

Overexpression of *VaPAT1*, a GRAS transcription factor from *Vitis amurensis*, confers abiotic stress tolerance in *Arabidopsis*

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Abstract

Key message *VaPAT1* functions as a stress-inducible GRAS gene and enhanced cold, drought and salt tolerance in transgenic *Arabidopsis* via modulation of the expression of a series of stress-related genes.

Abstract The plant-specific GRAS transcription factor family regulates diverse processes involved in plant growth, development and stress responses. In this study, *VaPAT1*, a GRAS gene from *Vitis amurensis* was isolated and functionally characterized. Sequence alignment and phylogenetic analysis showed that *VaPAT1* has a high sequence identity to *CmsGRAS* and *OsCIGR1*, which belong to PAT1 branch of GRAS family and function in stress resistance. The transcription of *VaPAT1* was markedly induced by stress-related phytohormone abscisic acid

(ABA) and various abiotic stress treatments such as cold, drought and high salinity, however, it was repressed by exogenous gibberellic acid (GA) application. Overexpression of *VaPAT1* increased the cold, drought and high salinity tolerance in transgenic *Arabidopsis*. When compared with wild type (WT) seedlings, the *VaPAT1*-overexpression lines accumulated higher levels of proline and soluble sugar under these stress treatments. Moreover, stress-related genes such as *AtSIZ1*, *AtCBF1*, *AtATR1/MYB34*, *AtMYC2*, *AtCOR15A*, *AtRD29A* and *AtRD29B* showed higher expression levels in *VaPAT1* transgenic lines than in WT *Arabidopsis* under normal growth conditions. Together, our results indicated that *VaPAT1* functions as a positive transcriptional regulator involved in grapevine abiotic stress responses.

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Keywords GRAS transcription factor · *VaPAT1* · *Vitis amurensis* · Abiotic stress · Gibberellic acid (GA) · Stress-related genes

Abbreviations

GRAS	GAI (gibberellin acid insensitive), RGA (repressor of GAI), and SCR (scarecrow)
PAT	Phytochrome A signal transduction
ABA	Abscisic acid
GA	Gibberellic acid
WT	Wild type
WRKY	DELLA name derived from the most prominent amino acid residues in the protein
SCL	Scarecrow-like
SHR	Short-root
Ls	Lateral suppressor
HAM	Hairy meristem
ROS	Reactive oxygen
CDS	Coding sequence

CIGR Chitin-inducible gibberellin-responsive
ICE Inducer of CBF expression

Introduction

Abiotic stresses, such as cold, drought and high salinity adversely affect plant growth and crop production (Shinozaki and Yamaguchi-Shinozaki 2000; Qiu and Yu 2009). In response to these environmental adversities, plants have developed a number of strategies to get better tolerance or resistance. One primary model of these strategies is to modulate the expression of genes involved in stress response pathways (Yang et al. 2015). Many transcription factors, such as DREB/CBF (dehydration responsive element/C-repeat binding factor), ERF (ethylene responsive factor), WRKY, bHLH (basic helix-loop-helix), MYB (myeloblastosis), MYC (myelocytomatosis) and NAC (NAM, ATAF1/2 and CUC2) families have been found to play critical roles in controlling the transcription of stress-responsive genes and therefore regulating plants adaptation to stresses separately or cooperatively (Singh et al. 2002). Overexpression of these transcription factors mentioned above could increase stress tolerance in corresponding plants (Yamaguchi-Shinozaki and Shinozaki 2006; Lindemose et al. 2013; Li et al. 2013).

GRAS transcription factor family comprises plant-specific proteins (Benfey et al. 1993). The acronym GRAS was named after the first three identified family members: GIBBERELLIN ACID INSENSITIVE (GAI), REPRESSOR of GA1 (RGA), and SCARECROW (SCR) genes (Pysh et al. 1999). Based on the 33 and 55 GRAS members from Arabidopsis and rice, respectively, the GRAS protein family can be divided into eight branches which include SCL9, SCR, DELLA, SHR, Ls, SCL4/7, PAT1 and HAM (Bolle 2004; Lee et al. 2008). GRAS proteins have been shown to be involved in various biological processes in plants such as root development (Benfey et al. 1993), axillary meristem initiation (Schumacher et al. 1999), shoot meristem maintenance (Helariutta et al. 2000), GA and phytochrome A signal transduction (Bolle et al. 2000; Boss and Thomas 2002; Fu et al. 2001; Heo et al. 2011). Moreover, recent studies have reported the participation of GRAS proteins in stress response in several species. For instance, *NtGRAS1* from tobacco was strongly induced by various stimulants that raise the intracellular reactive oxygen (ROS) levels (Czikkel and Maxwell 2007). *AtSCL14* is also involved in the activation of stress-inducible promoters and could increase plants tolerance to noxious chemicals (Fode et al. 2008). In addition, overexpressing of *PeSCL7* confers improved drought and salt tolerance in transgenic Arabidopsis plants (Ma et al. 2010),

while *OsGRAS23* could positively modulate drought tolerance in rice (Xu et al. 2015). However, the underlying mechanisms on how GRAS proteins are involved in stress response in plants are still largely unknown.

Vitis amurensis is a wild grapevine species with remarkable cold and drought tolerance and therefore widely used during cross breeding to increase the cold and drought tolerance for grapevine (Su et al. 2015; Li 2015). Previous study on whole genome transcriptome analysis of *V. amurensis* found that the expression of two GRAS genes was significantly increased under cold stress (Xin et al. 2013). The coding region of one of the GRAS genes, GSVIVT01014570001, was cloned from *V. amurensis*. Sequence alignment and phylogenetic analysis showed that the gene belongs to PAT1 branch of GRAS protein family, and thus we named it as *VaPAT1*.

In the present study, the expression pattern of *VaPAT1* induced by phytohormones ABA and GA, and subjected to various abiotic stresses was analyzed. Moreover, the *VaPAT1* overexpressed lines were constructed in Arabidopsis by the floral dip method to examine the effects of *VaPAT1* gene in response to cold, drought and high salinity stresses. To further understand the molecular mechanisms of enhanced stresses tolerance by *VaPAT1*, the expression levels of stress-related genes in *VaPAT1* transgenic Arabidopsis were tested. Together, the investigation increased the comprehensive understanding toward the function of *VaPAT1* in stress responses. These results suggest that *VaPAT1* play a positive role in plant stress responses and thus could be a promising candidate for commercial crop species stresses-tolerant trait improvement.

Materials and Methods

Plant materials and growth conditions

Tissue culture plantlets of *V. amurensis* (collected from Changbai Mountain in Jilin province in China) were grown on half-strength (1/2) Murashige and Skoog (MS) medium containing 1 % sucrose and 0.7 % agar in a growth chamber at constant 26 °C under a 16-h light/8-h dark photoperiod and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity.

Arabidopsis thaliana (ecotype Columbia Col-0) was used for gene transformation in this study. Seeds of Arabidopsis were vernalized at 4 °C in the dark for two days and then sown in same pots with equiponderate soil in a growth chamber at 22 °C under a 16-h light/8-h dark cycle and 60 % relative humidity.

Exogenous phytohormone and stress treatments

Forty-day-old grapevine plantlets with 5–6 totally expanded leaves were used for the expression analysis under

exogenous phytohormones and stress treatments. Phytohormones ABA and GA treatments were induced by carefully transferring plantlets to 1/2 MS liquid medium (without 0.7 % agar) containing 100 μ M ABA or 200 μ M GA. Moreover, leaves of the plantlets were separately sprayed with 100 μ M ABA or 200 μ M GA solutions. The control (no stress treatment) were transferred to liquid 1/2 MS medium with leaves sprayed with same amount of deionized water. For cold treatment, plantlets were transferred to a low-temperature chamber at 4 °C with a 16-h light/8-h darkness cycle. For drought and salt stress, roots of plantlets were immersed into liquid 1/2 MS medium containing 2.5 % PEG (polyethylene glycol) 6000 or 200 mM NaCl, respectively.

The shoot apex with the first fully expanded leaf was harvested at specific time after initiating the treatments. The collected samples at each time point has three independent replicates. Harvested samples were immediately frozen in liquid nitrogen and stored at -80 °C for later RNA extraction.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from collected samples using TIANDZ Column Plant RNAout 2.0 Kit (Tiandz, Beijing, China) following the manufacturer's procedure. A maximum of 1 μ g total RNA was used for synthesizing cDNA by TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China). The qRT-PCR experiment was performed on a StepOnePlus™ realtime PCR Instrument (Applied Biosystems) using FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany). The β -actin gene (*VvACT*, GenBank accession: EC969944) and malate dehydrogenase gene (*VvMDH*, GenBank accession: EC921711) were used as reference genes to normalize the expression of *VaPAT1* in *V. amurensis*. While *AtACT2* (GenBank accession: BAH20120.1) was used to normalize the expression level of genes in Arabidopsis. Gene specific primer pairs for qRT-PCR (listed in Table 1) were designed by Primer 5.0 and their specificities were tested by NCBI Primer BLAST. Three biological replicates were performed to ensure the accuracy of results. The relative expression was estimated via the relative quantization method ($2^{-\Delta\Delta C_t}$).

The cDNA cloning and sequence analysis

Full coding sequence of *VaPAT1* was obtained by homologous cloning using a primer pair (*VaPAT1*CDS-F/R, Table 1) designed according to the putative cDNA sequence of GSVIVT01014570001 from the published 12 \times *Vitis vinifera* cv. Pinot Noir genome sequences

(<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>). The fragment was cloned into pGEMT-easy vector (Promega, WI, USA) and sequenced using ABI3730.

ExpASy server (<http://www.expasy.org/>) was applied to translate the confirmed CDS sequence of *VaPAT1* into amino acids and calculate the theoretical isoelectric point (pI) and molecular weight of *VaPAT1* protein. Alignment of amino acid sequences was performed with the software DNAMAN 6.0. The phylogenetic tree was generated by the MEGA program (v.5.0) using the neighbor-joining (NJ) method (Hall 2013).

Vector construction and Arabidopsis transformation

The full coding sequence of *VaPAT1* was amplified with primers containing restriction sites *Bam*HI and *Eco*RI (*VaPAT1*-BamH and *VaPAT1*-EcoR, Table 1). The amplified fragment was digested with *Bam*HI and *Eco*RI and inserted into the binary vector pCAMBIA 1301, which contains 35S CaMV (cauliflower mosaic virus) promoter. The expression vectors of *VaPAT1* were introduced into *Agrobacterium tumefaciens* GV3101, and then transformed into Arabidopsis by the floral dip method (Clough and Bent 1998). Seeds of WT and transgenic Arabidopsis were harvested, surface-sterilized with 2 % sodium hypochlorite for 5 min and then washed with sterile water for 3 times. Transgenic seedlings were selected on 1/2 MS medium containing hygromycin (50 mg/L). Homozygous T3 transgenic lines were further tested by hygromycin resistance screening and used for subsequent stress tolerance assay experiments.

Abiotic stress tolerance assays of transgenic Arabidopsis

WT and T3 generation transgenic Arabidopsis seedlings at 2-week-old stage were employed for detection of the cold, drought and salt stress tolerance. Cold stress was induced by transferring Arabidopsis seedlings inside a cold treatment incubator at -1 °C for 8 h to initiate freezing, followed by the temperature decreased at a rate of 2 °C/h from -1 to -9 °C, each temperature point (-3, -5, -7, -9 °C) was kept for 3 h, respectively. After that, seedlings thawed overnight at 4 °C and then incubated at 22 °C for recovery. Drought stress was imposed to previously adequately watered plants by withholding water for 12 days. Finally, seedlings were rewatered to analyze the recovery from drought stress. For salt treatment, 2-week-old seedlings were watered with 200 mM NaCl twice at 5-days intervals.

About 20 plants per line (WT, 35S::*VaPAT1*-1, -2 or -4) were used in each stress treatment experiment. The seedlings exhibiting >50 % green on their above-ground

Table 1 List of PCR primers used in this study

No.	Name	Oligonucleotides (5' ~ 3')	Note
P1	VaPAT1CDS-F	CCCATAGCATGGACTCGCGC	For acquisition of <i>VaPAT1</i> fragment
	VaPAT1CDS-R	GACTGTCAATACCAGGCCG	
P2	VaPAT1RT-F	TCCAACCTCAGCCATCCTTTTCT	For qRT-PCR analysis of <i>VaPAT1</i>
	VaPAT1RT-R	GAGCCTCACCTGGTCTCACATCC	
P3 ^a	VaPAT1-BamH	<u>GACGGATCCCC</u> ATAGCATGGACTCGCGC	For full-length cloning of <i>VaPAT1</i> and expression vector construction
	VaPAT1-EcoR	<u>CAGGAATTC</u> GACTGTCAATACCAGGCCG	
P4	VvACT-F	CTTGCATCCCTCAGCACCTT	For qRT-PCR analysis of the reference genes in grape
	VvACT-R	TCCTGTGGACAATGGATGGA	
P5	VvMDH-F	CCATGCATCATCACCCACAA	
	VvMDH-R	GTCAACCATGCTACTGTCAACC	
P6	AtACT2-F	TTACCCGATGGGCAAGTCA	For qRT-PCR analysis of the reference genes in Arabidopsis
	AtACT2-R	AAACGAGGGCTGGAACAAGA	
P7	AtSIZ1-F	AGGATGCAGTGGCCTCAGTATG	
	AtSIZ1-R	GCCGTTGACTCCAAAAGC	
P8	AtCBF1-F	GTTTGGGATGCCGACTTTGTGTTGG	
	AtCBF1-R	GTCACCATCTCCTTCGCCGTCAT	
P9	AtATR1-F	GCGGGACGAAGTACAACGAAA	
	AtATR1-R	GCGGTTAAGAAGCCTAGCGGAA	
P10	AtMYC2-F	CGATTGGAACACCTGGATCTAACG	
	AtMYC2-R	GGGTTTTTCGGTTATTGTGCTTGAG	
P11	AtCOR15A-F	AAAGAGGCATTAGCAGATGGTGA	
	AtCOR15A-R	TTTTCTTTCTCCTCCACATACG	
P12	AtRD29A-F	CAAACCTCAAGTGGCGGGAAGTGT	
	AtRD29A-R	AGGTAACCTCGTCGTCACGGCAG	
P13	AtRD29B-F	TCCGACAAGAGGTGATGTGAAAGTAG	
	AtRD29B-R	GCTAACTGCTCTGTGTAGGTGCTTGG	
P14	AtProDH1-F	ACACATAACGCTGATTCGGGGAG	
	AtProDH1-R	GATACGGTATAGCGGTTGCGACG	

^a The underlined bases in primers are the restriction sites

tissues were judged as surviving plants. Survival rates of WT and transgenic Arabidopsis were calculated based on three independent repeats for each stress test (about 60 plants for per line).

Measurement of proline and soluble sugar contents

Leaves sampled from normal and stresses-treated WT and transgenic Arabidopsis were used for determination of proline and soluble sugar contents. Proline was assayed as described by Bates et al. (1973) with some modifications. Briefly, fresh leaf material (200 mg) of Arabidopsis was extracted with 3 ml of 3 % sulfosalicylic acid at 100 °C for 10 min. Thereafter, 2 ml of the extract was heated at 100 °C for 30 min mixing with same volume of glacial acetic acid and acid ninhydrin reagent. After cooling, 3 ml toluene was

added to partition the mix, and the absorbance of the supernatant extract containing proline was measured at 520 nm. The soluble sugar content was determined by an anthrone-sulfuric acid method (Dubois et al. 1956). Absorbance of proline and soluble sugar hereby were all measured using a Tecan Infinite M1000 PRO Multiskan Spectrum Microplate Spectrophotometer (TECAN, Austria).

Results

VaPAT1 encodes a GRAS protein that belongs to the PAT1 branch

Full coding sequence (CDS) of the *VaPAT1* was cloned from *V. amurensis* leaf cDNA using gene-specific primers

VaPAT1CDS-F and VaPAT1CDS-R (Table 1). The length of the nucleotide sequence of *VaPAT1* was 1752 bp, encoding a protein of 583 amino acid residues with a calculated molecular mass of 65.0 kDa and a pI (predicted theoretical isoelectric point) of 5.75. Alignment of *VaPAT1* with their homologues derived from other plant species showed that it contained a variable N-terminal and a conserved C-terminal domain similar to other members of the GRAS family. The conserved C-terminal part contains the typical motifs defined for GRAS proteins, namely two leucine-rich (LR) domains flanking a conserved V/I HIID domain (Fig. 1a). Furthermore, the motifs LXXLL, PFYRE, RVER and SAW were highly conserved. A phylogenetic tree based on the conserved domains was constructed. The result confirmed that VaPAT1 protein is a member of the PAT1-branch of the GRAS protein family (Fig. 1b). In the PAT1-branch, VaPAT1 presented close evolutionary relationship with CmsGRAS from *Citrus medica* and OsCIGR1 from *Oryza sativa* (74 and 52 % identities, respectively) (Shi et al. 2011; Day et al. 2003). Both CmsGRAS and OsCIGR1 were previously identified as positive components that functioned in stress resistance, which implies that *VaPAT1* may also have a similar role in stress-responsive processes.

***VaPAT1* responded to exogenous ABA and GA applications and could be induced by cold, drought and high salinity treatments**

ABA is a representative stress-related phytohormone, with a role in the regulation of stomatal aperture and genes expression when plants were subjected to environmental stress factors (Hubbard et al. 2010; Lindemose et al. 2013). GRASs were previously reported to be involved in GA signal transduction pathway. Upon GA application, DELLA-branch GRAS proteins bind to *GID1* (GA receptor) to form a GA-*GID1*-DELLA complex, thus induces the degradation of DELLAs via the ubiquitin proteasome pathway (Silverstone et al. 1998, 2001; Fu et al. 2001; Heo et al. 2011). The inducible expression profiles of *VaPAT1* in *V. amurensis* by exogenous ABA and GA applications were hereby investigated. The expression of *VaPAT1* was quickly induced by ABA application with an early peak at 2 h and the second peak at 24 h after the plantlets were subjected to ABA application, which suggests that *VaPAT1* might function via an ABA-dependent pathway (Fig. 2). On the contrary, the expression of *VaPAT1* was reduced gradually within the first 3 h (with a minimum level at 3 h, about one-third compared to the control), and then raised back to a relatively higher level (about two-thirds compared to the control) in the following time course after GA application (Fig. 2).

To confirm whether *VaPAT1* was involved in abiotic stress response in *V. amurensis*, its expression patterns under various stresses were investigated by real-time PCR. As shown in Fig. 2, the expression levels of *VaPAT1* were increased in all the stress conditions used. Under cold stress at 4 °C and drought stress simulated by PEG, *VaPAT1* expression was induced quickly and the level of the products peaked at 2 h or 4 h (about 5.5-fold or 3.4-fold that of the untreated control plants), respectively. As regard to high salinity treatment, the *VaPAT1* transcripts raised constantly and reached to a maximum at 48 h (up to 22-fold higher than that before salt treatment).

Overexpression of *VaPAT1* in Arabidopsis enhances cold, drought and salt tolerance

To examine the biological function of *VaPAT1*, transgenic Arabidopsis plants that overexpresses *VaPAT1* were constructed. Three independent homozygous transgenic lines of T3 transgenic plants (35S::*VaPAT1*-1, -2, and -4) with higher *VaPAT1* expression levels were selected to assess the cold, drought, or salt tolerance (Fig. S1). Moreover, there were no significant differences in morphological traits between the WT and transgenic plants during their life cycles.

After the cold treatment, all the leaves of WT and transgenic plants were flooded. Upon returning them back to normal growth conditions for five days, the leaves of most WT plants became dry and withered. However, most transgenic plants recovered and had green leaves (Fig. 3a1). The survival rates of transgenic lines were 78 % compared with 23 % of WT plants under cold stress. To determine their drought resistance, 2 weeks old WT and transgenic Arabidopsis plants were watered once deeply and then subjected to water withholding for an additional 12 days. The WT plants wilted more severely and only about 13 % plants could survive after rewatering. As regards *VaPAT1*-overexpressing lines, 72 % of plants recovered after rewatering (Fig. 3b). Moreover, the development of surviving WT plants was retarded by drought stress compared with *VaPAT1*-overexpressing lines (Fig. 3a2). *VaPAT1*-overexpressing lines bolted even during the drought stress treatment (Fig. 3a2). Similarly, after 200 μ M NaCl treatment for 10 days, the WT plants showed a much more severe etiolating phenotype than transgenic seedlings (Fig. 3a3). On average, 22 % of total WT plants were able to survive, whereas 71 % of transgenic lines continued growing and exhibited much bigger leaves than in normal growth conditions (Fig. 3b). These results indicated that the overexpression of *VaPAT1* could improve the tolerance to cold, drought and high salinity in Arabidopsis.

Fig. 1 Alignment and phylogenetic analysis of VaPAT1 with other GRAS proteins. **a** Alignment of amino acid sequences of VaPAT1 with other GRAS proteins. Sequences were from *Vitis vinifera* (VvPAT1, XP_002282942.1), *Arabidopsis thaliana* (AtPAT1, At5g48150), *Oryza sativa* (OsCIGR1, AAL61820.1 and OsCIGR2, Q8GVE1.1), *Citrus medica* var. *sarcodactylis* (CmsGRAS, JF440647.1), *Nicotiana tabacum* (NtGRAS1, ABE02823.1) and *Populus euphratica* (PeSCL7, AHZ13509.1). Conserved sequences are coloured. Gaps introduced to facilitate alignment are indicated as dashes. The five main conserved domains in the C-terminal are indicated with uppercase letters above the alignment. Asterisks mark the conserved Leucine residues in the leucine-rich domains flanking the VI/IIID motif. W(X)₇G, W(X)₁₀W, the two pairs of conserved residues in SAW domain is double-underlined. **b** Phylogenetic tree of the GRAS protein family. The red solid diamond represents VaPAT1. All Arabidopsis PAT1 branch proteins (AtPAT1; AtSCL5, At1g50600; AtSCL13, At4g17230; AtSCL21, At2g04890) as well as several stress-related proteins belong to PAT1-branch from other species (CmsGRAS, OsCIGR1, OsCIGR2) are presented. Representatives of other seven branches of the GRAS family are shown (AtSCL9, At2g37650; AtSHR, At4g37650; AtRGA, At2g01570; AtGAI, At1g14920; AtSCR, At3g54220; SILs, AAD05242.1; AtSCL4, At5g66770; AtSCL7, At3g50650; and PhHAM, AAM90848.1). *Sl* *Solanum lycopersicum*; *Ph* *Petunia × hybrid*

Overexpression of VaPAT1 led to significantly higher proline and soluble sugar contents in transgenic lines

To deeply uncover the physiological changes in transgenic Arabidopsis, several physiological index related to abiotic stress were measured in WT and transgenic plants. A total of six indexes including malondialdehyde (MDA),

superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), proline and soluble sugar contents were measured. The content of MDA, activity of SOD, POD and CAT did not show significant differences between WT and VaPAT1 transgenic plants either in normal growth conditions or exposed to stresses (Fig. S2). The proline concentration in overexpression lines was higher than that in WT plants under normal growth conditions (Fig. 4a), whereas soluble sugar contents in transgenic lines were similar to that in WT plants (Fig. 4b). When the seedlings were subjected to stresses including cold, drought and salt, the proline and soluble sugar contents increased distinctly in both VaPAT1-overexpression and WT plants. Meanwhile, VaPAT1-overexpression lines accumulated more proline and soluble sugar than in WT plants. Overall, these findings indicated that overexpression of VaPAT1 led to the increase of the proline and soluble sugar contents, which were two important factors for the enhanced cold, drought and salt stress tolerance in transgenic Arabidopsis.

Altered expression of stress-related genes in VaPAT1-overexpression lines

The expression profiles of a set of stress-related genes (Table S1) were analyzed to further elucidate the possible molecular mechanisms underlying enhanced stress tolerance by VaPAT1. Under normal conditions, the test genes including *AtSIZ1*, *AtCBF1*, *AtATRI1/MYB34*, *AtMYC2*, *AtCOR15A*, *AtRD29A* and *AtRD29B* showed significantly higher expression levels in VaPAT1 transgenic lines

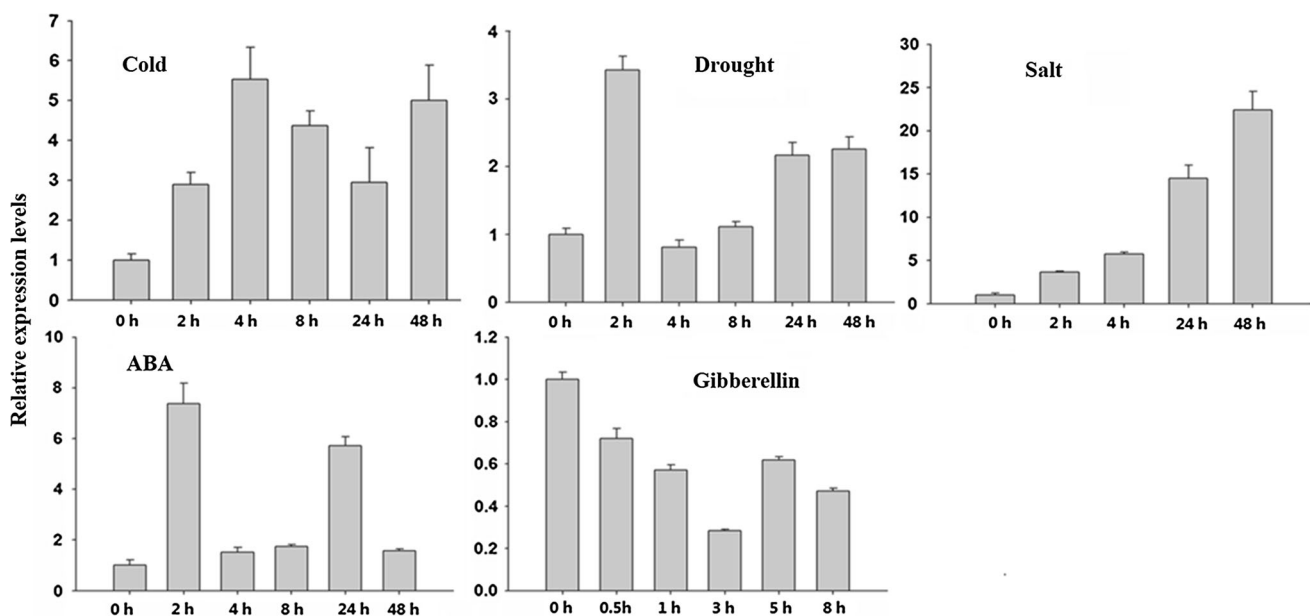


Fig. 2 Expression patterns of VaPAT1 in response to cold (4 °C), drought (2.5 % PEG6000), salt (200 mM NaCl), 100 μM ABA and 200 μM GA treatments. Error bars refer to three biological replicates.

The expression level of VaPAT1 in the controls was set at 1.0. The relative expression level of VaPAT1 in the control plant without treatment (0 h) was defined as 1 to evaluate the dynamic changes

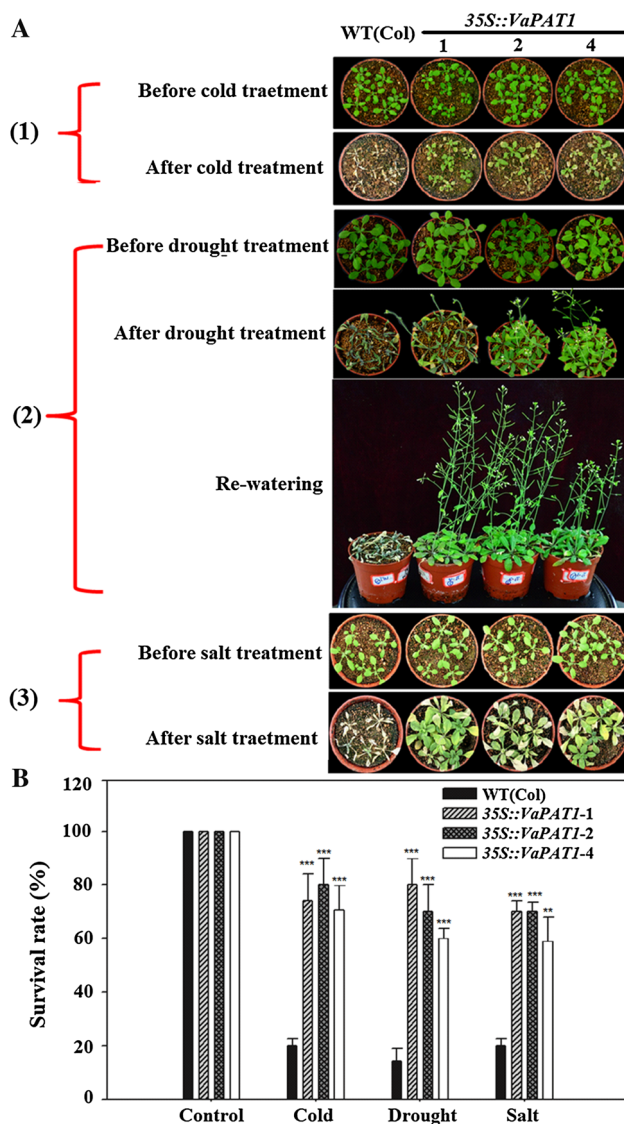


Fig. 3 Stress tolerance assays of WT and *VaPAT1*-overexpressing Arabidopsis plants: **a** Phenotypic differences between WT and transgenic Arabidopsis seedlings under cold, drought and salt stresses. **1** Cold treatment. Two weeks old seedlings grown at 22 °C under long days (16-h light/8-h dark) were incubated at −1 °C for 8 h, then followed by a gradient freezing process decreasing at a rate of −2 °C/h to −9 °C, each temperature point kept for 3 h. Seedlings were photographed after 5 days recovery at normal growth conditions (22 °C). **2** Drought treatment. Two weeks old WT and transgenic Arabidopsis were subjected to water withholding for 12 days then seedlings were rewatered for recovery. Photographs were taken 5 days after rewatering. **3** Salt treatment. Two weeks old seedlings were watered with 200 mM NaCl twice at an interval of 5 days. Photographs were taken 10 days after salt stress treatment. **b** Survival rate of WT and *VaPAT1*-overexpressing lines after stresses treatments. Control plants without stresses have a survival rate of nearly 100 %. Error bars represent standard deviation of three biological replicates, 20 plants per line (WT, 35S::VaPAT1-1, -2 or -4) in each experiment. ** and *** indicate significant differences between WT and *VaPAT1*-overexpressing lines at $P < 0.01$ and $P < 0.001$ level (Student's *t* test), respectively

compared to that of WT plants as shown in Fig. 5. These results suggest that *VaPAT1* could affect the expression of those genes. Moreover, *AtProDH*, a gene encoding a proline dehydrogenase was significantly down-regulated in *VaPAT1*-overexpression lines, which may partly confer to the higher proline contents in *VaPAT1*-overexpression lines even under normal growth conditions (Fig. 4a).

Discussion

In the present study, *VaPAT1*, a GRAS gene that participates in the cold, drought and high salinity stresses responses in *V. amurensis*, has been characterized. Further phylogenetic analysis demonstrated that VaPAT1 has high sequences similarity with CmsGRAS and OsCIGR1 and belongs to PAT1 branch of GRAS proteins. *CmsGRAS* from *Citrus medica* was significantly up-regulated under low-temperature stress (Shi et al. 2011). *OsCIGR1* was rapidly induced upon co-cultivation with rice blast fungus and N-acetylchitooligosaccharide perception, functioning in the early defense response in rice (Day et al. 2003). Other members such as *OsCIGR2* and *AtSCL13*, which also belongs to the PAT1 branch, were reported to have a role in stress responses (Day et al. 2003; Torres-Galea et al. 2006). In addition to PAT1 branch, genes from other branches of the GRAS family such as *PeSCL7* (SCL4/7-branch) in poplar (*Populus euphratica* Oliv.) and *OsGRAS23* (Ls-branch) in rice, were also shown to improve stress tolerance in corresponding overexpressed lines (Ma et al. 2010; Xu et al. 2015). Moreover, *AtSCL14*, a gene in SCL9 branch, were reported to interact with Class II TGA (TGACG MOTIF-BINDING FACTOR) transcription factors to activate the expression of genes involved in the detoxification of harmful chemicals (Fode et al. 2008). These results therefore indicate that GRAS gene family members widely participate in abiotic stress related signal transduction pathways in plants.

A promoter prediction of *VaPAT1* using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) showed it contains many stress and phytohormone responsive cis-acting regulatory elements, including the defense and stress responsiveness element, heat stress responsive element, anaerobic inducible element, GA and SA (salicylic acid) responsive element (data not shown). These motifs may be responsible for the dynamic transcription of *VaPAT1* during stress conditions and exogenous hormones applications. The expression of *VaPAT1* decreased under GA application, similar to a typical feature of DELLA-branch GRAS proteins (Silverstone et al. 1998; Wen and Chang 2002). Previous studies demonstrated that

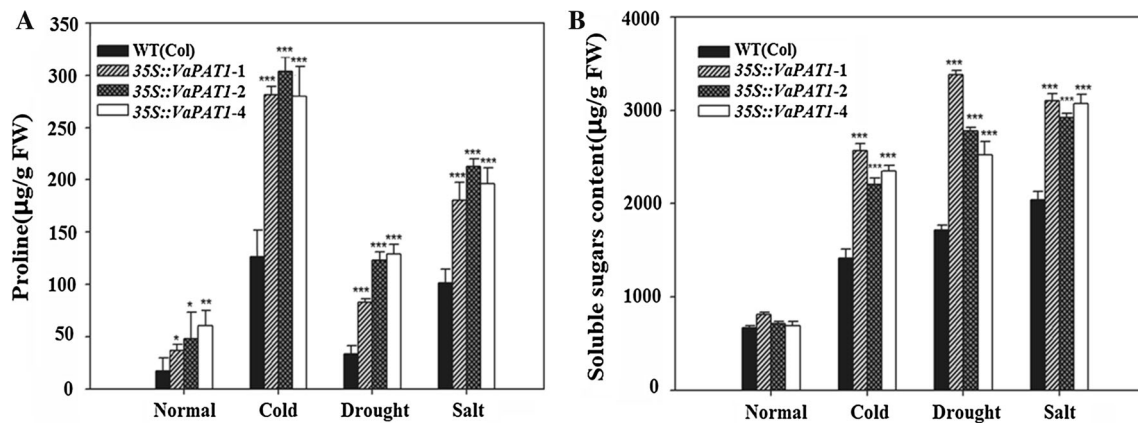


Fig. 4 Proline and soluble sugar contents of WT and *VaPATI*-overexpression Arabidopsis with or without stress treatments. **a** Proline content. **b** Soluble sugar content. Normal: three-week-old plants grown under normal conditions; Cold treatment: incubating seedlings at 4 °C for 3 days; Drought treatment: withholding water for 8 days;

Salt treatment: irrigated with 200 mM NaCl for a week. The bars represent standard deviation of three biological replicates. *, ** and *** indicate significant differences between WT and *VaPATI*-overexpressing lines at $P < 0.05$, $P < 0.01$ and $P < 0.001$ level based on Student's *t* test, respectively

high level expression of *DELLAs* confer reduced GA responses associated with dwarfism phenotypes (Fu et al. 2001; Boss and Thomas 2002; Achard et al. 2008). In this study however, *VaPATI*-overexpression Arabidopsis exhibited no phenotype differences compared with WT under normal growth conditions. In accordance with our findings is that, the expression of *PeSCL7* (a member of the SCL4/7-branch GRASs) decreased under GA application, but *PeSCL7*-overexpression Arabidopsis exhibited normal phenotypes (Ma et al. 2010). These results implied that *VaPATI* and *PeSCL7* may be involved in GA response pathway, but in a way different from that of *DELLAs*.

Proline and soluble sugar are common compatible osmolytes to protect cell membrane system from detrimental effects of abiotic stresses, which are widely used as physiological indicators estimating cellular damage upon various stresses (Ashraf and Foolad 2007; Karan and Subudhi 2012; Yu et al. 2013). The contents of proline and soluble sugar increased in both *VaPATI*-overexpression and WT Arabidopsis plants under various stresses, but accumulated significantly higher in *VaPATI*-overexpression lines than in WT. Moreover, it was found that the effect of *VaPATI* overexpression on the accumulation of proline was different from that on soluble sugar. Prior to subjection to stresses, transgenic Arabidopsis contained higher proline than WT plants, which suggests that overexpression of *VaPATI* could increase the proline content. With regard to soluble sugar, the increased content in overexpression lines was only found under stresses. This phenomenon indicates that soluble sugar maybe regulated by *VaPATI* with the help of other factors under stresses. Furthermore, *AtProDH* was found down-regulated in *VaPATI* transgenic Arabidopsis. This gene encodes proline dehydrogenase 1, an enzyme functioning in proline

metabolism process (Cecchini et al. 2011). Down-regulation of *AtProDH* may partly contribute to higher proline content in *VaPATI*-overexpression lines in normal growth conditions, which, in turn, resulted in increased stress tolerance in transgenic Arabidopsis.

In order to further study the molecular mechanisms underlying enhanced abiotic stress tolerance in *VaPATI* transgenic Arabidopsis, the expression levels of many stress-related genes were examined in WT and *VaPATI* transgenic Arabidopsis under normal growth conditions. From the real time RT-PCR analysis, among the examined genes, transcripts of seven genes, including *AtSIZ1*, *AtCBF1*, *AtATRI/MYB34*, *AtMYC2*, *AtCOR15A*, *AtRD29A* and *AtRD29B* were significantly higher in *VaPATI*-overexpression lines than in WT (Fig. 5). *AtSIZ1* and *AtCBF1* are upstream transcriptional regulation factors in the ICE-CBF-COR pathway under cold stress (Yamaguchi-Shinozaki and Shinozaki 2006; Novillo et al. 2007; Miura and Nozawa 2014). *AtSIZ1* encodes a small ubiquitin-like modifier (SUMO) E3 ligase, which mediates sumoylation of ICE1, thereby enhancing the stability of ICE1, leading to increased induction of *CBFs* and its target *COR* genes (*COR15A* and *RD29A*) (Miura et al. 2007). In addition, a study carried out by Catala et al. (2007) showed that *AtSIZ1* plays a role in Arabidopsis drought tolerance through the regulation of a set of drought-responsive genes, including *AtATRI/MYB34*, *AtMYC2*, *AtCOR15A* and *AtRD29B*. These genes were probably up-regulated by *AtSIZ1* in *VaPATI*-overexpression Arabidopsis plants in our investigation. *AtATRI/MYB34* and *AtMYC2* also function as transcription factors in stress response processes (Abe et al. 1997). *AtATRI/MYB34* is a R2R3 MYB gene which can respond to various hormone applications and salt stress treatment (Chen et al. 2006). *AtMYC2*, which encodes a bHLH transcription factor, is involved in activating ABA-

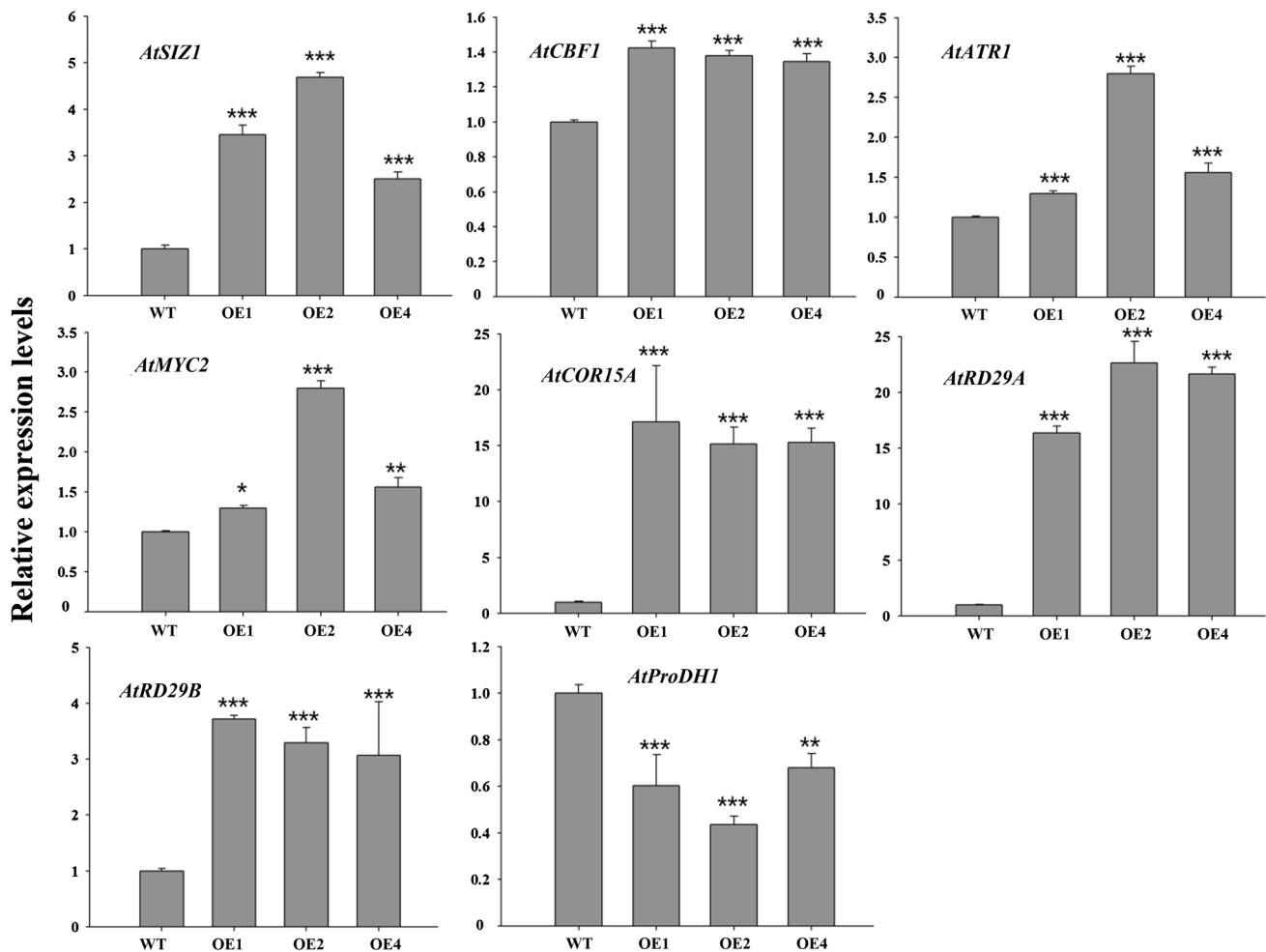


Fig. 5 Expression of stress-related genes in WT and *VaPAT1*-overexpression Arabidopsis plants under normal growth conditions. Total RNA was obtained from two-week-old WT and T3 generation *VaPAT1* transgenic seedlings (*35S::VaPAT1*-1, -2 and -4, which were also marked as OE1, OE2 and OE4 in the figure) without stress treatment. Expression values were calculated by real-time qRT-PCR using the gene-specific primers listed in Table 1. The putative downstream stress-related genes are *AtSIZ1* (AT5G60410), *AtCBF1*

(AT4G25490), *AtATRI/MYB34* (AT5G60890), *AtMYC2* (AT1G32640), *AtCOR15A* (AT2G42540), *AtRD29A* (AT5G52310), *AtRD29B* (AT5G52300) and *AtProDH1* (AT3G30775). Real-time RT-PCR quantifications were normalized to the expression of *AtACTIN2* (AT3G18780). Error bars indicate SD from three independent experiments. The asterisk *, ** and *** indicate significant differences between WT and *VaPAT1*-overexpressing lines at $P < 0.05$, $P < 0.01$ and $P < 0.001$ level based on Student's *t* test, respectively

inducible gene expression under drought stress (Abe et al. 2003; Nakata et al. 2013). It has been confirmed that *AtCOR15A*, *AtRD29A* and *AtRD29B* are downstream genes implicated in multiple stress responses (Thalhammer et al. 2014; Miyazaki et al. 2015; Kim et al. 2014) and regulated by various upstream transcription factors (Yamaguchi-Shinozaki and Shinozaki 2006). Taken together, *VaPAT1* triggered the constitutive expression of many stress-related genes and enhanced abiotic stresses in transgenic plants. These genes could be considered as direct or indirect downstream genes of *VaPAT1* and further works still need to uncover the *VaPAT1* related signal transduction during abiotic stress.

In summary, we obtained a GRAS transcription factor *VaPAT1* from *V. amurensis*. *VaPAT1* responds to exogenous ABA and GA, as well as cold, drought, and salt stress treatments. Ectopic expression of *VaPAT1* in Arabidopsis modulated the expressions of a series of stress-related genes, increased proline and soluble sugar contents and enhanced cold, drought and high salinity tolerance in *VaPAT1*-overexpression lines. Hence, it is concluded that *VaPAT1* is a novel stress-responsive GRAS gene playing positive roles in plants stress responses. This new gene identified can be used as a candidate gene in molecular breeding of commercial crop species to get better stress tolerance.

Author contribution statement YY and LF designed and conducted experiments. YY wrote the manuscript. LZ and YG contributed by helping some experiments presented in the manuscript. SKK helped to edit the manuscript. SL and HX supervised the studies and revised the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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