing and maintained in a greenhouse (approximately 25 °C, 16 h/8 h light period) with regular irrigation. After 2 weeks adaptation, leaves were detached from the fully hydrated plants and dried for 0 h (d0), 2 h (d2), 8 h (d8), 24 h (d24), 72 h (d72) under 50% relative humidity and 16 h/8 h light period at 25 °C in a climate chamber. For rehydration, the 72-h dried leaves were placed in Petri dishes with wet filter papers for 8 h (r8) and 48 h (r48).

To analyze the effect on gene expression of phytohormones, signaling molecules and other environmental stresses, *B. hygrometrica* leaves were incubated in 100 μM abscisic acid (ABA), 0.5 mM salicylic acid (SA), 40% ethephon, 5 mM $CaCl_2$, 10 mM EGTA and 200 μM H_2O_2, or subjected to 4 and 37 °C treatments in Petri dishes with wetted filter papers, respectively. These leaves were sampled before treatment (unt) and after treatment for 0.5 h (0.5), 8 h (8), and 24 h (24).

2.2. 5′-RACE-based cloning of BhDIR1

A cDNA fragment of *BhDIR1* was identified through a microarray-based screening of dehydration-inducible genes from a cDNA library prepared from dehydrated leaves of *B. hygrometrica*. The *BhDIR1* cDNA, with a complete open reading frame, was subsequently obtained (GenBank Accession No. EU122334) by 5′-RACE using a kit of System for Rapid Amplification of cDNA Ends (Invitrogen, USA). Synthesis of the cDNA used the primer R-1: 5′-GCTCCTCCTGGCAATCC-3′. For the first round PCR, the primers were Anchor primer-1: 5′-GGCCACGCGTCGACTAGTAC$(G)_{14}$-3′ and gene-specific primer R-2: 5′-CTCCAAGTCCGAGAAGGCTCCGAA-3′. The PCR conditions were 94 °C denaturation for 5 min, 35 cycles of 94 °C 30 s; 55 °C 30 s; 72 °C 1 min, and a final 72 °C extension for 10 min.

2.3. Cloning, sequencing and sequence analysis

The full length of the open reading frame (ORF) of *BhDIR1* was amplified with gene-specific primers 5′-CGGATCCATGGGAAAGCTTG-3′ and 5′-GACTCGAGATAATACGTAAGTACAG-3′, using the cDNA from total RNA of *B. hygrometrica* leaves dehydrated for 2 h as template. PCR was carried out with an initial denaturation at 95 °C for 5 min, 35 cycles of 94 °C 30 s; 57 °C 30 s; 72 °C 45 s, and a final extension at 72 °C for 10 min. The PCR product was analyzed on a 1.2% agarose gel. The corresponding fragment was purified using a gel purification kit (Beijing Daopu Biology Company) and ligated to the pBlueScript vector. The ligation product was transformed into *Escherichia coli* DH5α competent cells using the heat shock method. The plasmid DNA was isolated from the transformed bacteria, confirmed by PCR using gene-specific primers and sequenced (Beijing AuGCT Biotechnology Co. Ltd.). The amino acid sequence was deduced using ExPASy (http://au.expasy.org). DNA and amino acid sequence similarities were determined using BLAST programs (http://www.ncbi.nlm.nib.gov/blast). Alignment of BhDIR1 to dirigent proteins from *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor*, *Gossypium barbadense*, *Forsythia intermedia*, *Hordeum vulgare*, *Thuja plicata, Picea sitchensis* and *Picea glauca* was performed using Clustal W (version 1.81). A phylogenetic tree was constructed with MEGA 3.1 software using the Neighbor-Joining method. The stability of internal nodes was assessed by bootstrap analysis with 1000 replicates.

2.4. RNA extraction and semi-quantitative RT-PCR

Total RNA from *B. hygrometrica* leaves was extracted using the acid-guanidinium thiocyanate–phenol–chloroform method, and was reverse transcribed using M-MLV reverse transcriptase (Promega, USA) and random primers (Promega, USA). *BhDIR1* was amplified using primers as described in Section 2.3. The 18S rDNA was amplified as a control using primers 5′-TTGTGTTGGCTTCGGGATCGGAGTAAT-3′ and 5′-TGCACCACCACCCATAGAATCAAGAA-3′. The PCR program used for both *BhDIR1* and 18S rDNA was 95 °C 5 min, 24 cycles of 94 °C 30 s; 57 °C 30 s; 72 °C 45 s, and 72 °C 10 min. The cycle numbers for *BhDIR1* and 18S rDNA were determined individually according to the linear range of PCR products.

2.5. BhDIR1-GFP fusion protein construction and GFP signal detection

The *BhDIR1* cDNA was ligated upstream of the GFP coding sequence in pGJ280 (generously provided by Dr. Guido Jach, Max-Planck-Institute for Plant Breeding, Germany) under the control of the *CaMV 35S* promoter. The expression cassette was inserted into the region between the

T-DNA left and right borders of the pBIN19 vector. The construction was confirmed by sequencing before being transferred into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation. *N. benthamiana* leaf discs were transiently transformed with the transformed *Agrobacterium*. Epidermis strips were obtained from the transformed leaves to detect the GFP fluorescent signal at 488 nm wavelength using a confocal laser microscope (Zeiss, Germany). A 0.8 M mannitol solution was used to induce plasmolysis.

2.6. Measurement of acid-soluble lignin content

The acid-soluble lignin content was measured using the Klason method [13]. Fresh and fully dehydrated leaves of *B. hygrometrica* were incubated in an oven at 80 °C for 24 h, then powdered and extracted for 8 h with alcohol. The powder was air-dried and 12 ml of 72% H_2SO_4 was added per 0.8 g powder. The mixture was hydrated at 20 °C for 2 h (shaking once every 10 min). Subsequently, 448 ml water was added to the mixture and then the mixture was sterilized at 121 °C for 1 h. The mixture was filtered after cooling and rinsed with water (90 °C) several times to remove H_2SO_4. Finally, the powder was dried in an oven at 80 °C overnight and then weighed. The relative content of acid-soluble lignin was calculated as the percentage of weight of the powder to the dry weight of the plant material.

3. Results

3.1. Cloning and sequence analysis of BhDIR1

A 521 bp gene fragment was identified in the cDNA microarray screening of transcripts that was up-regulated after dehydration. This fragment, designated as *BhDIR1*, has a 35% similarity to a dirigent protein encoding gene reported from spruce [14]. We obtained a *BhDIR1* full length cDNA of 791 bp by 5′-RACE. *BhDIR1* cDNA contains a 600 bp open reading frame, an 88 bp of 5′-UTR, and a 95 bp of 3′-UTR ending with a poly A stretch. By comparing the size of the PCR products from genomic DNA and cDNA, it was found that *BhDIR1* gene did not contain any intron, which is the same gene structure as other reported dirigent protein genes [15]. *BhDIR1* encodes a protein composed of 199 amino acids. Homology analysis revealed that BhDIR1 has a 38% similarity to NbDIR1. An N-terminal signal peptide of 25 amino acids was predicted using the PSORT program, indicating that BhDIR1 is likely to be a secreted protein. BhDIR1 contains a dirigent protein domain (PF03018) and two N-glycosylation sites at the 68th (Asn) and 78th (Asn) amino acid positions (Fig. 1(a)). Such N-glycosylation sites are a feature of secreted proteins and have been found in FiDIR1, the first and best characterized dirigent protein [16], and

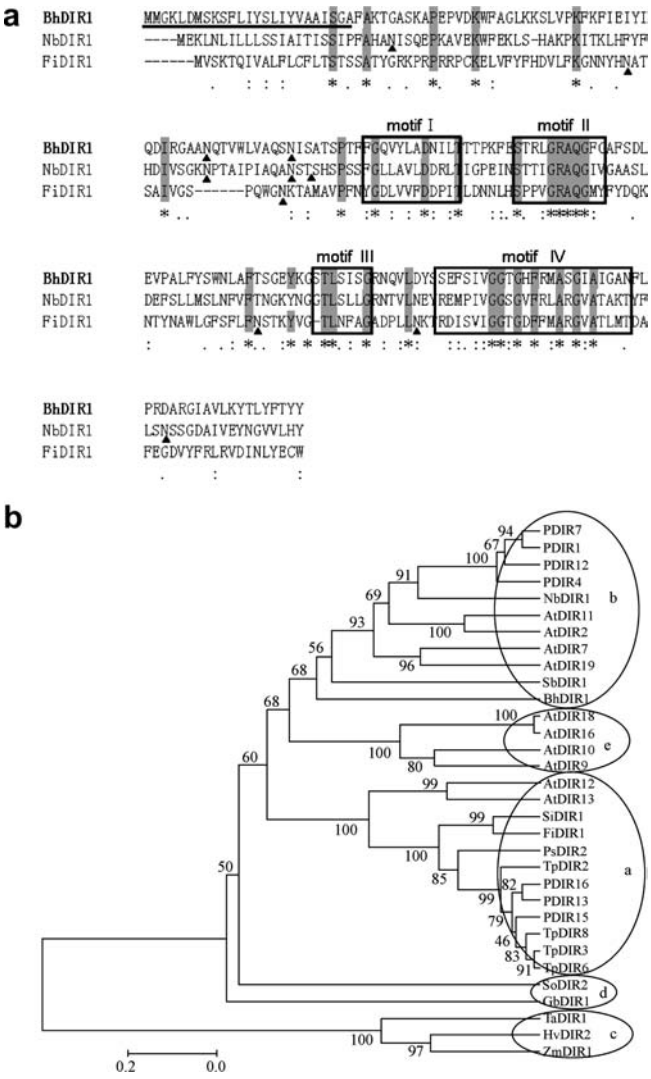

Fig. 1. Sequence alignment and phylogenetic tree of BhDIR1 and homologous proteins from other plants. (a) Amino acid sequence alignment of BhDIR1, FiDIR1 and NbDIR1. Identical amino acids are labeled with "*" and shown on a grey background; similar amino acids are labeled with "•"; spaces between amino acids are indicated by "-". Conserved motifs are boxed. Amino acids underlined indicate a signal peptide sequence; those marked with an asterisk are the deduced N-glycosylation sites. Sequences were aligned using the Clustal W program (http://www.ebi.ac.uk/clustalW). (b) An unrooted phylogenetic tree constructed based on sequences alignment using Mega software. Accession numbers of selected proteins are PDIR7 (*Picea glauca*) ABD52118; PDIR12 (*P. glauca*) ABD52123; PDIR4 (*P. glauca* × *engelmannii*) ABD52115; NbDIR1 (*Nicotiana benthamiana*) BAF02555; AtDIR2 (*Arabidopsis thaliana*) AY093095; AtDIR7 (*A. thaliana*) AK118030; AtDIR19 (*A. thaliana*) AK117899; SbDIR1 (*Sorghum bicolor*) AAM94289; AtDIR9 (*A. thaliana*) BT010722; AtDIR10 (*A. thaliana*) BT002889; AtDIR16 (*A. thaliana*) BT008336; AtDIR18 (*A. thaliana*) AY081267; AtDIR12 (*A. thaliana*) BT004016; AtDIR13 (*A. thaliana*) BT009718; SiDIR1 (*Sesamum indicum*) AAT11124; FiDIR1 (*Forsythia intermedia*) AAF25357; PsDIR1 (*Picea sitchensis*) AAD25355; TpDIR2 (*Thuja plicata*) AAF25360; PDIR15 (*P. sitchensis*) ABD52126; PDIR13 (*P. glauca* × *engelmannii*) ABD52124; TpDIR3 (*T. plicata*) AAF25361; TpDIR6 (*Thuja plicata*) AAF25364; TpDIR7 (*Thuja plicata*) AAF25365; TpDIR8 (*Thuja plicata*) AAF25366; SoDIR2 (*Saccharum officinarum*) CAF25234; GbDIR2 (*Gossypium barbadense*) AAY44415; TaDIR1 (*Triticum aestivum*) AAC49284; HvDIR1 (*Hordeum vulgare*) AAA87042; ZmDIR1 (*Zea mays*) AAF71261; PDIR1 (*P. glauca*) ABD52112.

NbDIR1, the protein with highest similarity with BhDIR1.

The alignment with FiDIR1 and NbDIR1 also showed that BhDIR1 has four conserved motifs characteristic for dirigent proteins (I–IV; boxed in Fig. 1(a)). Motif I (FGsasVhDDPaT) starts from the 87th amino acid position, motif II (SssVGRAQGhY) the 105th amino acid; motif III (uTashsG) the 133rd amino acid; and motif IV (RcaSVVGGTGcFhMARGaAsacT) the 156th amino acid (where h is hydrophobic, p is polar, a is aliphatic, s is small, u is tiny, c is charged and x is any). A phylogenetic tree of BhDIR1 and dirigent proteins from other plants showed that BhDIR1 belongs to the DIRb subfamily (Fig. 1(b)), but is distinct from other DIRb proteins.

3.2. Expression analysis of *BhDIR1*

Semi-quantitative RT-PCR data showed that *BhDIR1* was weakly expressed in untreated (d0) leaves, but was significantly induced at the beginning of dehydration (d0.5 h), and maintained a high expression level during the later dehydration and rehydration (Fig. 2(a)). High temperature (37 °C) induced the expression of *BhDIR1* rapidly (0.5 h), but low temperature induced *BhDIR1* expression relatively slowly (8 h) (Fig. 2(b)).

BhDIR1 began to accumulate significantly after 0.5 h incubation with $CaCl_2$, H_2O_2, EGTA, SA, ABA or ethephon, and expression was maintained at a relatively high level during the period of treatment (Fig. 2(b)). These results showed that *BhDIR1* rapidly responds to a wide range of environmental stresses, phytohormones and signaling molecules, therefore it may be involved in a variety of signaling pathways and stress responses.

3.3. Subcellular localization of BhDIR1-GFP

The BhDIR1-GFP fusion protein was transiently expressed in *N. benthamiana* leaves. The GFP fluorescent signal was detected using confocal laser microscopy. In contrast with the unfused GFP control, which was distributed throughout the transformed cells, the BhDIR1-GFP fusion protein signal was restricted to the cell periphery (Fig. 3). After plasmolysis, the BhDIR1-GFP signal mainly co-existed predominantly on the plasma membrane. Occasionally the BhDIR1-GFP signal was detected on the cell wall, but it was not observed in the intracellular space (Fig. 3). The GFP control signal was found throughout the cell, even after plasmolysis. These results show that the localization of BhDIR1-GFP fusion protein is at the cell periphery, notably the cell wall and plasma membrane.

3.4. Change of acid-soluble lignin content

Our experiment revealed that *BhDIR1* was induced in *B. hygrometrica* leaves during dehydration and rehydration, but neither potassium permanganate staining nor phloroglucinol staining showed any difference in lignin content between fresh and dehydrated leaves (data not shown). However, it was shown that the acid-soluble lignin content decreased significantly in fully dehydrated leaves using the Klason method (Fig. 4). Therefore we speculated that BhDIR1 may influence the acid solubility of lignin by changing the components and coupling of monolignols, subsequently affecting the content of the acid-soluble lignin in *B. hygrometrica* leaves depending on the plant hydration status.

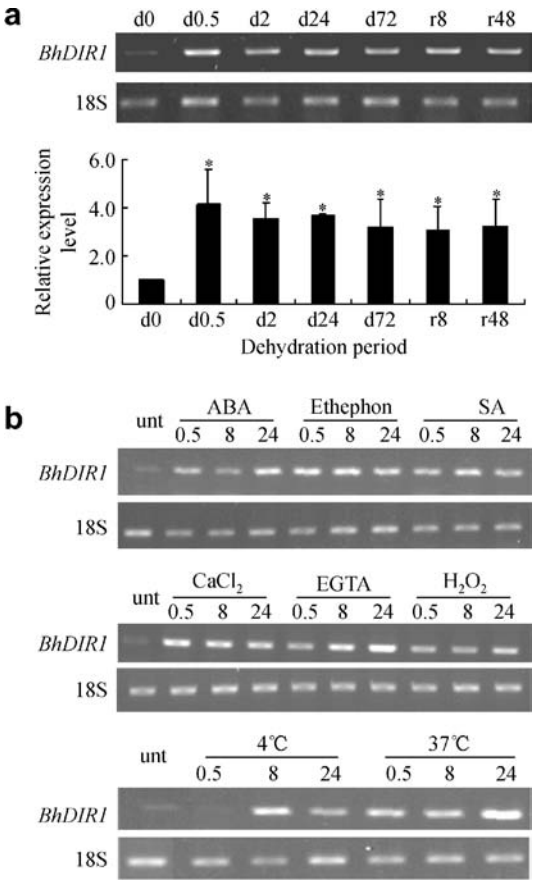

Fig. 2. Expression of *BhDIR1*. (a) Changes in the transcript level of *BhDIR1* in *B. hygrometrica* leaves dehydrated for 0 h (d0), 0.5 h (d0.5), 2 h (d2), 24 h (d24), 72 h (d72), and subsequently rehydrated for 8 h (r8) and 48 h (r48), respectively. 18S rRNA was amplified as control; "*" significant level $P < 0.05$. (b) Effect of phytohormones, signaling molecules and temperature stress on *BhDIR1* expression in *B. hygrometrica* detached leaves. unt, untreated; 18S rRNA was amplified as a control, all RT-PCRs were repeated at least twice.

4. Discussion

Dirigent protein, named after a Latin *dirigere* (to align or guide), was first found in *F. intermedia* by Davin et al. as a 26 kDa glycosylated protein that can form a 78 kDa polymer [17]. The polymer had no catalytic center and its function is speculated to capture free-radical lignin monolignols and help monolignol coupling in a stereo-selective manner to form dimers with the involvement of an oxidase enzyme [11]. The function was subsequently demonstrated *in vitro*, when FiDIR was shown to direct the coupling of

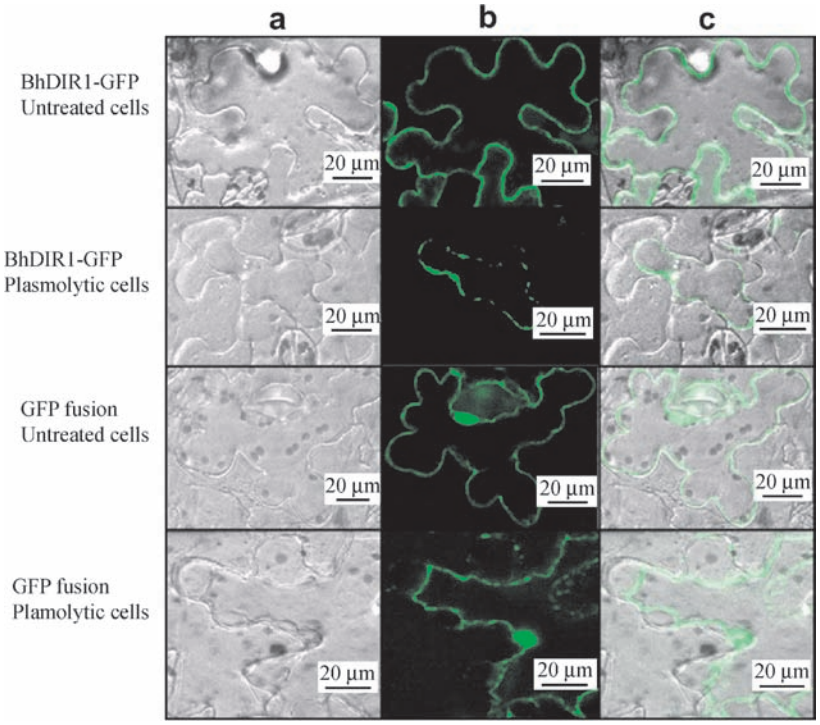

Fig. 3. Subcellular localization of BhDIR1-GFP fusion protein. Bright field images; (b) GFP fluorescence images; (c) merged bright field/GFP fluorescence images. GFP protein was used as control.

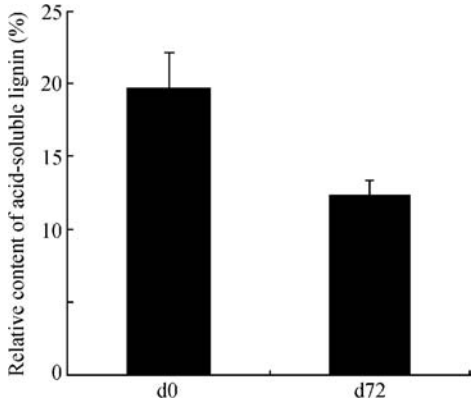

Fig. 4. Acid-soluble lignin content in *B. hygrometrica* leaves. d0, dehydrated for 0 h; d72, dehydrated for 72 h; "*" significant level $P < 0.05$.

two E-coniferyl alcohol molecules to produce the lignan (+)-pinoresinol in the presence of laccase [17]. On the basis of these observations, it was proposed that dirigent proteins may play a role in the capture of monolignols and their stereo-selective coupling, thus influencing the composition of monolignols in lignin, and affecting the physical properties including the mechanical strength and flexibility of the cell wall.

Dirigent proteins have been reported from many land plants including lichens, ferns, gymnosperms and angiosperms. The sequence similarity among dirigent proteins ranges from 19.5% to 98.4% [14]. BhDIR1 is similar to previously reported dirigent proteins, and shares conserved motifs (Fig. 1(a)). Steven et al. classified the dirigent proteins into five subfamilies: DIRa, DIRb, DIRc, DIRd and DIRe [11]. Members of DIRa have been shown to participate in the stereo-selective coupling of monolignols during synthesis of lignan and lignin. This function has not been reported for proteins from the other subfamilies, consequently classes DIRb–DIRe are referred to as dirigent-like proteins. BhDIR1 belongs to the DIRb subfamily (Fig. 1(b)). The phylogenetic tree we constructed showed that BhDIR1 is distinct from other DIRb class dirigent proteins, which may affect the structural and functional characters of this protein and could be linked with desiccation tolerance.

Recent reports have suggested that stress conditions influence the expression of DIR genes. For example, both *DIRa* and *DIRb* genes in spruce are dramatically induced by insects and mechanical wounding [14]. Similar responses were also observed with dirigent genes in *F. intermedia* [18]. These results indicate that dirigent proteins participate in plant defense processes. Our study showed that *BhDIR1* responded to dehydration and was maintained at a high mRNA level during rehydration, and it was also induced by high and low temperatures. These data demonstrate a possibility that *BhDIR1* may be involved in the response to a variety of stresses and play a common role in abiotic stress responses. Ethylene, ABA and H_2O_2 are phytohormone signals that regulate the signal transduction pathways in plants in response to stress conditions. In this study, it was shown that *BhDIR1* was up-regulated by exogenous application of

phytohormones as well as $CaCl_2$, EGTA (a chelator of calcium), suggesting that the expression of *BhDIR1* may be regulated by a number of signal pathways.

Many dirigent and dirigent-like genes are regulated in a developmental- and tissue-specific way. Myoung et al. reported that when individual promoters of dirigent genes from red cedar were fused to the glucuronidase (GUS) reporter gene and transformed into *Arabidopsis*, GUS activity could be detected mostly in the vascular bundle [19]. Dirigent proteins in *F. intermedia* were specifically detected in the cell wall of vascular tissue [20]. In agreement, we observed that BhDIR1 is localized on the cell membrane and cell wall (Fig. 3). The cell wall is the site where lignin is synthesized, highlighting the possibility that BhDIR1 may be involved in lignin synthesis. The composition and structure of lignin monolignols in different plants are different [21], and it has been hypothesized that this is due to the various stereo-selective linkages of monolignols that are guided by dirigent proteins in the synthesis of lignin. Although potassium permanganate and phloroglucinol staining failed to show an effect of dehydration on the lignin content of *B. hygrometrica* leaves (data not shown), the decrease in acid-soluble lignin content as well as the increase in *BhDIR1* during dehydration indicated that BhDIR1 might affect the composition and coupling of monolignols and consequently affect the acid solubility of lignin. The decrease in acid-soluble lignin content may change the flexibility and strength of the cell wall, providing the *B. hygrometrica* cell wall the ability of folding inward together with the cell membrane upon dehydration, which can reduce the damage caused by water loss.

In summary, we report the isolation and expression analysis of a dirigent protein from the resurrection plant, which was highly expressed in response to drought. The high level of this protein may change the physical nature of lignin through regulating the connections between lignin monolignols and the composition of lignin, thus affecting the mechanical strength and flexibility of cell wall, reducing drought-caused mechanical damage and allowing rapid resumption of living activity soon after water is available.

Acknowledgements

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