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Full Length Article

A Prohibitin Family Gene (*LpPHB3*) Enhances Salt and Oxidative Stress Tolerance when Overexpressed in *Lilium pumilum*

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Abstract

LpPHB3 was overexpressed in transgenic *Lilium pumilum* under stress caused by treatment with 20 m*M* $NaHCO_3$. Transgenic *L. pumilum* was more resistant to salt and oxidative stress than wild-type *L. pumilum*. The physiological indices of transgenic and wild-type *L. pumilum* were analysed and compared. To assess K^+/Na^+ homeostasis, the K^+/Na^+ ratios of the transgenic plants and wild-type plants under stress were compared. The intercellular ROS content and the expression of genes related to ROS (*APX*, *CAT*, *AOX*1a and *NDB*1) in transgenic plants and wild-type plants were compared. *LpPHB3 overexpression* reduced salt-induced damage in plants, possibly by reducing or eliminating excessive ROS in the plants. As *PHB3* in plants has been relatively poorly studied, further study of *LpPHB3* increases our understanding of the stress-associated function of this gene in *Lilium*. Furthermore, the stress tolerance of other plants can be improved by the introduction of this gene into these plants in the future. Two-month-old wild-type and transgenic seedlings were transferred to pots containing nutrient soil. After another 2 weeks of growth, the pots were irrigated with 50 mL of a 300 m*M* NaCl, 300 m*M* $NaHCO_3$ or 2 *M* H_2O_2 solution 3 times every 4 days. The pots were covered with a breathable plastic cover to minimize evaporation and maintain the concentration of the solution. Images of the plants were taken after 12 days of treatment. © 2020 Friends Science Publishers

Keywords: *Lilium pumilum*; Gene expression; Physiological change; Abiotic stresses; ROS

Introduction

The genus *Lilium* is an important ornamental plant. *Lilium* bulbs are considered Chinese medicine and a healthy food (Zhou *et al*. 2012; Tang *et al*. 2014). *Lilium pumilum* DC. (*L. pumilum*) is a wild lily species distributed in Northeast China. *L. pumilum* is valuable not only for its beautiful flowers, edible properties and medicinal usage but also for its high adaptability to soil conditions and resistance to drought and salinity (Zhao *et al*. 1996; Proscevičius 2010). Therefore, this research selected *L. pumilum* as an ideal material for investigating the gene salinity tolerance of *Lilium*.

The PHB1 protein was identified with anti-proliferative activity and was hence named prohibitin (McClung *et al*. 1989). Prohibitins constitute a family of evolutionarily conserved proteins, comprising two highly homologous PHB1 and PHB2 subunits. PHB not only controls cell lifespan and plant growth (Coates *et al*. 1997; Merkwirth *et al*. 2008; Merkwirth and Langer 2009; Lee *et al*. 2015) but also has some relationship with stress in plants, such as knockdown of *AtPHB3* and *AtPHB4*, which can improve stress-related transcript abundance (Aken *et al*. 2007). *Prohibitin* expression was induced by high or light metabolic stress (Vandenabeele *et al*. 2003; Sieger *et al*. 2005). The gene expression level of rice *prohibitin* was changed in a rice lesion-mimic mutant (Takahashi *et al*. 2003). *Arabidopsis atPHB3* mutants appeared more resistant to salt stress than the wild type under NaCl treatment (Wang *et al*. 2010). The *Caenorhabditis elegans phb* mutant caused increased sensitivity to oxidative stress (Artal-Sanz *et al*. 2003). *PHB*-silenced tobacco was more susceptible to H_2O_2 induced by oxidative stresses (Ahn *et al*. 2006). *AtPHB3* regulates salicylic acid biosynthesis, which is induced by stress (Seguel *et al*. 2018). Low *PHB1* or *PHB2* expression was associated with increased ROS (Zhou *et al*. 2014).

In this study, the *LpPHB3* gene was isolated from an *L. pumilum* bulb grown under 20 m*M* $NaHCO_3$ stress. *LpPHB3* was overexpressed in transgenic *L. pumilum*. Transgenic *L. pumilum* had more resistant to salt and oxidative stress than wild type. The physiological index between transgenic and wild type *L. pumilum* was analyzed. The homeostasis ratios of K^+ and Na^+ between transgenic plants and wild type plants under stress were compared. The

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intercellular ROS content and the expression of genes related to ROS (APX; CAT; AOX1a; NDB1) in transgenic plants and wild type plants were compared. *LpPHB3* overexpression reduces the damage of salt to plants maybe by reducing or eliminating excessive ROS in the plants. *PHB3* has been relatively little studied in plants. The study of *LpPHB3* not only benefited our understanding of the stress-associated function of this gene in *Lilium* but also can improve the stress tolerance of other plants by introducing this gene into plants in the future.

Materials and Methods

Obtaining the open reading frame (ORF) region of *LpPHB3*

Total RNA of two-month-old tissue culture seedlings and was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized by PrimeScript reverse transcriptase (Takara, Tokyo, Japan). The PCR product was obtained with the primers listed in Table 1 and ligated with pMD18-T (Takara, Tokyo, Japan). Then, sequences of the deduced protein were used as queries for conserved domain and blast studies in NCBI. A multiple sequence alignment was constructed with DNAMAN8. MEGA 3.0 was used for phylogenetic analysis. The new gene was named *LpPHB3* (NCBI number: MH319853).

LpPHB3 expression in *L. pumilum*

Total RNA of *L. pumilum* roots, leaves, shoots, flowers and seeds was extracted, and cDNA was synthesized. qPCR analysis was performed using SYBR Green (TaKaRa, Tokyo, Japan). The *lilyActin* gene (the NCBI number: JX826390) as the reference (Liang *et al*. 2013), the *LpPHB3* and *lilyActin* gene primers for qPCR are listed in Table 1.

Two-month-old *L. pumilum* seedlings grown on MS medium (Murashige and Skoog 1962) in culture bottles were transferred to fresh MS medium either with 200 m*M* NaCl, 30 m*M*,15 m*M* H_2O_2 or no stress. The expression of *LpPHB3* in bulbs after 0, 6, 12, 24, 36, and 48 h stress was detected by qPCR.

Identification of transgenic *L. pumilum*

pMD18T-*LpPHB3* plasmid DNA was amplified with primers *LpPHB3*-*BamH*1F and *LpPHB3*-*Sac*I R (listed in Table 1). The PCR product was digested by *Bam*HI / *Sac*I and ligated to the *Bam*HI/*Sac*I double digestion of vector pBI121. The pBI121-*LpPHB3* plasmid was transformed into strain *EHA105* (*Agrobacterium tumefaciens*), which was then transformed into *L. pumilum* scale through *Agrobacterium*-mediated transformation (Cáceres *et al*. 2011). Transgenic scale was germinated on kanamycin-selective MS+2 mg/L 6BA+0.5 mg/L NAA medium at first, when plants differentiated from scales, the region of the PBI121 vector carrying *LpPHB3* was amplified from the DNA of 6 independent transgenic *L. pumilum* leaves using the PBI121 forward and reverse primers we designed (primers are listed in Table 1). Finally, five transgenic lines (except #5) were selected and analyzed by qPCR to confirm that the transformation was successful; the *LpPHB3* and *lilyActin* gene primers for qPCR are listed in Table 1, and the protocol is described above. The transgenic lines (#2, #3, and #4) were used for all further analyses.

Stress tolerance compared with the wild type and the transgenic *L. pumilum*

The two-month-old wild type and transgenic lines (#2, #3, #4), which grew in culture bottles with the same growth status, were transplanted into MS medium either with 200 m*M* NaCl, 20 m*M* $NaHCO_3$ or 20 m*M* H_2O_2 or without for 48 h to observe the leaf phenotypic characteristics.

Two-month-old wild type and transgenic seedings were transferred to pots containing nutrient soil. After another 2 weeks of growth, the pots were irrigated with 50 mL solution of 300 mm NaCl, 300 mm $NaHCO_3$ or 2 *M* H_2O_2 for 3 times every 4 days. The pots container is covered with a breathable plastic cover to minimize evaporation and keep the concentration of the solution from changing too much. Images of the plant were taken after 12 days of treatment.

Measurements of physiological indices of *L. pumilum* under the stresses

Seedlings of two-month-old wild type and transgenic plants of the same size were placed in MS medium either with 200 m*M* NaCl, 20 m*M* $NaHCO_3$ or 20 m*M* H_2O_2 or without. The leaves were harvested after 48 h to measure the physiological indices, and the proline content was estimated as described by Bates (Bates *et al*. 1973). MDA content is estimated as describing by Heath (Heath and Packer 1968). The total chlorophyll content was determined as described by Arnon (Tu *et al*. 2016). Electrolyte leakage measured using the conductometer (Cen *et al*. 2016).

K^+ and Na^+ content in *L. pumilum* under stresses

Two-month-old wild type and transgenic *L. pumilum* were cultured in 200 m*M* NaCl or 20 m*M* $NaHCO_3$ or no stress MS medium for 48 h. The dried bulbs were digested with HNO_3 and HClO (87:13, v/v) then 2.5% HNO_3 diluted, and the ion content was measured by atomic absorption spectrophotometry (AA800, Perkin Elmer, USA).

Noninvasive microtest technology (NMT) measured net K^+ and Na^+ flux

Two-month-old wild type and transgenic seedlings were transplanted onto MS medium either with 200 m*M* NaCl or 20 m*M* $NaHCO_3$ or without for 48 h. NMT (Younger USA LLC, Amherst, U.S.A.), as well as with iFluxes/imFluxes

1.0 software, was used to measure the K^+ and Na^+ fluxes of plant roots as described previously (Xin *et al*. 2014).

Reaction to ROS stress in transgenic *L. pumilum*

To investigate whether the LpPHB3 protein is related to ROS stress, the two-month-old wild type and transgenic plants were treated with 0 (control), 200 m*M* NaCl, 20 m*M* $NaHCO_3$ or 20 m*M* H_2O_2 for 48 h. The accumulation of H_2O_2 and O_2^- in plant leaves was observed through staining with 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) (Hoffmann *et al*. 2005). The stained rosettes were observed under a microscope (Olympus).

The expression of four ROS stress-related genes (*APX*, *CAT*, *AOX*, *NDB*) in wild type and transgenic plants either with 200 m*M* NaCl, 20 m*M* $NaHCO_3$ or 15 m*M* H_2O_2 or without was examined by qPCR. Primers used for qPCR were designed according to the *L. pumilum* transcriptomes that were analyzed by the company. The primers for actin and the four stress-related genes used for qPCR are listed in Table 1. The same protocol in the "*LpPHB3* gene expression in *L. pumilum*" section was performed.

Statistical analysis

All treatments were performed in triplicates and analysing the variance using SPSS for Windows version 11.5, significant differences at a level $P < 0.05$.

Results

Cloning of the *LpPHB3* ORF region

The *LpPHB3* ORF containing 846 bp, encoding 281 amino acids, was obtained from *L. pumilum* cDNA. Conserved domain analysis revealed that the protein sequence possessed the conserved PHB domains (Fig. 1). From the alignment of the *LpPHB3* deduced amino acid sequence, the LpPHB3 protein had the highest similarity (83.33%) with the HaPHB3 protein from *Helianthus annuus* (XP_022011535.1) (Fig. 2). Phylogenetic tree analysis was used to compare the LpPHB3 protein with some known homologous PHB3 proteins from a variety of plants (Fig. 3).

Expression of the *LpPHB3* gene in *L. pumilum*

The highest expression of *LpPHB3* was found in the bulb, followed by young leaves, flowers, roots, seeds and mature leaves (Fig. 4A). Under the stress of 300 m*M* NaCl, the expression of *LpPHB3* reached its highest at 24 h, which was approximately 8 times the *LpPHB3* expression in the control group (Fig. 4B). The expression of *LpPHB3* increased suddenly at 12 h and reached its highest at 24 h, approximately 42 times the *LpPHB3* expression in the control group under 20 m*M* $NaHCO_3$ stress. (Fig. 4C). The expression of *LpPHB3* remained constant for 12 h and then reached its highest at 24 h, approximately 2.4 times the *LpPHB3* expression in the control group under 20 m*M* H_2O_2 stress (Fig. 4D).

Table 1: Names and sequences of forward and reverse primers for PCR amplification of *LpPHB3*

Name	Sequence (5′-3′)	Length (bp)
LpPHB3-F	ATGGGCTCCAACCCCCAAGC	846 bp
LpPHB3-R	TCACCGTCCTGCGGTGTTGA	
*LpPHB3-BamH*1F	GGATCCATGGGCTCCAACCCCCA	858 bp
*LpPHB3-Sac*I R	CCGCGGTCATTTGCAGGTGCAT	
PBI 121-F	TCATTTCATTTGGAGAGAACAC	1000bp
PBI 121-R	TTGCCAAATGTTTGAACGATC	
lilyActin-F	GCATCACACCTTCTACAACG	286 bp
lilyActin-R	GAAGAGCATAACCCTCATAGA	
qLpAPX-F	GTTGTTGCCGTGGAAGTGAC	226 bp
qLpAPX-R	CCTCATAGCCTGACCGTTCC	
qLpCAT-F	TGTGCTGATTTCATGCGTGC	292 bp
qLpCAT-R	GGCTTTCCGGATGGTGAGAA	
qLpAOX-F	ACAAGCTCGCGTTTTGGATG	263 bp
qLpAOX-R	GCGTTCGTACCATCTAGGCT	
qNDB-F	GCACGTAGCATTGTTGAGCC	239 bp
qNDB-R	TGACAATGCTCCTCCACACC	

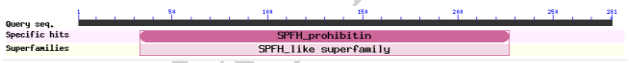

Fig. 1: Conserved domain analysis indicated that LpPHB3 has a conserved PHB domain and belongs to PHB family

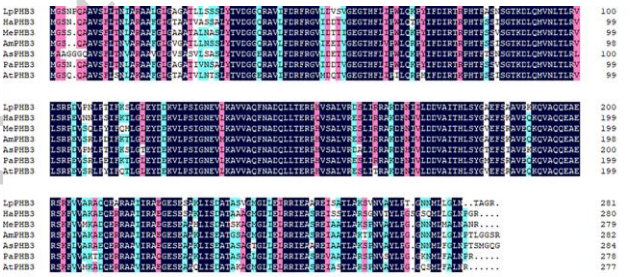

Fig. 2: Alignment of the *LpPHB3* deduced amino acid sequence with PHB3 proteins from other plant species. The amino acid sequence of this transcript was similar to that of the HaPHB3 protein (XP_022011535.1, 83.33%) from *Helianthus annuus*, MePHB3 protein (XP_021599581.1, 83.27%) from *Manihot esculenta*, AmPHB3 protein (XP_006842332.1, 83.10%) from *Amborella trichopoda*, AsPHB3 protein (PKA61440.1, 82.46%) from *Apostasia shenzhenica*, PaPHB3 protein (XP_021825041.1, 81.85%) from *Prunus avium*, and AtPHB3 (NP_198893.1, 78%) from *Arabidopsis thaliana*

Generation of an *LpPHB3*-overexpressing strain of *L. pumilum*

To investigate the function of *LpPHB3*, transgenic *L. pumilum* containing the construct 35S:*LpPHB3* was generated. The wild type strain produced no PCR product. Transgenic lines #1, #2, #3, #4, and #6 had approximately 800 bp PCR bands using the primers *PBI121*F and the *PBI121*R we designed (Fig. 5A). This result indicates that the transgenic plants are positive. These independent lines

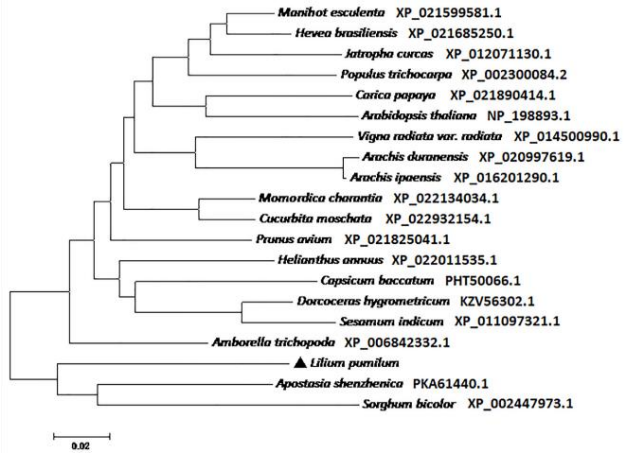

Fig. 3: Phylogenetic tree of 20 selected plant PHB3 proteins. The MEGA3 program was used for the construction of phylogenetic trees. Bar represents 0.1 amino acid substitutions per site

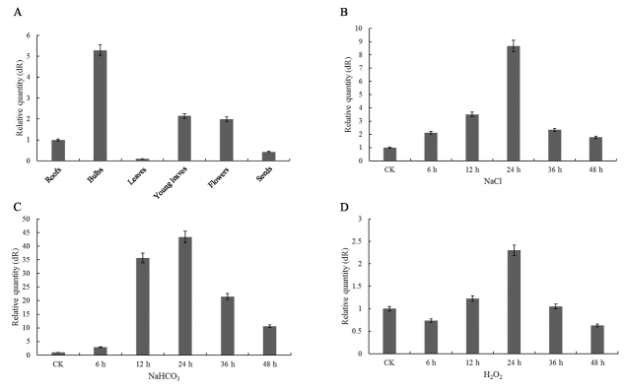

Fig. 4: Organ distribution of *LpPHB3* expression in *L. pumilum* and detection of *LpPHB3*-relative expression under the stresses. (**A**). *LpPHB3* relative expression in different organs of *L. pumilum*. (**B**). Relative expression of the *LpPHB3* gene under 200 mM NaCl treatment. (**C**). Relative expression of the *LpPHB3* gene under 20 mM $NaHCO_3$ treatment. (**D**). Relative expression of the *LpPHB3* gene under 20 mM H_2O_2 treatment. No treatment (CK= 0) was used as the control

were selected for qPCR, and the expression of *LpPHB3* in transgenic plants was higher than that in wild type plants. As shown in Fig. 5B, transgenic lines #2, #3 and #4 had higher *LpPHB3* expression levels and were selected for further research.

Comparison of stress resistance in transgenic plants and wild-type plants

The leaves showed signs of wilt under different levels of stress for 2 days-4 days. The leaves of the transgenic plants were green, while those of the wild-type plants were yellow under treatment with 200m*M* NaCl, 20m*M* $NaHCO_3$ or 20m*M* H_2O_2 which indicated the increased resistance of the transgenic plants compared to the nontransgenic plants (Fig. 6). The effects of NaCl, $NaHCO_3$ and H_2O_2 on the transgenic

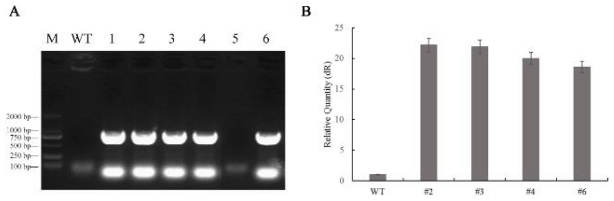

Fig. 5: A. The detection of *LpPHB3* transgenic *L. pumilum* by PCR; DNA of 6 independent transgenic *L. pumilum* leaves was amplified using the PBI121 forward and reverse primer. **B**. The detection of *LpPHB3* transgenic *L. pumilum* by qPCR analysis. Five transgenic lines (except #5) were selected for confirmation by qPCR analysis. WT, wild type; #2, #3, #4, #6, transgenic lines

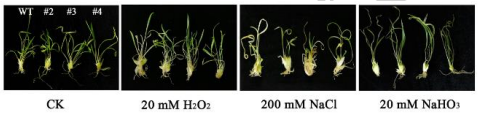

Fig. 6: Relative stress tolerance of wild type and transgenic plants (#2, #3, #4) at the tissue culture bottle stage. Two-month-old seedlings were grown on medium supplemented either with 20 mM H_2O_2, 200 mM NaCl, or 20 mM $NaHCO_3$ or without (CK). WT, wild type; #2, #3, #4, transgenic lines

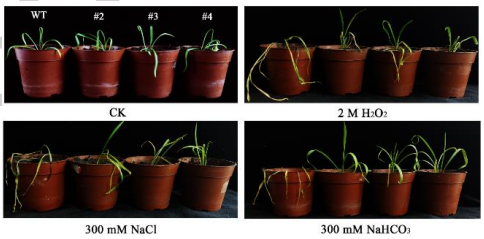

Fig. 7: Relative stress tolerance of wild type and transgenic plants (#2, #3, #4) at the spot culture stage. Plants were grown on soil supplemented either with 20 mM H_2O_2, 200 mM NaCl, or 20 m*M* $NaHCO_3$ or without (CK). WT, wild type; #2, #3, #4, transgenic lines

plants and wild-type plants on several parts of the plants were examined (Fig. 7). The wild-type and transgenic lines grew well in medium without stress. Under stress induced by 300 m*M* NaCl, 300 m*M* $NaHCO_3$ or 2 M H_2O_2, the wild-type plants died, while the transgenic plants survived; furthermore, approximately 50, 20 and 10% of the transgenic plant leaves, respectively, wilted.

Measurements of physiological indices

There was no difference between the physiological indices of the wild type and transgenic plants under no treatment. No significant difference was observed between the physiological indices of the three kinds of transgenic plants after treatment. However, there were significant differences between the wild type and transgenic plants ($P<0.05$) (Fig. 8). The content of chlorophyll in the wild type (21.12 $mg \cdot g^{-1}$) and transgenic plants (22.98 $mg \cdot g^{-1}$, 25.07 $mg \cdot g^{-1}$, and 24.13 $mg \cdot g^{-1}$) was similar under the usual conditions. The

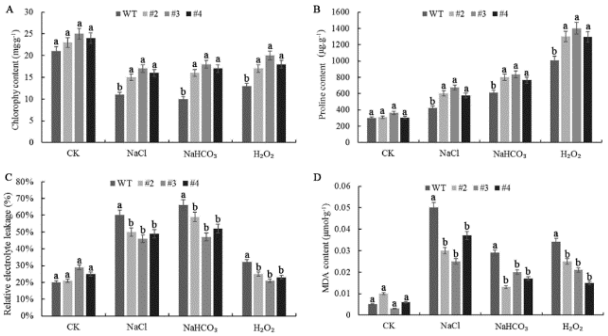

Fig. 8: Physiological changes associated with stress response in wild type and transgenic lines. Seedlings were grown in MS and subsequently transferred to MS medium either with 200 mM NaCl, 20 mM $NaHCO_3$ or 20 mM H_2O_2 or without, and samples were harvested 24 h later. Physiological indices were detected in the leaves of seedlings. (**A**) Chlorophyll content. (**B**) Proline content. (**C**) Relative electrolyte leakage. (**D**) MDA content. Each data point is the average of five replicates, and error bars represent ± SE. Lower case letters a and b indicate significant differences among mean values within each plant at $P < 0.05$

chlorophyll levels in the transgenic leaves were 15.01 mg·g^{-1}, 17.04 mg·g^{-1}, and 15.84 mg·g^{-1}, while that in the wild -type leaves was 10.31 mg·g^{-1} under 200 m*M* NaCl. The chlorophyll levels in the transgenic leaves were 16.10 mg·g^{-1}, 17.57 mg·g^{-1}, and 16.33 mg·g^{-1}, while that in the wild type leaves was 10.15 mg·g^{-1} under 20 m*M* $NaHCO_3$. The chlorophyll levels in the transgenic leaves were 16.34 mg·g^{-1}, 18.72 mg·g^{-1}, and 17.32 mg·g^{-1}, while that in the wild type leaves was 13.01 mg·g^{-1} under 20 m*M* H_2O_2 treatment (Fig. 8A). Under control conditions, the proline content of the wild type (300 μg·g^{-1}) and transgenic plants (310 μg·g^{-1}, 364 μg·g^{-1}, and 305 μg·g^{-1}) was similar. The proline contents of the transgenic plants were 604 μg·g^{-1}, 674 μg·g^{-1}, and 578 μg·g^{-1}, while that of the wild type plants was 425 μg·g^{-1} under 200 m*M* NaCl. The proline contents of the transgenic plants were 800 μg·g^{-1}, 836 μg·g^{-1}, and 763 μg·g^{-1}, while that of the wild type plants was 610 μg·g^{-1} under 20 m*M* $NaHCO_3$. The proline content of transgenic plants was 1300 μg·g^{-1}, 1402 μg·g^{-1}, and 1294 μg·g^{-1}, while that of the wild type plants was 1006 μg·g^{-1} under 20 m*M* H_2O_2 (Fig. 8B).

The levels of electrolyte leakage in the transgenic plants (20%) and wild type plants (21, 29 and 25%) were similar under control conditions. The electrolyte leakage levels in the transgenic plants were 50, 46 and 49%, while that in the wild type plants was 60% under 300 m*M* NaCl. The electrolyte leakage levels in the transgenic plants were 59, 47 and 52%, while that in the wild type plants was 66% under 20 m*M* $NaHCO_3$. The electrolyte leakage levels in the transgenic plants were 25, 21 and 23%, while that in the wild type plants was 32% under 20 m*M* H_2O_2 (Fig. 8C).

The MDA content of the wild type (0.005 mol·g^{-1}) and transgenic plants (0.01 mol·g^{-1}, 0.003 mol·g^{-1}, and 0.006 mol·g^{-1}) was similar under no stress conditions. Then, the MDA content in the wild type and transgenic plants increased under stress. The MDA content of the transgenic plants was 0.03 mol·g^{-1}, 0.025 mol·g^{-1}, and 0.037 mol·g^{-1}, while that of the wild type plants was 0.05 mol·g^{-1} under 300 m*M* NaCl. The MDA content of the transgenic plants was 0.013 mol·g^{-1}, 0.02 mol·g^{-1}, and 0.017 mol·g^{-1}, while that of wild type plants was 0.029 mol·g^{-1} under 20 m*M* $NaHCO_3$. The MDA content of the transgenic lines was 0.025 mol·g^{-1}, 0.021 mol·g^{-1} and 0.015 mol·g^{-1}, while that of the wild type lines was 0.034 mol·g^{-1} under 20 m*M* H_2O_2 treatment for 24 h (Fig. 8D).

Na^+, K^+ accumulation and Na^+, K^+ flux

There was no significant difference in Na^+ content between wild type root systems (1.763 mg·g^{-1}) and transgenic root systems (0.999 mg·g^{-1}, 1. 635 mg·g^{-1}, 1. 1.925 mg·g^{-1}) under normal culture conditions. However, Na^+ accumulation in the roots of plants increased when exposed to stresses; the Na^+ content in the transgenic plants increased to 7.886 mg·g^{-1}, 9.236 mg·g^{-1} and 8.325 mg·g^{-1}, which was apparently lower than that in wild type, 12.082 mg·g^{-1}, under 200 m*M* NaCl stress. The Na^+ content in the transgenic plants increased to 1.626 mg·g^{-1}, 1.356 mg·g^{-1}, and 1.466 mg·g^{-1}, which was apparently lower than that in wild type, 2.004 mg·g^{-1}, under 20 m*M* $NaHCO_3$ stress (Fig. 9A).

The K^+ levels in the roots of the wild type (73.717 mg·g^{-1}) and transgenic plants (75.912 mg·g^{-1}, 77.463 mg·g^{-1}, and 76.235 mg·g^{-1}) were similar under normal culture conditions. The transgenic plants had significantly higher K^+ contents (74.781 mg·g^{-1}, 77.436 mg·g^{-1}, and 75.246 mg·g^{-1}) than the wild type plants (55.260 mg·g^{-1}) under 200 m*M* NaCl stress (Fig. 9B). The transgenic plants had significantly higher K^+ contents (73.979 mg·g^{-1}, 70.464 mg·g^{-1} and 75.426 mg·g^{-1}) than the wild type plants (60.414 mg·g^{-1}) under 20 m*M* $NaHCO_3$ stress (Fig. 9B). Under 200 m*M* NaCl or 20 m*M* $NaHCO_3$ stress, the K^+/Na^+ homeostasis ratios of *LpPHB3* transgenic plants were 1.417 and 1.744 times higher than that of wild type plants, respectively.

NMT flux data showed that Na^+ efflux in the root tips of all plants was significantly higher under salt conditions. Compared with the roots of wild type plants, the exudation rate of Na^+ in the roots of the transgenic plants was significantly higher under 200 m*M* NaCl or 20 m*M* $NaHCO_3$ treatment for 24 h (Fig. 10A). We investigated the K^+ flux in plants, and salt shock induced K^+ efflux under 200 m*M* NaCl or 20 m*M* $NaHCO_3$ treatment. The average rate of K^+ efflux in the transgenic strains was lower than that in the wild type strain (Fig. 10B). These observations indicated that *LpPHB3* is involved in the regulation of K^+/Na^+ homeostasis under salt stress. In the figure, the positive values indicate outflows and the negative values indicate inflows.

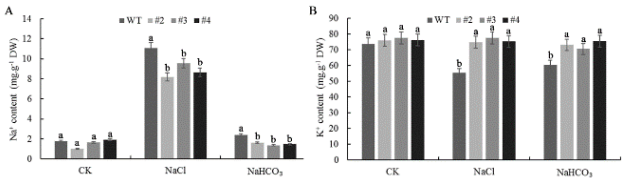

Fig. 9: Na^+ and K^+ content in wild type and transgenic lines. Two-month-old seedlings were grown on MS medium either with 200 mM NaCl or 20 m*M* $NaHCO_3$ or without, and samples were harvested 24 h later. **A**. Na^+ contents in the roots of plants. **B**. K^+ contents in the roots of plants. DW, dry weight

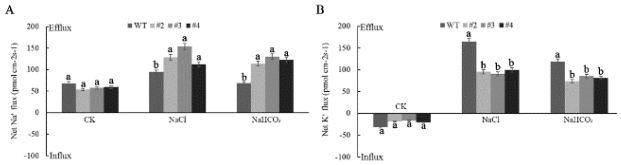

Fig. 10: Net K^+ and Na^+ flux in the root tips of wild type and transgenic *L. pumilum*. Two-month-old seedlings of wild type and transgenic *L. pumilum* were transferred to MS either with 200 m*M* NaCl or 20 m*M* $NaHCO_3$ or without for 24 h, and the seedlings were collected for NMT measurements. Each column shows the mean of six independent seedling flux rates within the measuring period of 0-20 min

ROS ($O_2^-$and H_2O_2) production in plant leaves under stresses

The stained leaf color of the wild type and transgenic plants showed no difference under no stress (Fig. 11). The wild type plant leaves showed a darker blue than the transgenic plant leaves under stresses (200 m*M* NaCl, 20 m*M* $NaHCO_3$ and 20 m*M* H_2O_2) for 24 h (Fig. 11A). The wild type plant leaves showed darker brown staining than the transgenic plant leaves under stresses (200 m*M* NaCl, 20 m*M* $NaHCO_3$ and 20 m*M* H_2O_2) for 24 h (Fig. 11B). This showed that more intercellular O_2^- and H_2O_2 accumulated in the leaves of the wild type plants than in the transgenic plants.

ROS stress-related gene expression in *L. pumilum*

Under normal conditions, ROS stress-related gene (*AOX*, *NDB*, *APX*, *CAT*) expression levels were very low, and no significant difference was observed in any of the plants. However, all four gene transcripts were increased under stress (Fig. 12).

Discussion

LpPHB3 was cloned from *L. pumilum*, and its transcriptional patterns were analysed to understand its function. Leaf growth is more sensitive to salinity than root growth, and while the root regulates full expansion of the leaves of the shoot (Munns and Termaat 1986), the bulb carries out the same function as the root. Therefore, the bulb plays a particularly important role in the salt tolerance of

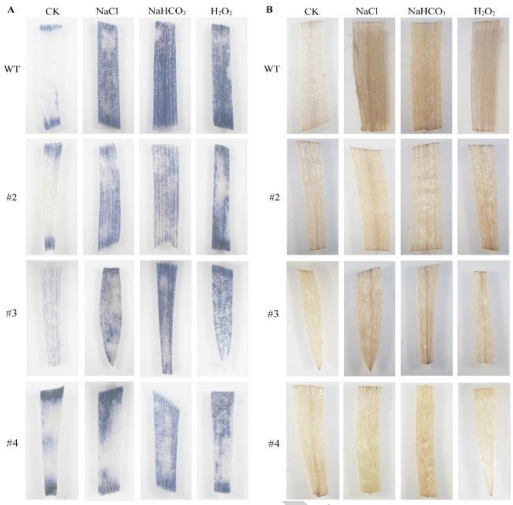

Fig. 11: Histochemical staining assay detection of O_2^- and H_2O_2 accumulation in leaves under 200 m*M* NaCl, 20 m*M* $NaHCO_3$, and 20 m*M* H_2O_2. A. Detection of O_2^- accumulation with NBT. B. Detection of H_2O_2 accumulation with DAB

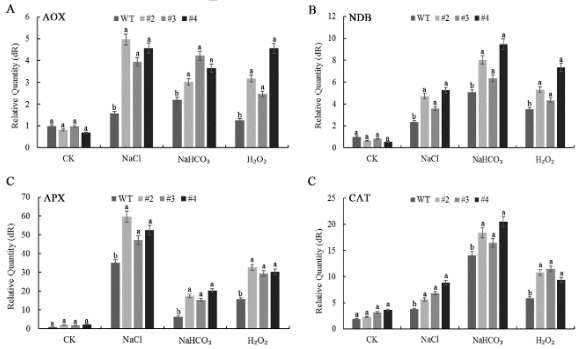

Fig. 12: Expression of stress-responsive genes in wild type and transgenic lines. **A**. Relative expression of the *AOX* gene under stress treatment. **B**. Relative expression of the *NDB* gene under stress treatment. **C**. Relative expression of the *APX* gene under stress treatment. **D**. Relative expression of the *CAT* gene under stress treatment. CK, no treatment. NaCl, 200 m*M* NaCl; $NaHCO_3$, 20 m*M* $NaHCO_3$; H_2O_2, 20 m*M* H_2O_2

plants. The highest expression of *LpPHB3* was found in the bulbs of *L. pumilum*, and LpPHB3 expression was beneficial for improving plant tolerance.

LpPHB3 transcript levels were increased under stress conditions. This shows that salt can promote the expression of *LpPHB3* and that the increased expression of *LpPHB3* can protect against an adverse environment. In addition, this result shows that *LpPHB3* is primarily related to salt stress.

We compared the stress tolerance of wild-type and transgenic plants grown in culture bottles and pots. The leaves of wild-type plants wilted and turned yellow, while the leaves of the transgenic plants grew normally and remained green. Transgenic *L. pumilum* appeared to exhibit more resistance to stress than the wild-type plants. *LpPHB3* plays a role in improving tolerance to salt and oxidant stress. To study the stress tolerance mechanism induced by

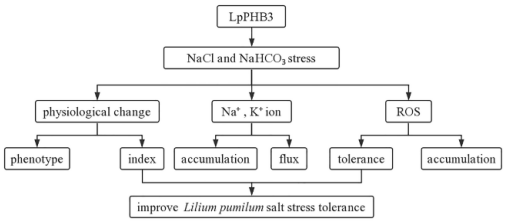

Fig. 13: Model of *LpPHB3* gene involvement in the stress response. Overexpressing *LpPHB3* changed the physiological index, regulated the content and flux of K^+ and Na^+, and repressed ROS accumulation under salt stress conditions to tolerate elevated salt stress

LpPHB3, we measured the physiological indices of wild-type and transgenic *L. pumilum*. The transgenic plants had higher chlorophyll and proline contents than the wild-type plants after stress treatment. Chlorophyll is the main pigment involved in photosynthesis, and proline is an important osmotic regulatory substance in plants (Delauney and Verma 1993; Cen *et al*. 2016; Kandoi *et al*. 2018). The results showed that compared to the wild-type lines, the transgenic lines could maintain a higher chlorophyll content to reduce photosynthetic system damage and a higher proline content to avoid excessive water loss caused by stress. MDA levels reflect the extent of membrane damage (Draper and Hadley 1990). The MDA content in *LpPHB3* transgenic *L. pumilum* was significantly lower than that in wild-type *L. pumilum* after stress, which indicates less damage to the transgenic plant membrane than to the wild-type plant membrane.

The degree of electrolyte leakage was used to evaluate abiotic stress tolerance, and the electrolyte leakage of the transgenic plants was lower than that of the wild-type plants, which indicated that stress-induced impairment of the transgenic lines was less pronounced than that of the wild-type lines.

Salinity stress causes K^+ deficiency (Maathuis and Amtmann 1999). Na^+ and K^+ have similar binding sites, and Na^+ competes with K^+ in plants (Shabala and Cuin 2008). The transgenic plants exhibited a higher K^+/Na^+ ratio than the wild-type plants (Fig. 9).

LpPHB3 overexpression in *L. pumilum* activated a major salt tolerance mechanism through limiting the accumulation of Na^+ to a high concentration. To further understand the role of *LpPHB3* in K^+/Na^+ homeostasis, we used NMT to study stress-induced K^+ and Na^+ flux around the root tips of wild-type and transgenic plants; the results revealed that the net Na^+ efflux was higher, but K^+ efflux was lower in the transgenic lines than in the wild-type lines under 200 m*M* NaCl or 20 m*M* $NaHCO_3$ treatment (Fig. 10). These results suggested that *LpPHB3* is involved in the regulation of K^+/Na^+ homeostasis under salt stress. Stress can lead to increased ROS production and cause oxidative damage to cellular components (Mittler 2002; Aken *et al*. 2009; Aken *et al*. 2010).

To understand whether the LpPHB3 protein can eliminate ROS produced by stress or reduce damage to plants caused by excess ROS, the cellular O_2^- and H_2O_2 levels in transgenic plants and wild-type plants were assessed by DAB and NBT staining. The O_2^- and H_2O_2 levels were higher in the wild-type plants than in the transgenic plants under stress (Fig. 11). Free radical-induced damage to the transgenic plants was less pronounced than that to the wild-type plants, indicating that the excessive expression of *LpPHB3* improved the salt tolerance of the transgenic plants by increasing their antioxidant capacity.

Plants have a variety of antioxidant enzymes to balance ROS levels, preventing ROS from accumulating to toxic levels. These enzymes include ascorbate peroxidases (APXs) and catalases (CATs) (Jardimmesseder *et al*. 2018). The *APX* and *CAT* expression levels in transgenic plants were significantly higher than those in wild-type plants under the same stress conditions. *AOX* and *NDB* expression in plant mitochondria has been widely used as a model to study ROS stress. PHB protein levels were found to be upregulated in cultured tobacco cells with induced *AOX* expression, suggesting that PHB helps control the ROS content in the presence of *AOX* (Sieger *et al*. 2005). The loss of *AtPHB2* and *AtPHB6* resulted in the activation of other respiratory pathways (Piechota *et al*. 2015). We compared the levels of *AOX* and *NDB* transcripts in the transgenic plants and wild-type plants; based on the results, we speculate that *LpPHB3* is related to *AOX* and *NDB*. The overexpression of *LpPHB3* triggered the relative response to stress.

As shown by comparisons of the phenotype, physiological indices, ion storage and transportation, stress-related gene expression and the ROS content of plants overexpressing the *LpPHB3* gene and wild-type plants under adverse conditions, the resistance of the transgenic plant system to stress was obviously higher than that of the wild-type plant system. *LpPHB3* may directly or indirectly affect plant stress signals. Under saline-alkali stress, the expression of *LpPHB3* may induce the expression of other salt tolerance-related genes, which together upregulate, reduce or eliminate excessive ROS produced by saline-alkali stress, thereby improving the salt-alkali resistance and antioxidation capacity of the plant.The exact mechanism for *LpPHB3* participation in the stress response is not yet known, and we suggest the following model based on research results (Fig. 13): Overexpression of *LpPHB3* enhances the salt stress tolerance of transgenic *L. pumilum*, and this may be associated with (1) changes in physiological indexes, (2) improved K^+ and Na^+ homeostasis under salt stress, and (3) repression of ROS accumulation.

Conclusion

LpPHB3 is mainly expressed in bulbs of *L. pumilum.* Through the comparison of transgenic and wild type

physiological indices, Na^+ and K^+ accumulation, and ROS content, transgenic plants improved salt and oxidative resistance than wild type.

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