Phosphate-induced-1 gene from *Eucalyptus* (*EgPHI-1*) enhances osmotic stress tolerance in transgenic tobacco

A.O. Sousa, E.T.C.M. Assis, C.P. Pirovani, F.C. Alvim and M.G.C. Costa

Centro de Biotecnologia e Genética, Departamento de Ciências Biológicas,
Universidade Estadual de Santa Cruz, Ilhéus, BA, Brasil

Corresponding author: M.G.C. Costa
E-mail: marciogc.costa@gmail.com

ABSTRACT. Environmental stresses such as drought, freezing, and high salinity induce osmotic stress in plant cells. The plant response to osmotic stress involves a number of physiological and developmental changes, which are made possible, in part, by the modulation of the expression of specific genes. *Phosphate-induced-1* gene (*PHI-1*) was first isolated from phosphate-treated phosphate-starved tobacco cell cultures as a stress-inducible gene, which is presumably related to intracellular pH maintenance; however, the role of the *PHI-1* gene product has not yet been clarified. A gene encoding a predicted protein with high similarity to tobacco PHI-1, named *EgPHI-1*, was previously identified in *Eucalyptus* by comparative transcriptome analysis of xylem cells from species of contrasting phenotypes for wood quality and growth traits. Here, we show that the overexpression of *EgPHI-1* in transgenic tobacco enhances tolerance to osmotic stress. In comparison with wild-type plants, *EgPHI-1* transgenic plants showed a significant increase in root length and biomass dry weight under NaCl-, polyethylene glycol, and mannitol-induced osmotic stresses. The enhanced stress tolerance of transgenic plants was correlated

with increased endogenous protein levels of the molecular chaperone binding protein BiP, which in turn was correlated with the *EgPHI-1* expression level in the different transgenic lines. These results provide evidence about the involvement of *EgPHI-1* in osmotic stress tolerance via modulation of BiP expression, and pave the way for its future use as a candidate gene for engineering tolerance to environmental stresses in crop plants.

Key words: Intracellular pH; Phosphorylation; Abiotic stress; Chaperone; Endoplasmic reticulum; Cell death

INTRODUCTION

Environmental stresses, such as drought, freezing, and high salinity, are often interconnected since they may induce similar cellular damage that is primarily manifested as osmotic stress. As environmental stress conditions seriously affect crop production in many parts of the world, the molecular and cellular processes underlying the acclimation of plants to osmotic stress have attracted much research interest recently (Zhu, 2002; Mahajan and Tuteja, 2005; Vásquez-Robinet et al., 2010). To cope with osmotic stress, plants have developed a number of physiological and developmental mechanisms, including alteration of life cycle, inhibition of shoot growth and enhancement of root growth, adjustment of ion transport, carbon metabolism, and the synthesis of compatible solutes (Xiong and Zhu, 2002). Many of these changes are made possible by modulations of the expression of specific genes, which provides an important approach for engineering tolerance in plants (Brinker et al., 2010).

A complex apparatus of signaling factors is required for the transcription of stress-inducible genes. These factors perceive environmental variations and transduce these signals through cells. Changes in intracellular pH are one of the first indicators of osmotic stress perceived by cells (Netting, 2000). Metabolism, transport, and signaling are homeostatic processes that are fully dependent on the regulation of intracellular pH in plant cells (Sakano, 1998). For example, the *phosphate-induced-1* gene (*PHI-1*), which was first identified in cell cultures of tobacco (Sano et al., 1999), has attracted considerable interest. Despite its sequence similarity to the *EXORDIUM* (*EXO*) gene of *Arabidopsis*, *PHI-1* and *EXO* do not show similarities to any gene with known function. Tobacco *PHI-1* was initially presumed to play a role in phosphate-induced cell cycle re-entry (Sano et al., 1999). Subsequently, the *PHI-1* gene product was inferred to play a role in alleviating changes of intracellular pH caused by stress conditions in cells (Sano and Nagata, 2002). However, the exact role of the *PHI-1* gene product has not yet been clarified.

Comparative analysis between the xylem transcriptomes of *Eucalyptus grandis* and *Eucaplytus globulus*, using high-density microarrays, led to the identification of a differentially expressed gene encoding a predicted protein with high similarity to tobacco *PHI-1* (Pasquali et al., 2005). This gene, named *EgPHI-1*, was expressed 7.5 times more in *E. globulus* than in *E. grandis* (Pasquali et al., 2005), two *Eucalyptus* species with contrasting growth and wood characteristics. *E. grandis* is a species native to tropical and subtropical regions that exhibits rapid growth, but produces a low-density wood. On the other hand, *E. globulus* is a species from temperate climates that produces a high-density wood, but exhibits slow growth

when cultivated in tropical regions (Eldridge et al., 1993; Bernard, 2003; Myburg et al., 2007). Considering the economic and ecological relevance of the genus *Eucalyptus*, understanding the cellular function of stress-inducible genes is of central interest for biotechnological applications in forestry.

In this study, we investigated the relationship between *EgPHI-1* and osmotic stress. Transgenic tobacco plants overexpressing *EgPHI-1* were generated and subjected to osmotic stress induced by NaCl, mannitol, and polyethylene glycol (PEG). The results indicated that *EgPHI-1* enhances osmotic stress tolerance of transgenic plants through a mechanism that involves the modulation of the expression of the molecular chaperone binding protein, BiP, an important component of the endoplasmic reticulum (ER) that protects the cell against stresses.

MATERIAL AND METHODS

Plant material

The *EgPHI-1*-coding sequence, which was identified in a xylem cDNA library of *E. globulus*, was released from the pSPORT1 cloning vector by *Sal*I and *Not*I digestion. The purified ~1.2-kb fragment was subcloned in the sense orientation between the promoter and terminator sequences of the cauliflower mosaic virus (CaMV) 35S of the pUC118 vector using the *Xho*I and *Not*I restriction sites. The resulting 35S::*EgPHI-1* construct was released from pUC118 by *Bam*HI/*Hin*dIII double-digestion and inserted into the same restriction sites of the binary vector pCAMBIA 2301 (CAMBIA, Australia), which also contains the neomycin phosphotransferase (*nptII*) and β-glucuronidase (*uidA*) genes under the control of the CaMV 35S promoter. The plasmid construct was introduced into *Agrobacterium tumefaciens* EHA105 by direct DNA uptake. The construction was transferred to wild-type (WT) tobacco (*Nicotiana tabacum* cv. Havana) by *A. tumefaciens*-mediated genetic transformation, as previously described (Horsch et al., 1985). Three transgenic lines (L1, L2, and L3), representing distinct transformation events, were selected on appropriate media by their resistance to kanamycin, positive reaction in GUS histochemical assays (Jefferson, 1989), polymerase chain reaction (PCR) amplification of *npt*II and *uid*A, and by reverse transcription-quantitative real-time PCR (RT-qPCR) (Sousa AO, Assis ETCM, Silva DC, et al., unpublished results). Seeds from WT and kanamycin-resistant T_1 transgenic plants used in the osmotic stress experiments were derived from plants grown in a greenhouse under identical conditions.

Osmotic stress experiments

Seeds from WT and transgenic plants were sterilized and plated on MS medium (Murashige and Skoog, 1962) solidified with 0.7% agar. After 2 days in a cold room (4°C), the plates were transferred to a growth chamber with a long day light regime (16 h day, 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 27° ± 2°C; 8 h night, 27° ± 2°C), and grown in a randomized manner over 15 days. After this period, nine plants of similar size from each of the WT and transgenic lines were transferred to MS medium supplemented with the stress inductors, NaCl, mannitol, or PEG, in concentrations sufficient to impose a water potential of -1 MPa. The WT and transgenic plants were transferred to MS medium without stress inductors in the control treatment. The plants were maintained in a growth chamber over the next 25 days under the same con-

ditions described above. At the end of this period, phenotypic traits, including leaf wilting, chlorosis, and necrotic lesions, were analyzed, along with the root length and the biomass dry weight of individual plants. To determine the biomass dry weight, plants were kept in an air-circulation stove at 75°C until reaching a constant weight. All treatments were performed in triplicate.

Protein extraction and Western blot analysis

Leaves of control and stressed WT and transgenic plants were used for BiP immunoblot analyses. These plants were grown for 60 days in a greenhouse under identical normal conditions. Lyophilized leaves were macerated in liquid nitrogen and 1% (w/w) polyvinyl-pyrrolidone by the phenol/SDS method (Pirovani et al., 2008). The concentration of total protein was determined using the 2-D Quant Kit (GE Healthcare, USA) according to manufacturer instructions. Equivalent amounts of total protein (20 µg) were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using the iBlot Dry Blotting System (Invitrogen, USA) according to manufacturer instructions. Membranes were probed with the polyclonal antibody against the carboxyl region of soybean BiP (Figueiredo et al., 1997) at a 1:2000 dilution. As secondary antibody, the goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, USA) was used. The 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitroblue tetrazolium (NBT; Promega, USA) were used as substrates for the colorimetric reaction from alkaline phosphatase activity. BiP levels were quantified from membrane images using the GelQuant.Net 1.8.0 software (www.biochemlabsolutions.com) and the values of transgenic lines were calculated in relation to WT protein levels.

Statistical analysis

All measurements were repeated at least three times from different individual plants. Statistical differences between WT and transgenic lines were assessed based on analysis of variance (ANOVA) and differences in means were determined with the Student t-test, with critical values of $P \leq 0.05$ and $P \leq 0.01$.

RESULTS

Overexpression of *EgPHI-1* enhances osmotic stress tolerance in transgenic tobacco

We analyzed the effects of *EgPHI-1* overexpression, at the whole plant level, on the response of plants to osmotic stress induced by NaCl, mannitol, and PEG. The transgenic lines showed increased tolerance to the different stresses compared with WT tobacco, even 25 days after the stress treatments (Figure 1). While the WT tobacco exhibited small leaves with chlorotic aspects and reduced growth in the NaCl treatment compared with the control treatment, growth of transgenic plants was not affected. Although PEG and mannitol treatments affected the growth of transgenic plants to some extent as compared with the control treatment, these effects were more severe in WT tobacco. PEG promoted slight chlorosis in the transgenic plants, while WT plants became completely necrotic under this treatment. Mannitol promoted

a more severe chlorosis and reduced growth in the transgenic plants compared with the control treatment. It is interesting to note that under control conditions (without stress inductor), the transgenic plants exhibited higher growth than the WT plants.
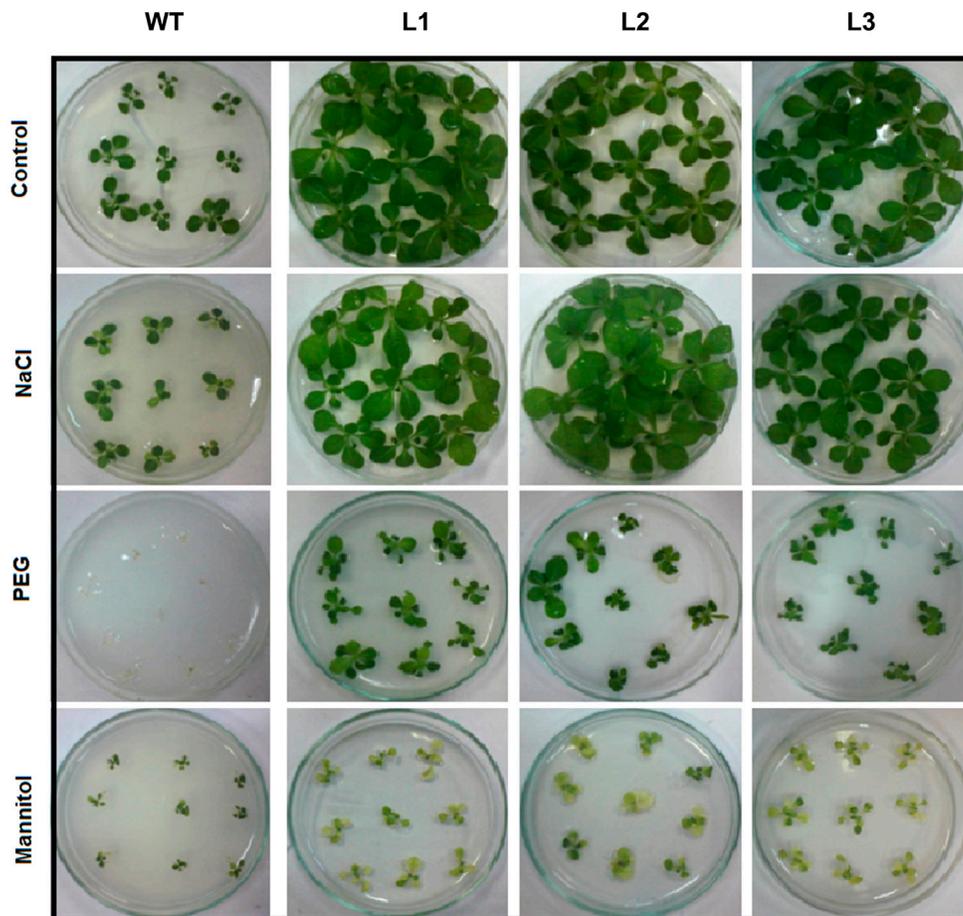

Figure 1. Phenotypes of tobacco plants exposed to different osmotic stress treatments. Fifteen-day-old wild-type (WT) and transgenic (L1, L2 and L3) tobacco plants were subjected to osmotic stress (-1.0 MPa) induced by NaCl, polyethylene glycol (PEG) and mannitol. Plants were maintained in medium without stress inductors in the control treatment. Photographs were taken 25 days after the treatments.

A direct comparison between stressed WT and transgenic plants demonstrated significantly higher root length and biomass dry weight in the transgenic plants than in the WT plants under all stress conditions (Figure 2). The only exception was found in the mannitol treatment, in which the root length of transgenic plants was smaller than that of WT plants. However, the biomass dry weight of transgenic plants was ~3-fold higher than that of WT plants under this treatment. The transgenic lines, L1, L2, and L3, showed root lengths that were 3.7-, 4.7-, and 4.1-fold higher, respectively, than those of WT plants under NaCl treatment, whereas their

biomass dry weights under this same treatment were, respectively, 3.7-, 6.4-, and 3.5-fold higher than WT plants. For the PEG treatment, the transgenic lines, L1, L2, and L3, showed a 123-, 142-, and 81-fold increase in root length, respectively, and a 7-, 12-, and 9-fold increase in biomass dry weight compared with WT plants.
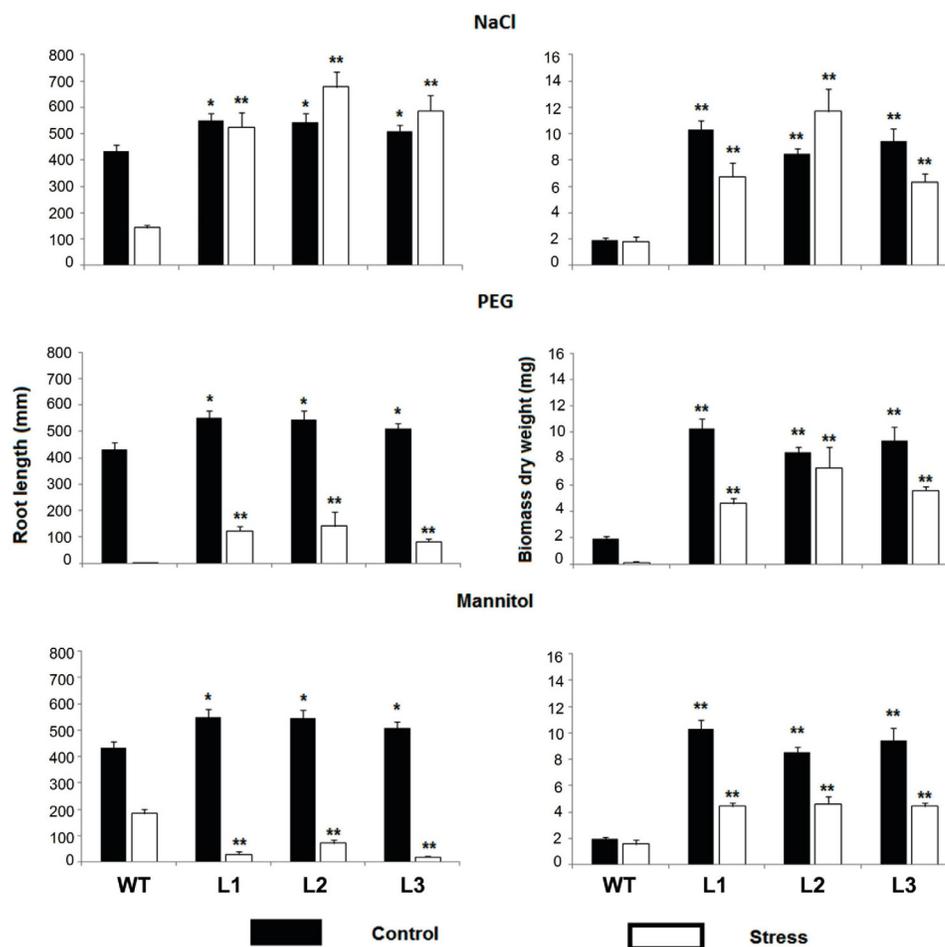

Figure 2. Root length (left) and biomass dry weight (right) of *EgPHI-1* expressing transgenic tobacco plants under different stress treatments. Measurements were performed in wild-type (WT) and transgenic (L1, L2 and L3) plants subjected to osmotic stress induced by NaCl, polyethylene glycol (PEG) and mannitol (open bars) or maintained under control (non-stress) conditions (filled bars), 25 days after the treatments. Bars represent the mean ± SE of the three replicates (N = 27). Statistically significant differences at *P ≤ 0.05 and **P ≤ 0.01 between the WT and transgenic plants grown under control or stress conditions are indicated.

EgPHI-1-mediated osmotic stress tolerance correlates with BiP expression

The deduced amino acid sequence of EgPHI-1 is predicted to contain a signal

peptide in its N-terminal portion targeting the ER (data not shown). Therefore, the constitutive overexpression of *EgPHI-1* in the transgenic plants may disrupt ER homeostasis. This phenomenon usually results in the induction of ER-molecular chaperones, such as BiP, which has been demonstrated to mediate drought tolerance in transgenic plants (Alvim et al., 2001; Valente et al., 2009). To examine whether the observed osmotic stress tolerance of transgenic tobacco plants overexpressing *EgPHI-1* correlates with BiP accumulation in plant tissues, BiP concentration was determined in leaves of WT and transgenic plants by Western blot analysis. All transgenic plants evaluated showed a remarkable increase in BiP accumulation as compared with WT tobacco (Figure 3), showing increases of 21, 45, and 85% in L1, L2, and L3, respectively. Interestingly, the increase in BiP accumulation was proportional to the expression levels of *EgPHI-1* in the transgenic lines. The transgenic line L3 was shown to accumulate the highest levels of *EgPHI-1* transcripts, L2 had an intermediate level, and L1 showed the lowest transcript levels (Sousa AO, Assis ETCM, Silva DC, et al., unpublished results).
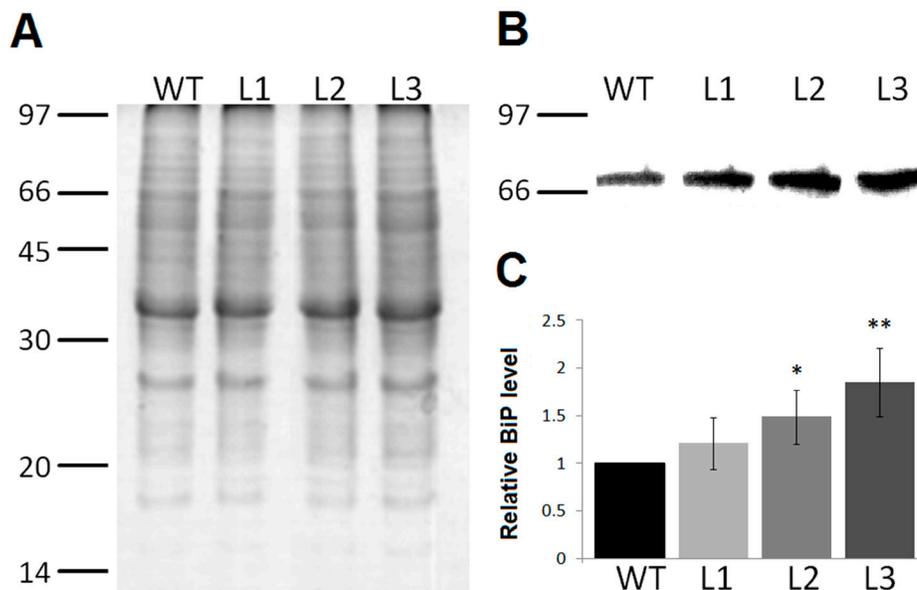

Figure 3. Binding protein (BiP) levels in wild-type (WT) and transgenic tobacco. **A.** Total protein extract. Equal amounts of total protein isolated from leaves of WT and transgenic (L1, L2 and L3) plants were separated by SDS-PAGE. **B.** Western blot of BiP protein. Total protein extracts were immunoblotted with anticarboxyl BiP antibody. **C.** Relative BiP level. BiP levels were quantified from membrane images using the GelQuant.Net 1.8.0 software and the values of transgenic lines (L1, L2 and L3) were calculated in relation to WT protein levels. Bars represent the mean ± SE of the three replicates (N = 3). Statistically significant differences at $*P \leq 0.05$ and $**P \leq 0.01$ between the WT and transgenic plants are indicated.

DISCUSSION

The results presented here show that *EgPHI-1* expression modulates plant development and response to hyperosmotic conditions. The transgenic plants showed higher root length and biomass dry weight than the WT plants in control (non-stress) conditions. A similar

phenotype was observed in transgenic *Arabidopsis* plants overexpressing *EXO*, another member of the *PHI-1* family. *EXO* induced high growth of transgenic plants at the first stages of development by promoting the cellular expansion process (Coll-Garcia et al., 2004). Thus, our observations suggest a connection between *EXO* and *EgPHI-1* functions.

NaCl, mannitol, and PEG induce hyperosmotic stress in different ways. NaCl penetrates through membranes and causes ionic stress and toxicity due to Na^+ excess. Mannitol also has the ability to penetrate through membranes, but it does not induce ionic stress, whereas PEG is a non-ionic and non-penetrating agent that decreases the water potential of the nutrient solution without causing toxicity (Gangopadhyay et al., 1997a,b). Therefore, the use of these three stress inducers allowed comparison of plant responses to osmotic stress by disturbance of water relationships, changes in the absorption and utilization of essential nutrients, and accumulation of toxic ions (Yokoi et al., 2002).

Transgenic tobacco plants expressing *EgPHI-1* showed a significantly improved tolerance to osmotic stress induced by NaCl, mannitol, and PEG as compared with WT plants. They displayed increased root lengths and biomass dry weights under all osmotic stress conditions, whereas WT tobacco showed progressive chlorosis, severe growth inhibition, and necrosis under the same stress conditions tested. The only exception was observed in the mannitol treatment, which inhibited the root length of transgenic plants relative to WT plants. This result suggests that EgPHI-1 might facilitate the penetration of mannitol through the root cell membranes and/or be involved in cellular processes in the root that are negatively affected by the presence of mannitol.

The tolerance of plants to salt stress has generally been attributed to their ability to accumulate Na^+ in intracellular compartments (He and Yu, 1995; Lutts et al., 1996). This mechanism allows better plant growth under salt stress (Thomas et al., 1992). The enhanced tolerance of *EgPHI-1*-expressing transgenic tobacco plants to NaCl treatment may be related to the increased intracellular compartmentalization of Na^+ or to the alleviation of intracellular pH changes caused by salt stress, as suggested for tobacco *PHI-1* (Sano and Nagata, 2002). Tobacco *PHI-1* was postulated to play a role in alleviating changes of intracellular pH that are caused by stress, such as high concentrations of phosphate (Sano and Nagata, 2002), through its involvement in one of two possible mechanisms: i) intracellular proton exclusion from cells via an ATP-driven proton pump, and ii) proton consumption through pH-sensitive decarboxylation reactions in organic acid metabolism (Sakano, 1998).

The most interesting observation of the present study was that BiP protein levels increased proportionally with *EgPHI-1* expression levels in the transgenic lines. Thus, our results provide evidence that EgPHI-1 enhances osmotic stress tolerance through a mechanism that involves, at least partially, the modulation of BiP expression. BiP is an ER resident molecular chaperone that plays a central role in ER stress signaling by sensing alterations that affect protein folding and assembly in the ER environment (Malhotra and Kaufman, 2007). In addition to its role as an ER molecular chaperone, BiP overexpression in plants has also been shown to increase tolerance to drought stress (Alvim et al., 2001; Valente et al., 2009). BiP-mediated drought tolerance has not been associated with the typical short-term and long-term avoidance responses or with other well-known tolerance mechanisms (Valente et al., 2009), but has instead been associated with the prevention of stress-induced cell death by negatively regulating the stress-induced N-rich protein-mediated cell death response (Reis et al., 2011). The increased BiP levels in transgenic plants overexpressing *EgPHI-1* may be

a result of ER stress triggered by the input of EgPHI-1 protein in the ER. High protein input in the ER overloads the folding machinery and generates an elevation of unfolded proteins in the ER lumen, resulting in the induction of ER-molecular chaperones such as BiP (Malhotra and Kaufman, 2007). The induction of many chaperone and co-chaperone genes was observed in transgenic rice expressing *BiP1*, and was associated with the ER stress response (Wakasa et al., 2011). These observations provide a fundamental basis for EgPHI-1-mediated osmotic stress tolerance that can be exploited for biotechnological applications in forestry, which can also be extended to other economically important crops.

ACKNOWLEDGMENTS

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