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ORIGINAL ARTICLE

A WRKY transcription factor participates in dehydration tolerance in *Boea hygrometrica* by binding to the W-box elements of the galactinol synthase (*BhGolS1*) promoter

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Abstract Accumulation of compatible osmolytes, such as soluble sugars, in plants is an important osmoprotective mechanism. Sugars play a role in osmotic adjustment and are associated with stabilization of proteins and cell structures, reactive oxygen species scavenging, signaling functions or induction of adaptive pathways. Galactinol is the galactosyl donor for the synthesis of raffinose family oligosaccharides (RFOs) and its synthesis by galactinol synthase (GolS) is the first committed step of the RFOs biosynthetic pathway. GolS genes are induced by a variety of stresses in both stress-sensitive and tolerant-plant species; however, the mechanism of transcriptional regulation is not fully established. In this paper, we characterized a GolS gene (*BhGolS1*) that was dehydration and ABA-inducible in the resurrection plant *Boea hygrometrica* and conferred dehydration tolerance in a transgenic tobacco system. Four W-box *cis*-elements were identified in the *BhGolS1* promoter and shown to be bound by an early dehydration and ABA-inducible WRKY gene (*BhWRKY1*). These data suggest a mechanism where *BhWRKY1* is likely to function in an ABA-dependent signal pathway to regulate *BhGolS1* expression, which leads to the accumulation of RFOs in desiccation-tolerant *B. hygrometrica* leaves.

Keywords *Boea hygrometrica* · Drought tolerance · Galactinol synthase · Resurrection plant · WRKY transcription factor

Abbreviations

GolS	Galactinol synthase
Gol	Galactinol
Raf	Raffinose
RFO	Raffinose family oligosaccharide

Introduction

Drought is the most severe threat to crop yield worldwide. Plants have evolved complex strategies to reduce potential damage of drought stress. Soluble sugars, including sucrose, trehalose and raffinose family oligosaccharides (RFOs) have been found to accumulate during drought stress in many plants (Albini et al. 1999; Collett et al. 2004; Farrant 2007; Peters et al. 2007). RFOs have multiple functions in plants, serving as transport carbohydrates in the phloem (Ayre et al. 2003) and as storage reserves and protectants in plant organs (Koster and Leopold 1988; Bachmann et al. 1994; Brenac et al. 1997; Sprenger and

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Keller 2000; Taji et al. 2002; Pennycooke et al. 2003), and playing a role as compatible solutes in the acquisition of desiccation tolerance and storability as they accumulate in maturing seeds (Horbowicz and Obendorf 1994).

RFOs composed mainly of raffinose, stachyose and verbascose are biosynthesized by the reversible addition of galactose units from galactinol (Gol) to sucrose/raffinose (Raf)/stachyose (Peterbauer and Richter 2001). Gol synthase (GolS; EC 2.4.1.123), catalyzing the first step of Gol synthesis from *myo*-inositol and UDP-galactose, has been proposed to be the key enzyme of the pathway (Handley et al. 1983; Keller and Pharr 1996; Peterbauer et al. 2002). *GolS* genes are found in diverse plant species and associated with multiple developmental and environmental responses. For example, in common bugle (*Ajuga reptans*), two distinct cold inducible *GolS* genes are transcribed in discrete locations (*GolS-1* in mesophyll cells and *GolS-2* in companion cells of the phloem) (Sprenger and Keller 2000). Ten *GolS* genes were identified in *Arabidopsis thaliana* genome, three of which are stress responsive. *AtGolS1* and *AtGolS2* transcripts accumulate in mature dry seeds and are up-regulated in response to water and salinity stress but not cold, whereas *AtGolS3* is induced only during cold stress (Taji et al. 2002; Nishizawa et al. 2008). Overexpression of *AtGolS2* in transgenic *Arabidopsis* plants conferred drought tolerance and caused an increase in Gol and Raf (Taji et al. 2002; Nishizawa et al. 2008). The link, however, between *GolS* gene expression/enzyme activity and RFOs accumulation and stress tolerance remains unclear. For example, the overexpression of a *GolS* gene from cucumber resulted in a 10- and 80-fold increase of Gol and Raf, respectively, but did not alter the degree of cold tolerance *in planta* (Zuther et al. 2004).

Two types of transcription factors regulate *GolS* gene expression in *Arabidopsis*. heat shock factors (HSFs) can activate expression of *AtGolS1* (Panikulangara et al. 2004; Nishizawa et al. 2006; Ogawa et al. 2007); DREB1A/CBF3 (dehydration-responsive element binding factor 1A/cold responsive-element binding factor 3) can activate *AtGolS3* expression and enhance drought and freeze tolerance (Taji et al. 2002). The participation of other classes of transcription factor in the regulation of *GolS* expression is unknown.

WRKY genes encode a large group of transcription factors, defined by a domain of 60 amino acids, which contains the amino acid sequence WRKY at its amino-terminal end and a putative zinc finger motif at its carboxy-terminal end. There are over 70 *WRKY* genes in *Arabidopsis* (Eulgem et al. 2000; Dong et al. 2003) and rice (*Oryza sativa*) (Goff et al. 2002; Zhang et al. 2004). WRKY proteins mostly bind to the cognate *cis*-acting element (C/T)TGAC(T/C) in the promoter (Eulgem et al. 2000) and play a variety of developmental and physiological roles in plants. Studies on *WRKY* genes predominantly point to an involvement in salicylic acid (SA) signaling and disease responses (Dellagi et al. 2000; Du and Chen 2000; Eulgem et al. 2000; Kim et al. 2000; Asai et al. 2002). In addition, *WRKY* genes are involved in plant responses to freezing (Huang and Duman 2002), wounding (Hara et al. 2000), oxidative stress (Rizhsky et al. 2004), drought, salinity, cold, and heat (Pnueli et al. 2002; Rizhsky et al. 2002; Seki et al. 2002; Qiu et al. 2004; Zhou et al. 2008). Knowledge is lacking, however, regarding the identity of natural target genes of WRKY factors that function in dehydration tolerance.

Boea hygrometrica is native to China and belongs to a group of so-called "resurrection plants", which have evolved the ability to recover from severe water loss in vegetative tissues (Deng et al. 2003). The acquisition of desiccation tolerance in resurrection angiosperms requires the induction of a co-ordinated program of genetic and biochemical processes during drying. This mechanism contrasts with desiccation-tolerant bryophytes and lichens, where drying occurs rapidly and survival is based largely on rehydration-induced repair processes (Farrant et al. 2009). Protection mechanisms are broadly constitutive in lower plants, minimal during dehydration and no novel transcripts are synthesized before rehydration. Desiccation-tolerant angiosperms have, however, established complex protection mechanisms, thus minimizing the need for extensive cellular repair upon rehydration (Farrant et al. 2009). The most prominent metabolic changes that take place during drying are the de novo synthesis of proteins and sugars, which are postulated to form the basis of protective mechanisms that limit damage to cellular constituents (Oliver et al. 2000; Scott 2000; Phillips et al. 2002; Vicre et al. 2004). This unique ability makes resurrection plants a good system for investigating of drought tolerance mechanisms.

Previously we reported that cDNA fragments with homology to galactinol synthase (*J-57*, also designated as *BhGolS1*) and WRKY factors represent dehydration-inducible genes in *B. hygrometrica* leaves (Wang et al. 2009). Here, we investigate the function of *BhGolS1* in Gol/Raf accumulation leading to enhanced drought tolerance using a transgenic approach and demonstrate a specific interaction between a WRKY transcription factor and the *BhGolS1* promoter in *B. hygrometrica* using chromatin immunoprecipitation and yeast one-hybrid assays.

Materials and methods

Plant materials and stress treatments

Boea hygrometrica plants were collected from their natural habitat in Beijing and maintained in a greenhouse with regular irrigation. In dehydration experiments, excised leaves

of *B. hygrometrica* or tobacco seedlings were dehydrated for 0–48 h and rehydrated as described (Jiang et al. 2007). For hormone treatment, detached leaves or tobacco seedlings were placed in solutions containing 100 μM ABA in a climate chamber for 0.5, 8 and 24 h, using the ABA-solvent (ethanol) as a control to monitor the effect of ethanol and wounding. For cold- and heat-stress, plants were kept on pre-wetted filter papers in Petri dishes for indicated periods at 4 and 37°C. Tobacco (*N. tabacum* cv. SR-1 and *N. benthamiana*) used for stable and transient transformation were grown in growth chamber with regular irrigation and light conditions (187.5 μmol m^{-2} s^{-1}; light/dark, 16/8 h) at 22°C.

Molecular cloning and sequence analysis

cDNA fragments of *BhGolS1* (Accession No. FJ 222452) and *BhWRKY1* (Accession No. FJ 222453) were identified by differential screening of dehydration-inducible genes from a cDNA library prepared from desiccated leaves of *B. hygrometrica* using a macroarray hybridization method (Wang et al. 2009). Complete coding regions were subsequently obtained using the System for Rapid Amplification of cDNA Ends Kit (Invitrogen, Carlsbad, CA). Reverse transcription was performed using gene-specific primers 5′-GACGAACTAAAGCAGAGCTG-3′ (*BhGolS1*) and 5′-ATGACTCGGCTTAGATT-3′ (*BhWRKY1*). Gene-specific primers 5′-AAGTGTAGTCCAGCGTTTCG-3′ and 5′-CCTTCACCTCCTCCAGATTCACATT-3′ were used for amplification of *BhGolS1*; 5′-AGGATCCCTGGTCAC CTTCTGT-3′ and 5′-TGGATCCAATCTTCGGTTTGA CG-3′ for amplification of *BhWRKY1*. A poly C tail was then added to the 3′-end of the cDNA using terminal deoxynucleotidyl transferase. Anchor primer (5′-GGCCAC GCGTCGACTAGTACG_{14}-3′) that recognized the poly C tail was used in a second round of PCR.

Genomic DNA was extracted using a cetyltrimethyl ammonium bromide (CTAB) based method (Doyle and Doyle 1990). The 5′-flanking region of *BhGolS1* was cloned by inverse PCR (Ochman et al. 1988) using primers 5′-GCG GATCCGGTGGTGTTCATTT-3′, 5′-ACCATGGCCAGC CAAGAATGTG-3′; and 5′-GCTCGAGAATAGATGAT GCCTAG-3′, 5′-ACCATGGCGGCGTTAGCAATC-3′. DNA and protein sequences were analyzed using the BLAST program (Altschul et al. 1990). Amino acid comparison and multiple alignments were performed using the ClustalW program (Chenna et al. 2003). The *cis*-elements analysis was performed using PLACE software (Higo et al. 1999).

RNA isolation and RT–PCR analysis

Total RNA was extracted from *B. hygrometrica* using an acidic guanidinuim thiocyanate–phenol–chloroform based procedure (Chomczynski and Sacchi 1987) and from tobacco using Trizol (Invitrogen, CA). Two micrograms of total RNA was heated to 70°C for 5 min and reverse transcribed with random primers using M-MLV Reverse Transcriptase (Promega, WI) for 60 min at 42°C in a volume of 25 μL. Semi-quantitative PCR was performed with cDNA samples (1:5 dilution) using rTaq polymerase (Takara Bio, Japan). Gene-specific primers are 5′-TGAATTCATGGCC CCGGAGATTG-3′ and 5′-CCTTCACCTCCTCCAGATT CACATT-3′ for amplification of *BhGolS1*; and 5′-GTGG TCGACATGGAGTTCACTAGTC-3′ and 5′-GCCGTCG ACACATCTTCTCATTTT-3′ for amplification of *BhWRKY1*.

Semi-quantitative RT-PCR was repeated at least four times for each sample. 18S rRNA was amplified as a control as described (Wang et al. 2009). The linear range of detection for each transcript was monitored and samples run for 30 cycles for *BhGolS1* and *BhWRKY1*, and 24 cycles for 18S rRNA were compared.

Production of the GST recombinant protein in *E. coli* and enzyme activity

The coding regions of *BhGolS1* and *BhWRKY1* were in-frame cloned into pGEX-4T-1 downstream to GST coding region (Novagen, Madison, WI) and transformed into BL21 (DE3) and BL21 codon plus cells (Stratagene, CA), respectively. The *E. coli* strains were grown in Luria–Bertani broth with 100 μg mL^{-1} of ampicillin to an OD_{600} of 0.4 before induction with isopropylthio-*β*-galactoside at a final concentration of 1 mM for 2 h at 37°C. The cells were collected, resuspended in PBS buffer (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and disrupted by mild sonication on ice. The soluble protein was separated by 12% SDS-PAGE to check the molecular weight of the recombinant protein, which was then purified using Glutathione Sepharose™ 4B (Amersham Biosciences, IL) according to the manufacturer's instructions. The purified proteins were used for generation of polyclonal antiserum. Galactinol synthase assays were performed with purified recombinant protein of GST-BhGolS1 by high-resolution gas chromatography. Galactinol synthase assays included 20 mM UDP-Gal as the galactosyl donor, 20 mM myo-inositol as the galactosyl acceptor, 50 mM Hepes buffer, pH 7.0, 2 mM dithiothreitol, 5 mM $MnCl_2$ and 0.5–1 mg purified enzyme protein (estimated by the Bradford assay) in 50 μL total volume. Assays were run at 30°C for 0–300 min. Reactions were stopped by addition of 50 μL of 100% ethanol. After addition of 25 mg of phenyl a-D-glucoside as internal standard, the reaction mixture was heated at 80°C for 30 min, passed through a 10,000 MW cutoff filter, and evaporated to dryness under a stream of nitrogen gas. Residues were stored overnight in a desiccator with

phosphorus pentoxide to remove traces of water, derivatized with trimethylsilylimidazole:pyridine (1:1, v/v) at 80°C for 45 min, and analyzed for soluble carbohydrate products by high-resolution gas chromatography as described by Ueda et al. (2005).

Protein extraction and Western blot analysis

Total proteins were extracted from leaves with protein extraction reagent [625 mM Tris–HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) β-ME] (Liu et al. 1998). Concentrations of proteins were measured using the Bradford assay (Bradford 1976). Twenty micrograms of protein was subjected to 12.5% SDS-PAGE and then transferred onto a nitrocellulose membrane using semi-dry transfer method (BIO-RAD, CA). The efficiency of transfer was monitored by Ponceau S staining. The membrane was incubated with the rabbit anti-BhGolS1 (or mouse anti-BhWRKY1) antiserum (1:400). Immunoblots were incubated with goat anti-rabbit (or anti-mouse) IgG (1:10,000, Jackson Immuno Research Laboratories, PA). Anti-BhGolS1 and anti-BhWRKY1 polyclonal antiserum were generated by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Coomassie Brilliant Blue R-250 stains of SDS-PAGE gels were used as loading controls.

Yeast one-hybrid assays

Yeast one-hybrid assays were performed according to the manufacturer's instructions (Clontech, CA). The bait plasmids were constructed into pHISi with fragments containing triple tandem copies of the W-box palindrome (construct *a)* and the corresponding mutated version (construct *b*) as described (Xu et al. 2004), or 414-bp fragment of *BhGolS1* promoter (from −566 to −152) containing 4 copies of W-box elements (construct *c*), 231-bp fragment of *BhGolS1* promoter (from −383 to −152) containing 3 copies of W-box elements or mutated sequences (construct *d–k*), as shown in Fig. 6b. The fragments *c–g* were amplified using primers 5′-GGAATTCGTTGCCTTAGTTGCTA-3′ and 5′-GGTCTAGATCTTACGGTCACATG -3′ (*c*); 5′-GGAATTCACCAAACTGACCAGTG-3′ and 5′-GTCGACCATGATGATCGATTTCCAG-3′ (*d*); 5′-GAATTCACCAAACTGAACAGTGGCTCAGA-3′ and 5′-TCTAGATCTTACGTTCACATGAAGGTGC-3′ (*e*);5′-GGAATTCACCAAACTGACCAGTG-3′ and 5′-TCTAGATCTTACGTTCACATGAAGGTGC-3′ (*f*); 5′-GAATTCACCAAACTGAACAGTGGCTCAGA-3′ and 5′-GTCGACCATGATGATCGATTTCCAG-3′ (*g*); fragment *h* was obtained by ligation of two *Sac*I-digested fragments amplified by 5′-GGAATTCACCAAACTGACCAGTG-3′ and 5′-AAGCTTGGAAGATTCACAGTTG-3′ and by 5′-GAATTCAAGCTTAGTGTATTCACCAATTTAAA-3′ and 5′-GTCGACCATGATGATCGATTTCCAG-3′ respectively; fragments *i* to *k* were amplified using construct *h* as a template by primers 5′-GGAATTCACCAAACTGACCAGTG-3′ and 5′-TCTAGATCTTACGTTCACATGAAGGTGC-3′ (*i*); 5′-GAATTCACCAAACTGAACAGTGGCTCAGA-3′, and 5′-GTCGACCATGATGATCGATTTCCAG-3′ (*j*); 5′-GAATTCACCAAACTGAACAGTGGCTCAGA-3′ and 5′-TCTAGATCTTACGTTCACATGAAGGTGC-3′ (*k*). The W-box and mutated core sequences are underlined. The coding region of the *BhWRKY1* and *AtWRKY60* were amplified with the gene-specific primer pairs (5′-GTGGTCGACATGGAGTTCACTAGTC-3′ and 5′-GCCGTCGACACATCTTCTCATTTT-3′ for *BhWRKY1*, 5′-CGAATTCATGGACTATGATCCCAA-3′ and 5′-GCCTCGAGTCATGTTCTTGAATGC-3′ for *AtWRKY60*, and were in-frame fused with the GAL4 activation domain of the pGAD-GAL4 vector to generate prey plasmids. Each pair of bait and prey plasmids were co-transformed into yeast YM4271 cells using a lithium acetate method (Clontech, CA) and analyzed for yeast growth on selective medium containing 50 mM 3-AT but lacking histidine and leucine.

Generation of transgenic plants, GUS staining and GFP visualization

BhGolS1 and *BhWRKY*1 coding regions were amplified and inserted downstream of the CaMV 35S promoter in a pBin19 series binary vector (Frisch et al. 1995) to generate overexpression constructs. The GFP coding region was in-frame fused upstream of *BhWRKY1* to generate the *GFP-BhWRKY1* construct. For the Pro_{Rd29A}*:BhGolS1* construct, the 946 bp *Rd29A* promoter was amplified from *Arabidopsis* genomic DNA using primers 5′-GGAATTCCCATAGATGCAATTCAA-3′ and 5′-CGAATTCCAAAGATTTTTTTCTTTCCAA-3′ to replace the CaMV 35S promoter in the pBin19 vector. The 659-bp 5′-flanking region of *BhGolS1* amplified with primers 5′-GGAATTCGTTGCCTTAGTTGCTA-3′ and 5′-GTCGACCATGATGATCGATTTCCAG-3′ was in-frame inserted into the pBI101 vector (Clontech, CA) upstream of the *GUS* coding sequence to generate the $Pro_{BhGolS1}$*:GUS* fusion. These constructs were introduced into *Agrobacterium* strain LBA4404 by electroporation and transformed into tobacco (SR1) via a leaf disc method (Horsch et al. 1985). Transgenic T0 and T1 plants were screened on MS media containing 100 mg L^{-1} kanamycin and confirmed by PCR with gene-specific primers. Homozygous lines were selected through two further rounds of selection on plates containing 100 mg L^{-1} kanamycin. Three independent lines of $Pro_{BhGolS1}$*:GUS* transgenic tobacco were used for GUS staining using 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as substrate. After staining, the tissues were soaked in several

changes of 95% ethanol and examined under a stereoscope (Jefferson 1987). Transgenic tobacco plants harboring *Pro*$_{BhGolS1}$*:GUS* were crossed with WT, *Pro*$_{35S}$*:BhWRKY1* and *Pro*$_{35S}$*:antiBhWRKY1*. Crossed progeny were selected on kanamycin plates and the incorporation of both transgenic events was confirmed by PCR using gene-specific primers for *Pro*$_{BhGolS1}$ and *BhWRKY1*. The plasmids containing *GFP-BhWRKY1* and *GFP* were transiently transformed into *N. benthamiana* leaves via an *Agrobacterium*-mediated method (Kapila et al. 1997). Visualization of GFP was conducted using a Zeiss confocal microscope (LSM 510 META) (excitation wavelength 480 ± 20 nm, emission wavelength 510 ± 20 nm) after cultivation for 36 h. Zeiss LSM Image Browser software (version 3.2.0.70) was used for image acquisition.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed with the ChIP Assay Kit (Upstate, CA) according to Wang et al. (2002). Nuclei were extracted from *B. hygrometrica* leaves dehydrated for 0.5 h. PCR was performed as follows: 95°C for 5 min, 5 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s and 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s, and 72°C for 10 min. Two fragments of the *BhGolS1* promoter, Pro-b and Pro-nb, were amplified using primers as shown in Fig. 3.

Drought tolerance analysis of transgenic plants

Seeds (T3 generation) of transgenic plants were sown and germinated in pots in parallel with wild-type (WT) seeds. Seedlings were grown for 6 weeks with regular irrigation prior to drought stress. Drought stress experiments were conducted by withholding water for 14 days. Plants were re-watered for 6 days to determine the survival rate. Survival rate was calculated by counting viable plants, i.e., plants that displayed green, turgid leaves and had resumed growth. Leaf RWC was estimated according to RWC (%) = (fresh weight − dry weight)/(turgid weight − dry weight) × 100. Soil water content was calculated according to soil water content (%) = (soil fresh weight − soil dry weight)/dry weight × 100. Quantum efficiency (Fv/Fm) and the extent of electrolyte leakage were measured as described (Jiang et al. 2007); the experiments were performed twice with three independent leaves for each treatment at each time point. For measurement of Gol and Raf content, 200 mg of leaves tissue was frozen and homogenized in 2 mL of ethanol: water (1:1,v:v), containing 100 μg of phenyl a-D-glucoside as internal standard in a 10-mL microfuge tube, heated at 80°C for 30 min to inactivate enzymes, and centrifuged at 15,000 × *g* for 20 min. The residue was re-extracted with 1 mL of ethanol–water. The supernatants were pooled and aliquots (0.5 mL) were passed through a 10,000 Mr cutoff filter and were analyzed by HPLC as described (Peters et al. 2007).

Statistical analyses

For physiological and biochemical data, an analysis of variance (ANOVA) was performed to investigate whether there was a significant difference between the samples, and, if a significant difference was found, a post hoc Tukey's honestly significantly different (HSD) test was also performed to determine which samples were responsible for the significant differences.

Results and discussion

BhGolS1 encodes a functional galactinol synthase protein

BhGolS1 (Accession No. FJ222452) encodes a 334-amino acid protein with 72% identity to AtGolS1 from *Arabidopsis* (Accession No. AAB63818) and XvGolS from the resurrection plant *Xerophyta viscosa* (Accession No. ABK27907) (Taji et al. 2002; Peters et al. 2007). Alignment of these proteins revealed that BhGolS1 contains a conserved region common to glycosyl transferase, family 8 proteins (Glyco_transf_8), a characteristic manganese-binding motif DXD/DGD and a serine phosphorylation site (Sprenger and Keller 2000), but lacks a carboxyl terminal pentapeptide APSAA (Taji et al. 2002) (Animation 1). *BhGolS1* mRNA and protein accumulated in leaves dehydrated for 2–48 h (RWC 45–25%), and disappeared after rehydration (Fig. 1a). Furthermore, *BhGolS1* was also rapidly induced by ABA at the mRNA level after 0.5 h and at the protein level after 8 h (Fig. 1a). No detectable expression was observed in the absence of ABA (CK), except after prolonged incubation (24 h). At this extended time point a very faint band was observed in the RT-PCR analysis; however, this was not confirmed at the protein level. A purified BhGolS1-GST fusion protein was shown to be able to catalyze galactinol synthesis from UDP-Gal and *myo*-inositol (Fig. 1b), with an enzyme activity of 3.8 μmol min^{-1} mg^{-1} protein, which is broadly similar to the level reported for GolS enzymes from kidney bean cotyledon and zucchini leaf (Liu et al. 1995). This result supports the enzymatic function of BhGolS1 as a galactinol synthase, in a manner similar to other reported GolS proteins (Liu et al. 1998; Keller and Pharr 1996; Zhao et al. 2004; Ueda et al. 2005; Peters et al. 2007). These data also suggest that the pentapeptide (APSAA) conserved in many GolS proteins at the carboxy-terminal ends may not be crucial for enzyme activity.

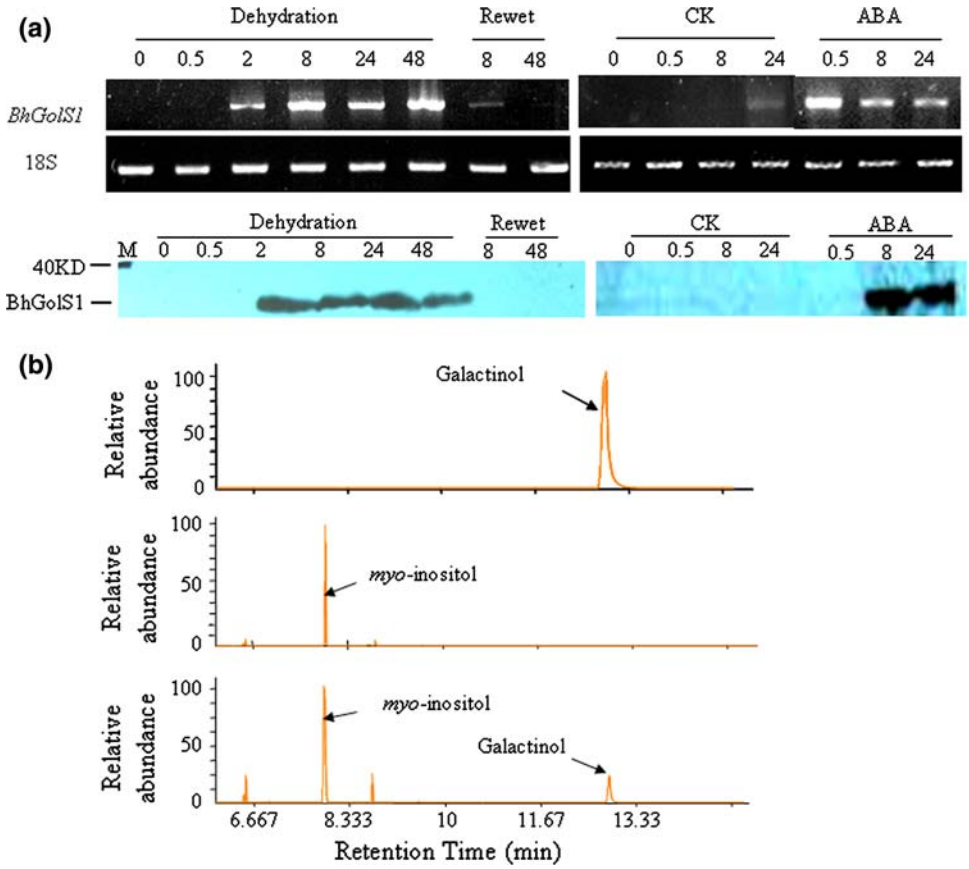

Fig. 1 *BhGolS1* expression and function. **a** Transcript (*upper*) and protein (*lower*) accumulation of *BhGloS1* in *Boea hygrometrica* leaves. Equal loading was verified by 18S rRNA amplification and CBB stain, respectively. *M* protein molecular weight marker. *CK* treatment in the presence of ABA-solvent (ethanol) only. *ABA* treatment with ABA (100 μM) solution. **b** Enzyme activity of purified GST-BhGolS1 recombinant protein using a high-resolution gas chromatography assay with *myo*-inositol as substrate. *Top* galactinol (standard reagent from Sigma, St Louis, MO); *middle* product from the reaction with purified GST protein; *bottom* product from the reaction with purified GST-BhGolS1 recombinant protein

BhGolS1 activity enhances drought tolerance in transgenic tobacco

To examine the role of *BhGolS1* in drought tolerance, transgenic tobacco plants where *BhGolS1* was constitutively expressed (Pro_{35S}:*BhGolS1*) or drought-induced (Pro_{Rd29A}:*BhGolS1*) were generated. Tobacco is an ideal testing system, because no internal accumulation of galactinol is detected in unstressed tobacco (Ayre et al. 2003) and no *GolS* genes from tobacco have been reported in GenBank so far. Three homozygous lines (S10-1 and S22-5 for Pro_{35S}:*BhGolS1*; and Rd15-6 for Pro_{Rd29A}:*BhGolS1*) were selected for further investigation. No difference in growth and morphology between these transgenic plants and wild-type (WT) tobacco was observed when grown under normal conditions (Fig. 2a). However, when exposed to drought stress by withholding water supply, improved drought tolerance in transgenic plants was observed, as indicated by the significantly higher levels of Fv/Fm and lower levels of electrolyte leakage as synchronous measured during dehydration (Fig. 2a, b). A higher survival rate of transgenic plants after rewatering (84.8% for S10-1; 72.6% for S22-5; 61.5% for Rd15-6), compared to WT (38.4%) (Fig. 2c) was observed, although the synchronous measured relative water content was not preserved to higher level in transgenic plants with exception of S10-1 (Fig. 2b). In agreement, *BhGolS1* transcripts and proteins were detected in leaves of untreated and stressed S10-1 and S22-5 plants, relatively higher in S10-1 than in S22-5; whereas *BhGolS1* transcripts were only induced after dehydration for 6–12 days and protein was only detected in 12 days dehydrated samples in Rd15-6 (Fig. 2d). Changes in galactinol and raffinose content (Fig. 2e, f) indicated that WT plants do not accumulate galactinol and raffinose in response to drought stress; the ectopic/drought-induced expression of *BhGolS1*, however, led to significant accumulation of galactinol before and during dehydration, part of which was converted to raffinose by the endogenous activity of raffinose synthase in tobacco (Ayre et al. 2003). Thus, our data support the importance of galactinol and raffinose in osmoprotection, and the key role of GolS in the production of galactinol and raffinose.

The *BhGolS1* promoter responds to abiotic stress and is developmentally regulated

To investigate the transcriptional regulation of *BhGolS1*, the 659 bp proximal promoter region of *BhGolS1* was isolated (Fig. 3). Analysis of transgenic tobacco plants harboring $Pro_{BhGolS1}$:*GUS* revealed that the *BhGolS1* promoter was active in germinating seeds and in 3- to 5 -day-old seedlings, but not in 10-day-old seedlings (Fig. 4a–e).

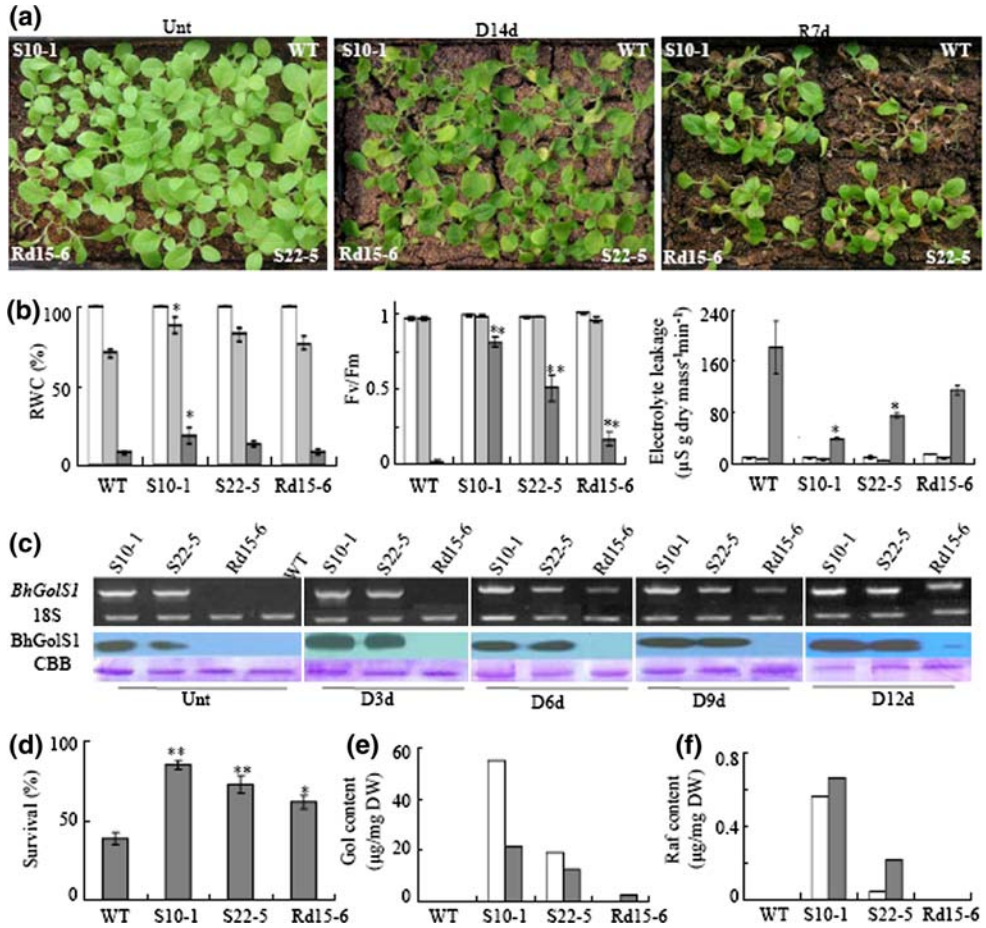

Fig. 2 Phenotype of *BhGolS1* transgenic tobacco. **a** Drought tolerance of *BhGolS1* transgenic and WT tobacco. T3 seeds were grown for 6 weeks in greenhouse with light (16 h) at 25°C (*Unt*), then withheld irrigation for 14 days (*D14d*) and re-watered for 7 days (*R7d*). The soil relative water content was 100, 3.5 and 77.9%, respectively. **b** Leaf RWC, Fv/Fm, electrolyte leakage of plants before (blank) and after drought for 7 (*grey*) and 14 days (*black*). All the experiments were performed twice with three independent leaves for each treatment at each time point. **c** Survival rate of plants after dehydration for 14 days and rehydration for 6 days. The values were the mean of three independent experiments. The bars indicate the SD; *single and double asterisks* marked significant level of $P = 0.05$ and 0.01 between different transgenic groups and WT, respectively; ANOVA and a post hoc Tukey's honestly significantly different (HSD) test was performed for analyzing the statistical significance (**b**, **c**). **d** Expression of *BhGloS1* in plants untreated (*unt*) and dehydrated for 3–12 days (*d3d–d12d*). Equal loading was verified by 18S rRNA amplification and CBB stain, respectively. **e**, **f** Contents of galactinol (Gol, **e**) and raffinose (Raf, **f**) in plants untreated (blank) and dehydrated for 14 days (*grey*) by HPLC

Drought, ABA (100 μM), cold (4°C) and heat (37°C) can induce GUS signals in leaves and roots, but not in the stems (Fig. 4f–k), indicating that the drought responsiveness was in agreement with the accumulation pattern of *BhGolS1* in *B. hygrometrica* (Fig. 1a), and triggered independently in each organ. Furthermore, GUS reporter gene activity in 2-h dehydrated leaves was weak, and become stronger in leaves that were dried for longer periods (8–24 h) (Fig. 4f–h), consistent with the data obtained by RT-PCR analysis in *B. hygrometrica*.

Several types of dehydration-related *cis*-elements were identified in the *BhGolS1* promoter region, including abscisic acid-responsive elements (ABRE), dehydration-responsive elements (DRE) and W-box elements (Fig. 3), indicating potential regulation by ABRE-binding bZIP factors (ABF), DRE box binding factors (DREB), and WRKY transcription factors. Four W-box elements were found to cluster in the promoter (165–470 bp upstream of the putative transcription initiation site), suggesting the possible interaction of WRKY factors with *BhGolS1* promoter.

Identification of a dehydration-inducible group II WRKY factor from *B. hygrometrica*

Coincident with the finding of W-boxes clustered in the stress responsive promoter region of *BhGolS1*, WRKY transcription factors were detected in a screen of dehydration-induced genes from a cDNA library prepared from dried *B. hygrometrica* leaves (Wang et al. 2009). The

Fig. 3 *BhGolS1* promoter sequence analysis. The *cis*-elements predicted by PLACE software are indicated in *red letters*; TATA box is shown with *dashed line*; the putative transcription start site is *underlined*; putative start codon (ATG) is labeled with a *rectangle*

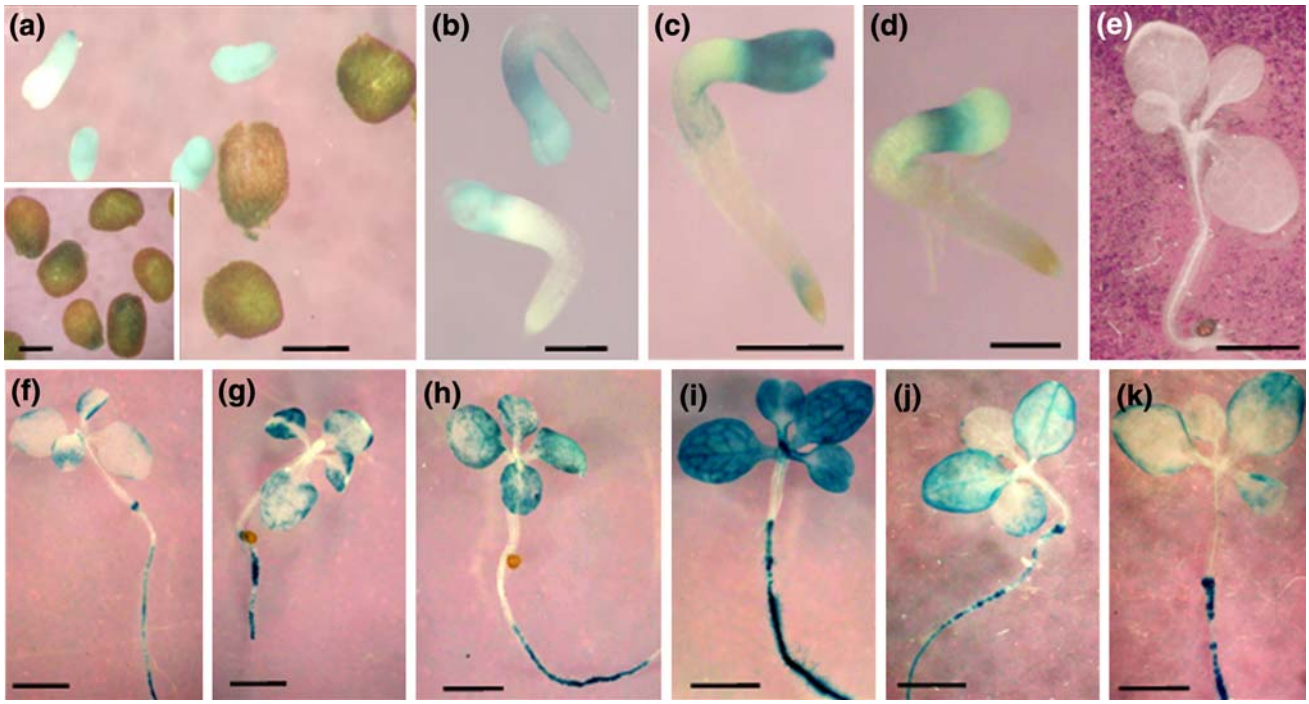

Fig. 4 *BhGolS1* promoter activity in tobacco. Histochemical analysis of untreated 1- (**a**), 2- (**b**), 3- (**c**), 5- (**d**), 10- (**e**) -day-old $Pro_{BhGolS1}$*:GUS* transgenic tobacco seedlings. The *inset* to panel **a** shows seed from $Pro_{BhGolS1}$*:GUS* transgenic tobacco. Histochemical analysis of 10-day-old $Pro_{BhGolS1}$*:GUS* transgenic tobacco dried for 2- (**f**), 8- (**g**) and 24 h (**h**), ABA treatment (100 μM) (**i**), cold stress (4°C) (**j**) and heat stress (37°C) (**k**) for 24 h. *Bar* 5 μm (**a**–**d**) and 5 mm (**e**–**k**)

full-length cDNA sequence was obtained by 5′RACE, and designated *BhWRKY1* (Accession No. FJ222453). *BhWRKY1* encodes a protein containing a single WRKY domain and a C_2H_2-type zinc finger motif, and shares homology with AtWRKY60 (Accession No. NP 180072, 40%) and OsWRKY71 (Accession No. BK005074, 37%) (Xu et al. 2004), two Group II WRKY proteins (Animation 2). Nuclear localization sequences were identified in the BhWRKY1 protein and subcellular localization in nuclei was confirmed by expression of a GFP-BhWRKY1 fusion protein in plant cells (Fig. 5).

WRKY factors are commonly found in early response to disease (Xie et al. 2005). Group II WRKY proteins have been shown to be responsive to wounding, biotic infection, abiotic stresses including drought, salt and cold, as well as senescence (Hara et al. 2000; Robatzek and Somssich 2001;

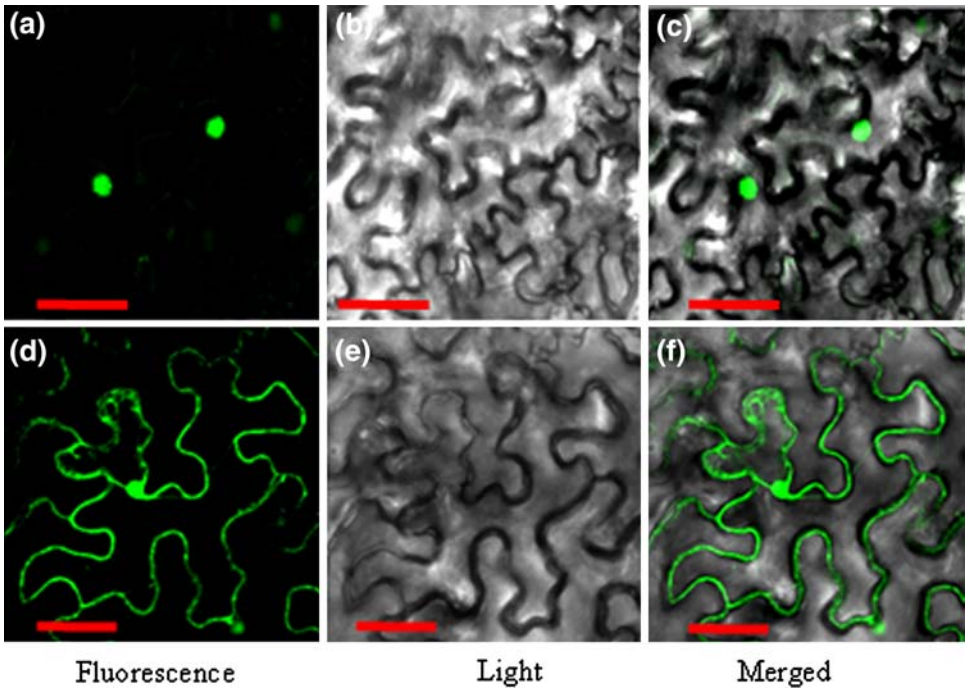

Fig. 5 Nuclear localization of GFP-BhWRKY1 in plant cells. Tobacco epidermal cells transiently expressing GFP-BhWRKY1 (**a**–**c**) and GFP alone (**d**–**f**) proteins were observed by confocal microscopy. **a**, **d** Fluorescence imaging (excitation wavelength 480 ± 20 nm; emission wavelength 510 ± 20 nm). **b**, **e** Bright field. **c**, **f** Fluorescence and bright field images merged. *Bar* 50 μm

Yu et al. 2001; Zhou et al. 2008). In our study, *BhWRKY1* is rapidly and transiently induced by dehydration and ABA (Fig. 6a), prior to *BhGolS1* induction (Fig. 1a), indicating that BhWRKY1 may function in signal transduction pathways to activate the expression of *BhGolS1* in response to dehydration and ABA. No detectable BhWRKY1 expression was observed in the absence of ABA, i.e., in detached leaves (control, CK) incubated in solution containing the ABA-solvent (ethanol), indicating that the expression in response to exogenously applied ABA is specific (Fig. 6a).

BhWRKY1 binds to *BhGolS1* promoter

To explore the possible interaction of BhWRKY1 and the W-boxes present in the *BhGolS1* promoter, a yeast one-hybrid assay was performed using AtWRKY60 as a positive control and GAL4 as a negative control. Results have showed that BhWRKY1 was capable of binding the W-box core sequence (TGAC) (construct *a*) (Xie et al. 2005) but failed to bind the mutated W-box (TGAA) (construct *b*) upstream of the *HIS* reporter gene; was capable of binding the *BhGolS1* promoter containing 2–4 W-box elements (constructs *c*, *d*, *f*–*h*) or a single W-box with a relatively lower affinity (constructs *i*, *j*) but failed to bind the *BhGolS1* promoter containing solely mutated W-box elements (construct *k*) or only a single complementary W-box (construct *e*) (Fig. 6b). The negative control, GAL4 alone, failed to activate the reporter gene in all cases. These data suggest that BhWRKY1 can specifically bind to the W-box elements in the *BhGolS1* promoter, and at least two W-box elements are needed for efficient binding, indicating the potential involvement of BhWRKY1 in the transcriptional regulation of *BhGolS1*.

In vivo binding of BhWRKY1 to *BhGolS1* promoter

To investigate the possibility that BhWRKY1 interacts with the *BhGolS1* promoter in vivo, chromatin immunoprecipitation (ChIP) was used to purify in vivo formed complexes of DNA binding protein(s) and associated DNA (Wang et al. 2002) using a polyclonal antibody generated against the BhWRKY1-GST fusion protein. The accumulation of BhWRKY1 protein during dehydration and the specificity of the antibody was confirmed by the detection of a single protein of 35 kDa, the predicted molecular weight of BhWRKY1, in nuclear protein extracts from *B. hygrometrica* leaves dehydrated for 0.5 h (leaf RWC 79%) but not from untreated leaves (Fig. 6c). Thus, leaves dehydrated for 0.5 h were used for ChIP assay. Using specific primers flanking the W-box cluster region of *BhGolS1* promoter, the corresponding fragment was amplified from nuclear DNA before immunoprecipitation and purified DNA immunoprecipitated by BhWRKY1-GST antibody, but not from DNA purified in parallel without incubation with the primary antibody (Fig. 6d).

BhWRKY1 regulates the expression of *BhGolS1 in planta*

To investigate the regulation of *BhGolS1* expression by BhWRKY1 *in planta*, Pro_{35S}*:BhWRKY1* and Pro_{35S}*:anti-BhWRKY1* transgenic tobacco were individually crossed with transgenic tobacco harboring $Pro_{BhGolS1}$*:GUS*, respectively,

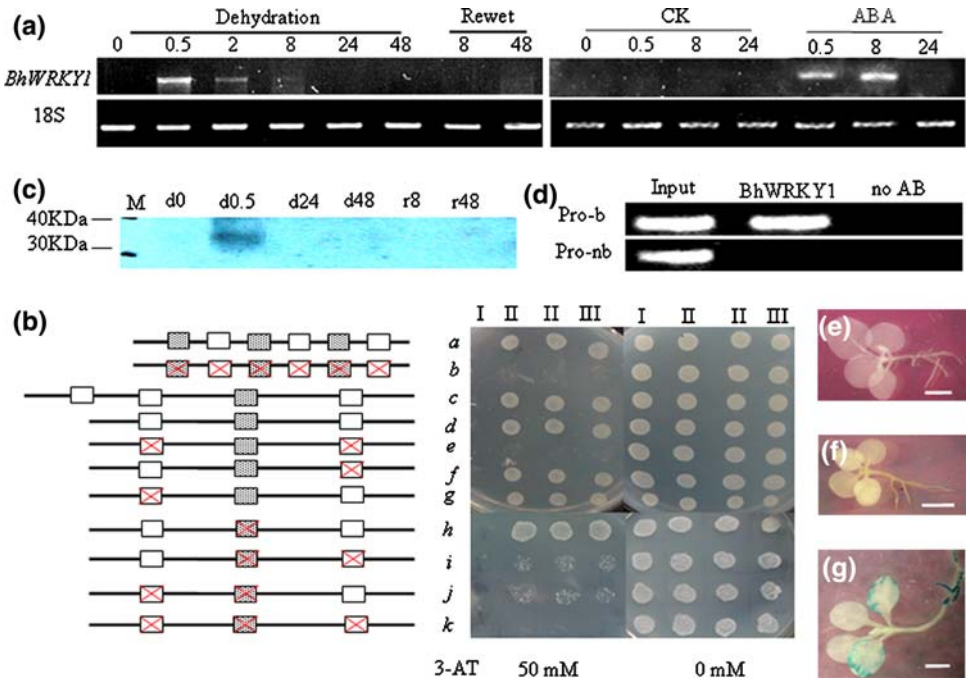

Fig. 6 BhWRKY1 expression and interaction with the *BhGolS1* promoter. **a** Dehydration-inducible expression of *BhWRKY1* in *B. hygrometrica* leaves. *CK* treatment in the presence of ABA-solvent (ethanol) only. *ABA* treatment with ABA (100 μM) solution. **b** Yeast one-hybrid assay of BhWRKY1 binding to W-box elements. *Left* schematic diagrams of W-box elements upstream of the *His* reporter gene, including synthetic (*a*, *b*) and *BhGolS1* promoter sequences (*c*–*k*). *White rectangular* W-box, *grey rectangular* complementary W-box, *hatched rectangular with a red cross* mutant W-box. *Right* photographs of the yeast harboring corresponding plasmids growing on 50 and 0 mM 3-AT plate (SD/-His-Leu). Negative control plasmid GAL4 (I). Prey plasmid containing *BhWRKY1* (II). Positive control prey plasmid containing *AtWRKY60* (III). **c** Immunoblot analysis showing BhWRKY1 protein accumulation in *B. hygrometrica* leaves during dehydration. *M* protein molecular weight marker. **d** ChIP assay showing BhWRKY1 binding to the $Pro_{BhGolS1}$ fragment in *B. hygrometrica* leaves dehydrated for 0.5 h. *Pro-b* W-box rich fragment of $Pro_{BhGolS1}$, *Pro-nb* downstream fragment of Pro-b. DNA pool before immunoprecipitation (Input), immunoprecipitated with BhWRKY1 antibody (BhWRKY1) or without antibody (no AB) were used as templates. Primers are shown in Fig. 3. **e**–**g** BhWRKY1 regulates the expression of $Pro_{BhGolS1}$*:GUS* in transgenic tobacco. 10-day-old T1 progeny of $Pro_{BhGolS1}$*:GUS* crossed with WT (**e**), Pro_{35S}*:antisenseBhWRKY1* (**f**) and Pro_{35S}*:BhWRKY1* (**g**) were stained directly. *Bar*s 5 mm

using WT as a control. T1 seeds were germinated and grown on MS media for 10 days before staining. A constitutive expression pattern was observed in the line containing both Pro_{35S}*:BhWRKY1* and $Pro_{BhGolS1}$*:GUS* without dehydration stress, but in the other lines only after dehydration for 2 h (data no shown). These results demonstrate that ectopic expression of BhWRKY1 is sufficient to transcriptionally activate *BhGolS1* expression (Fig. 6e–g).

In summary, our paper reports the cloning of full-length *BhGolS1* and *BhWRKY1* genes from the resurrection plant *B. hygrometrica*. Both are induced rapidly by dehydration and ABA. BhGolS1 exhibits enzyme activity, in the form of galactinol synthesis from UDP-Gal and *myo*-inositol, and confers drought tolerance and Gol/Raf accumulation when overexpressed. These data support the view that GolS is crucial for RFO accumulation and plays a protective role during drought stress (Taji et al. 2002; Peters et al. 2007). Previously, HSF and CBF-type of transcription factors have been shown to regulate GolS expression in *Arabidopsis* (Taji et al. 2002; Panikulangara et al. 2004). Here, we provide evidence that a WRKY transcription factor can interact, via W-boxes, with the *BhGolS1* promoter and activate BhGolS1 expression. At least two copies of W-boxes are needed for the interaction with BhWRKY1. Since both are induced rapidly by dehydration and ABA, it is likely that *BhWRKY1* is involved in the ABA signal pathway to activate its target gene *BhGolS1* expression in *B. hygrometrica*. To our knowledge, this is the first report that WRKY factors may be involved in the ABA-dependent signal pathway to regulate *GolS* gene expression in response to dehydration. In rice seeds, the ABA-induced OsWRKY51 factor was found to interact with OsWRKY71, which is also ABA-inducible, and promotes OsWRKY71 to compete with GA-induced OsGAMYB in binding the promoter of a GA-induced α-amylase, subsequently inhibiting the expression of α-amylase (Zhang et al. 2004). A similar regulatory network was also demonstrated in barley (Sun et al. 2003). Whether or not such a mechanism exists in leaves of *B. hygrometrica* is not known, however, the presence of MYB binding sites in the *BhGolS1* promoter does not rule out this possibility. All these indicated the complexity of the regulation process of *BhGolS1* gene in *B. hygrometrica*. The next

steps towards understanding desiccation tolerance will be to reconstruct the network of involvement of different transcription factor classes and then verify the roles these transcription factors play in the network.

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