



**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 mM NaHCO<sub>3</sub>. 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<sup>+</sup>/Na<sup>+</sup> homeostasis, the K<sup>+</sup>/Na<sup>+</sup> 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*, *AOX1a* and *NDB1*) 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 mM NaCl, 300 mM NaHCO<sub>3</sub> or 2 M H<sub>2</sub>O<sub>2</sub> 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; Prosevičius 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<sub>2</sub>O<sub>2</sub> 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 mM NaHCO<sub>3</sub> 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<sup>+</sup> and Na<sup>+</sup> between transgenic plants and wild type plants under stress were compared. The

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 mM NaCl, 30 mM, 15 mM H<sub>2</sub>O<sub>2</sub> 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-BamHI*F and *LpPHB3-SacI* 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 mM NaCl, 20 mM NaHCO<sub>3</sub> or 20 mM H<sub>2</sub>O<sub>2</sub> or without for 48 h to observe the leaf phenotypic characteristics.

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 solution of 300 mM NaCl, 300 mM NaHCO<sub>3</sub> or 2 M H<sub>2</sub>O<sub>2</sub> 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 mM NaCl, 20 mM NaHCO<sub>3</sub> or 20 mM H<sub>2</sub>O<sub>2</sub> 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 described 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<sup>+</sup> and Na<sup>+</sup> content in *L. pumilum* under stresses

Two-month-old wild type and transgenic *L. pumilum* were cultured in 200 mM NaCl or 20 mM NaHCO<sub>3</sub> or no stress MS medium for 48 h. The dried bulbs were digested with HNO<sub>3</sub> and HClO (87:13, v/v) then 2.5% HNO<sub>3</sub> diluted, and the ion content was measured by atomic absorption spectrophotometry (AA800, Perkin Elmer, USA).

### Noninvasive microtest technology (NMT) measured net K<sup>+</sup> and Na<sup>+</sup> flux

Two-month-old wild type and transgenic seedlings were transplanted onto MS medium either with 200 mM NaCl or 20 mM NaHCO<sub>3</sub> or without for 48 h. NMT (Younger USA LLC, Amherst, USA), 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 mM NaCl, 20 mM  $NaHCO_3$  or 20 mM  $H_2O_2$  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 mM NaCl, 20 mM  $NaHCO_3$  or 15 mM  $H_2O_2$  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

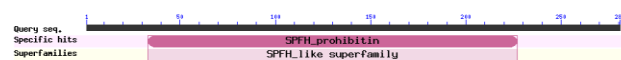
The *LpPHB3* ORF containing 846 bp, encoding 281 amino acids, was obtained from *L. pumilum* cDNA. Conserved domain analysis revealed that the protein sequence possessed the conserved PHB domains (Fig. 1). From the alignment of the *LpPHB3* deduced amino acid sequence, the *LpPHB3* protein had the highest 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 mM 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 mM  $NaHCO_3$  stress (Fig. 4C). The expression of *LpPHB3* remained constant for 12 h and

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

Name	Sequence (5'-3')	Length (bp)
<i>LpPHB3</i> -F	ATGGGCTCCAACCCCAAGC	846 bp
<i>LpPHB3</i> -R	TCACCGTCCTGCGGTGTGA	
<i>LpPHB3-BamHI</i> F	GGATCCATGGGCTCCAACCCCA	858 bp
<i>LpPHB3-SacI</i> R	CCGCGGTCATTTGACGGTGCAT	
<i>PBI121</i> -F	TCATTTTCATTTGGAGAGAACAC	1000bp
<i>PBI121</i> -R	TTGCCAAATGTTTGAACGATC	
<i>lilyActin</i> -F	GCATCACACCTTCTACAACG	286 bp
<i>lilyActin</i> -R	GAAGAGCATAACCCCTCATAGA	
<i>qLpAPX</i> -F	GTTGTTGCCGTGGAAGTGAC	226 bp
<i>qLpAPX</i> -R	CCTCATAGCCTGACCCGTCC	
<i>qLpCAT</i> -F	TGTGCTGATTTTCATGCGTGC	292 bp
<i>qLpCAT</i> -R	GGCTTCCGGATGGTGAGAA	
<i>qLpAOX</i> -F	ACAAGCTCGCGTTTTGGATG	263 bp
<i>qLpAOX</i> -R	GCGTTCGTACCATCTAGTGT	
<i>qNDB</i> -F	GCACGTAGCATTGTTGAGCC	239 bp
<i>qNDB</i> -R	TGACAATGCTCTCCACACC	



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

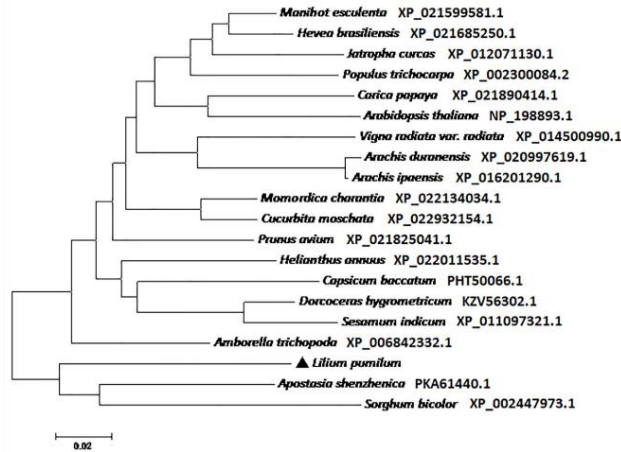


**Fig. 2:** Alignment of the *LpPHB3* deduced amino acid sequence with PHB3 proteins from other plant species. The amino acid sequence of this transcript was similar to that of the HaPHB3 protein (XP\_022011535.1, 83.33%) from *Helianthus annuus*, MePHB3 protein (XP\_021599581.1, 83.27%) from *Manihot 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*

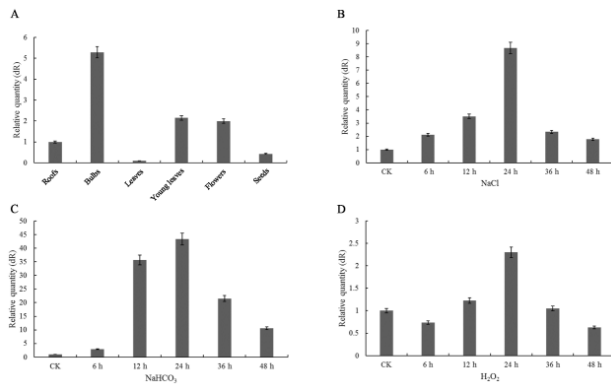
then reached its highest at 24 h, approximately 2.4 times the *LpPHB3* expression in the control group under 20 mM  $H_2O_2$  stress (Fig. 4D).

### 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

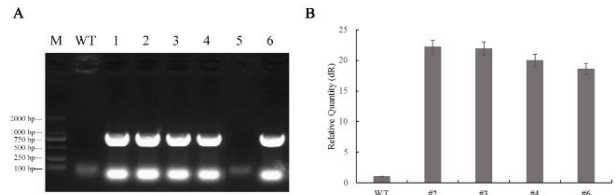


**Fig. 4:** Organ distribution of *LpPHB3* expression in *L. pumilum* and detection of *LpPHB3*-relative expression under the stresses. (A). *LpPHB3* relative expression in different organs of *L. pumilum*. (B). Relative expression of the *LpPHB3* gene under 200 mM NaCl treatment. (C). Relative expression of the *LpPHB3* gene under 20 mM NaHCO<sub>3</sub> treatment. (D). Relative expression of the *LpPHB3* gene under 20 mM H<sub>2</sub>O<sub>2</sub> treatment. No treatment (CK= 0) was used as the control

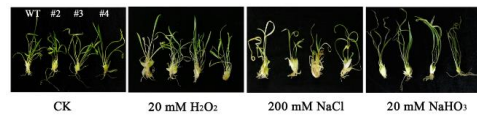
were selected for qPCR, and the expression of *LpPHB3* in transgenic plants was higher than that in wild type plants. As shown in Fig. 5B, transgenic lines #2, #3 and #4 had higher *LpPHB3* expression levels and were selected for further research.

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

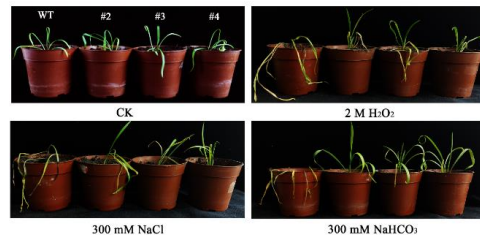
The leaves showed signs of wilt under different levels of stress for 2 days-4 days. The leaves of the transgenic plants were green, while those of the wild-type plants were yellow under treatment with 200 mM NaCl, 20 mM NaHCO<sub>3</sub> or 20 mM H<sub>2</sub>O<sub>2</sub> which indicated the increased resistance of the transgenic plants compared to the nontransgenic plants (Fig. 6). The effects of NaCl, NaHCO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> on the



**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<sub>2</sub>O<sub>2</sub>, 200 mM NaCl, or 20 mM NaHCO<sub>3</sub> or without (CK). WT, wild type; #2, #3, #4, transgenic lines

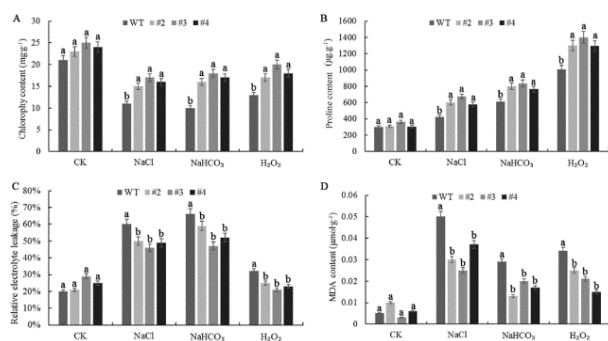


**Fig. 7:** Relative stress tolerance of wild type and transgenic plants (#2, #3, #4) at the spot culture stage. Plants were grown on soil supplemented either with 20 mM H<sub>2</sub>O<sub>2</sub>, 200 mM NaCl, or 20 mM NaHCO<sub>3</sub> or without (CK). WT, wild type; #2, #3, #4, transgenic lines

transgenic 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 mM NaCl, 300 mM NaHCO<sub>3</sub> or 2 M H<sub>2</sub>O<sub>2</sub>, 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·g<sup>-1</sup>) and transgenic plants (22.98 mg·g<sup>-1</sup>, 25.07 mg·g<sup>-1</sup>, and 24.13 mg·g<sup>-1</sup>) 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<sub>3</sub> or 20 mM H<sub>2</sub>O<sub>2</sub> 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  $\pm$  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<sup>-1</sup>, 17.04 mg·g<sup>-1</sup>, and 15.84 mg·g<sup>-1</sup>, while that in the wild type leaves was 10.31 mg·g<sup>-1</sup> under 200 mM NaCl. The chlorophyll levels in the transgenic leaves were 16.10 mg·g<sup>-1</sup>, 17.57 mg·g<sup>-1</sup>, and 16.33 mg·g<sup>-1</sup>, while that in the wild type leaves was 10.15 mg·g<sup>-1</sup> under 20 mM NaHCO<sub>3</sub>. The chlorophyll levels in the transgenic leaves were 16.34 mg·g<sup>-1</sup>, 18.72 mg·g<sup>-1</sup>, and 17.32 mg·g<sup>-1</sup>, while that in the wild type leaves was 13.01 mg·g<sup>-1</sup> under 20 mM H<sub>2</sub>O<sub>2</sub> treatment (Fig. 8A). Under control conditions, the proline content of the wild type (300 μg·g<sup>-1</sup>) and transgenic plants (310 μg·g<sup>-1</sup>, 364 μg·g<sup>-1</sup>, and 305 μg·g<sup>-1</sup>) was similar. The proline contents of the transgenic plants were 604 μg·g<sup>-1</sup>, 674 μg·g<sup>-1</sup>, and 578 μg·g<sup>-1</sup>, while that of the wild type plants was 425 μg·g<sup>-1</sup> under 200 mM NaCl. The proline contents of the transgenic plants were 800 μg·g<sup>-1</sup>, 836 μg·g<sup>-1</sup>, and 763 μg·g<sup>-1</sup>, while that of the wild type plants was 610 μg·g<sup>-1</sup> under 20 mM NaHCO<sub>3</sub>. The proline content of transgenic plants was 1300 μg·g<sup>-1</sup>, 1402 μg·g<sup>-1</sup>, and 1294 μg·g<sup>-1</sup>, while that of the wild type plants was 1006 μg·g<sup>-1</sup> under 20 mM H<sub>2</sub>O<sub>2</sub> (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 mM 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 mM NaHCO<sub>3</sub>. The electrolyte leakage levels in the transgenic plants were 25, 21 and 23%, while that in the wild type plants was 32% under 20 mM H<sub>2</sub>O<sub>2</sub> (Fig. 8C).

The MDA content of the wild type (0.005 mol·g<sup>-1</sup>) and transgenic plants (0.01 mol·g<sup>-1</sup>, 0.003 mol·g<sup>-1</sup>, and 0.006

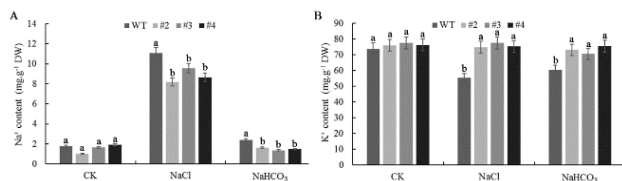
mol·g<sup>-1</sup>) was similar under no stress conditions. Then, the MDA content in the wild type and transgenic plants increased under stress. The MDA content of the transgenic plants was 0.03 mol·g<sup>-1</sup>, 0.025 mol·g<sup>-1</sup>, and 0.037 mol·g<sup>-1</sup>, while that of the wild type plants was 0.05 mol·g<sup>-1</sup> under 300 mM NaCl. The MDA content of the transgenic plants was 0.013 mol·g<sup>-1</sup>, 0.02 mol·g<sup>-1</sup>, and 0.017 mol·g<sup>-1</sup>, while that of wild type plants was 0.029 mol·g<sup>-1</sup> under 20 mM NaHCO<sub>3</sub>. The MDA content of the transgenic lines was 0.025 mol·g<sup>-1</sup>, 0.021 mol·g<sup>-1</sup> and 0.015 mol·g<sup>-1</sup>, while that of the wild type lines was 0.034 mol·g<sup>-1</sup> under 20 mM H<sub>2</sub>O<sub>2</sub> treatment for 24 h (Fig. 8D).

### Na<sup>+</sup>, K<sup>+</sup> accumulation and Na<sup>+</sup>, K<sup>+</sup> flux

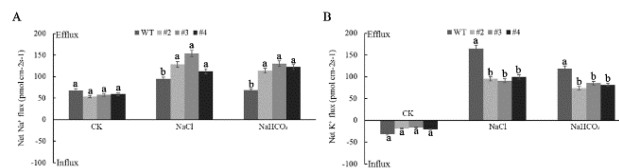
There was no significant difference in Na<sup>+</sup> content between wild type root systems (1.763 mg·g<sup>-1</sup>) and transgenic root systems (0.999 mg·g<sup>-1</sup>, 1.635 mg·g<sup>-1</sup>, 1.1925 mg·g<sup>-1</sup>) under normal culture conditions. However, Na<sup>+</sup> accumulation in the roots of plants increased when exposed to stresses; the Na<sup>+</sup> content in the transgenic plants increased to 7.886 mg·g<sup>-1</sup>, 9.236 mg·g<sup>-1</sup> and 8.325 mg·g<sup>-1</sup>, which was apparently lower than that in wild type, 12.082 mg·g<sup>-1</sup>, under 200 mM NaCl stress. The Na<sup>+</sup> content in the transgenic plants increased to 1.626 mg·g<sup>-1</sup>, 1.356 mg·g<sup>-1</sup>, and 1.466 mg·g<sup>-1</sup>, which was apparently lower than that in wild type, 2.004 mg·g<sup>-1</sup>, under 20 mM NaHCO<sub>3</sub> stress (Fig. 9A).

The K<sup>+</sup> levels in the roots of the wild type (73.717 mg·g<sup>-1</sup>) and transgenic plants (75.912 mg·g<sup>-1</sup>, 77.463 mg·g<sup>-1</sup>, and 76.235 mg·g<sup>-1</sup>) were similar under normal culture conditions. The transgenic plants had significantly higher K<sup>+</sup> contents (74.781 mg·g<sup>-1</sup>, 77.436 mg·g<sup>-1</sup>, and 75.246 mg·g<sup>-1</sup>) than the wild type plants (55.260 mg·g<sup>-1</sup>) under 200 mM NaCl stress (Fig. 9B). The transgenic plants had significantly higher K<sup>+</sup> contents (73.979 mg·g<sup>-1</sup>, 70.464 mg·g<sup>-1</sup> and 75.426 mg·g<sup>-1</sup>) than the wild type plants (60.414 mg·g<sup>-1</sup>) under 20 mM NaHCO<sub>3</sub> stress (Fig. 9B). Under 200 mM NaCl or 20 mM NaHCO<sub>3</sub> stress, the K<sup>+</sup>/Na<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> in the roots of the transgenic plants was significantly higher under 200 mM NaCl or 20 mM NaHCO<sub>3</sub> treatment for 24 h (Fig. 10A). We investigated the K<sup>+</sup> flux in plants, and salt shock induced K<sup>+</sup> efflux under 200 mM NaCl or 20 mM NaHCO<sub>3</sub> treatment. The average rate of K<sup>+</sup> 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<sup>+</sup>/Na<sup>+</sup> homeostasis under salt stress. In the figure, the positive values indicate outflows and the negative values indicate inflows.



**Fig. 9:** Na<sup>+</sup> and K<sup>+</sup> content in wild type and transgenic lines. Two-month-old seedlings were grown on MS medium either with 200 mM NaCl or 20 mM NaHCO<sub>3</sub> or without, and samples were harvested 24 h later. **A.** Na<sup>+</sup> contents in the roots of plants. **B.** K<sup>+</sup> contents in the roots of plants. DW, dry weight



**Fig. 10:** Net K<sup>+</sup> and Na<sup>+</sup> 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 mM NaCl or 20 mM NaHCO<sub>3</sub> 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<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>) production in plant leaves under stresses

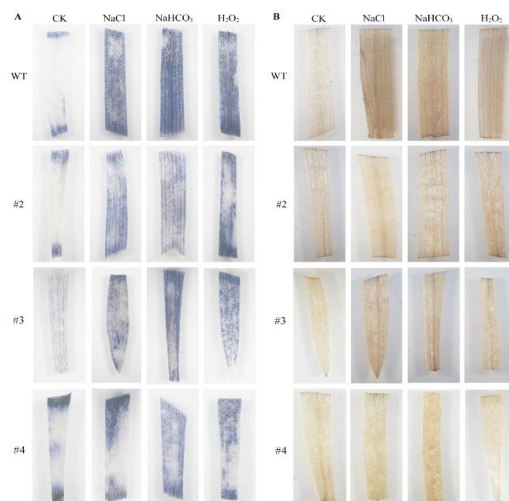
The stained leaf color of the wild type and transgenic plants showed no difference under no stress (Fig. 11). The wild type plant leaves showed a darker blue than the transgenic plant leaves under stresses (200 mM NaCl, 20 mM NaHCO<sub>3</sub> and 20 mM H<sub>2</sub>O<sub>2</sub>) for 24 h (Fig. 11A). The wild type plant leaves showed darker brown staining than the transgenic plant leaves under stresses (200 mM NaCl, 20 mM NaHCO<sub>3</sub> and 20 mM H<sub>2</sub>O<sub>2</sub>) for 24 h (Fig. 11B). This showed that more intercellular O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> accumulated in the leaves of the wild type plants than in the transgenic plants.

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

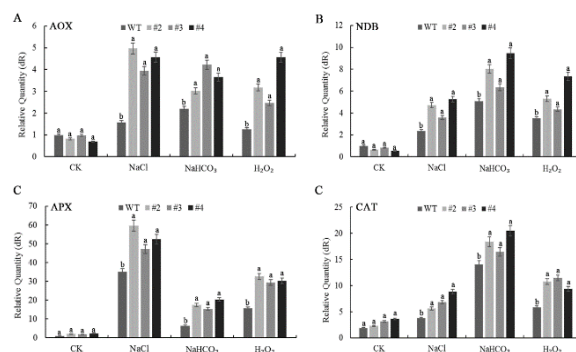
Under normal conditions, ROS stress-related gene (*AOX*, *NDB*, *APX*, *CAT*) expression levels were very low, and no significant difference was observed in any of the plants. However, all four gene transcripts were increased under stress (Fig. 12).

### 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<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> accumulation in leaves under 200 mM NaCl, 20 mM NaHCO<sub>3</sub>, and 20 mM H<sub>2</sub>O<sub>2</sub>. **A.** Detection of O<sub>2</sub><sup>-</sup> accumulation with NBT. **B.** Detection of H<sub>2</sub>O<sub>2</sub> accumulation with DAB

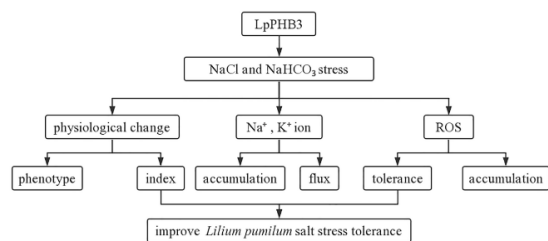


**Fig. 12:** Expression of stress-responsive genes in wild type and transgenic lines. **A.** Relative expression of the *AOX* gene under stress treatment. **B.** Relative expression of the *NDB* gene under stress treatment. **C.** Relative expression of the *APX* gene under stress treatment. **D.** Relative expression of the *CAT* gene under stress treatment. CK, no treatment. NaCl, 200 mM NaCl; NaHCO<sub>3</sub>, 20 mM NaHCO<sub>3</sub>; H<sub>2</sub>O<sub>2</sub>, 20 mM H<sub>2</sub>O<sub>2</sub>

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 mM NaCl or 20 mM  $NaHCO_3$  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) (Jardimmeseder *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<sup>+</sup> and K<sup>+</sup> accumulation, and ROS content, transgenic plants improved salt and oxidative resistance than wild type.

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