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ORIGINAL ARTICLE

Overexpression of *PSK1*, a *SKP1*-like gene homologue, from *Paeonia suffruticosa*, confers salinity tolerance in Arabidopsis

Qing Hao[3] · Hongxu Ren[1] · Jin Zhu[1,2] · Liangsheng Wang[1] · Shouchen Huang[3] · Zheng'an Liu[1] · Zhimin Gao[4] · Qingyan Shu[1]

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Abstract

Key message **Our study is the first to demonstrate that *PSK1*, a *SKP1*-like gene homologue, is involved in salinity tolerance. Our functional characterization of *PSK1* provides new insights into tree peony development.**

Abstract A homologous gene of S-phase kinase-associated protein1 (*SKP1*) was cloned from tree peony (*Paeonia suffruticosa*) and denoted as *PSK1*. The 462-bp open reading frame of *PSK1* was predicted to encode a protein comprising 153 amino acids, with a molecular mass of 17 kDa. The full-length gene was 1,634 bp long and included a large 904-bp intron. *PSK1* transcription was detected in all tissues, with the highest level observed in sepals, followed by leaves. Under salinity stress, overexpression of *PSK1* in Arabidopsis resulted in increased germination percentages, cotyledon greening, and fresh weights relative to wild-type plants. Furthermore, transgenic Arabidopsis lines containing *35S::PSK1* displayed increased expression of genes that would be essential for reproduction and growth under salinity stress: *ASK1*, *LEAFY*, *FT,* and *CO* involved in flower development and flowering time as well as *P5CS*, *RAB18*, *DREB,* and *SOD1-3* contributing to salinity tolerance. Our functional characterization of *PSK1* adds to global knowledge of the multiple functions of previously explored *SKP1*-like genes in plants and sheds light on the molecular mechanism underlying its role in salinity tolerance. Our findings also provide information on the function and molecular mechanism of *PSK1* in tree peony flower development, thereby revealing a theoretical basis for regulation of flowering and conferral of salinity tolerance in tree peony.

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Qing Hao and Hongxu Ren contributed equally to this work.

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✉ Zhimin Gao
gaozhimin@icbr.ac.cn

✉ Qingyan Shu
shuqy@ibcas.ac.cn

Qing Hao
haoqing5608@163.com

Hongxu Ren
hxren@ibcas.ac.cn

Jin Zhu
zhujinb916@163.com

Liangsheng Wang
wanglsh@ibcas.ac.cn

Shouchen Huang
oppo183@163.com

Zheng'an Liu
liuzhengan@ibcas.ac.cn

[1] Key Laboratory of Plant Resources/Beijing Botanical Garden, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

[3] Landscape and Forestry College, Qingdao Agricultural University, Qingdao 266109, China

[4] International Center for Bamboo and Rattan, Key Laboratory on the Science and Technology of Bamboo and Rattan, Beijing 100102, China

Keywords *SKP1* · *Paeonia suffruticosa* · *PSK1* · Salinity tolerance · Flowering

Abbreviations

CaMV	Cauliflower mosaic virus
DREB	Dehydration-responsive element-binding protein
MDA	Malondialdehyde
MS	Murashige and Skoog
ORF	Open reading frame
P5CS	Δ1-Pyrroline-5-carboxylate synthase
qPCR	Quantitative real-time PCR
RAB18	Ras-related protein
RACE	Rapid amplification of cDNA ends
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-PCR
SOD	Superoxide dismutase
WT	Wild type
CO	*CONSTANS*
FT	Flowering locus T

Introduction

S-phase kinase-associated protein1 (SKP1) was initially identified in yeast (Kong et al. 2004). Although only one functional SKP1 protein present in *Homo sapiens* and yeast (Kong et al. 2004), many animal and plant species possess multiple homologues, including 7 in *Drosophila melanogaster*, 21 in *Caenorhabditis elegans*, 21 in Arabidopsis (denoted as ASKs), and 32 in rice (denoted as OSKs) (Vierstra 2009; Nayak et al. 2002; Kong et al. 2007). SKP1 proteins found in various organisms have diverse functions associated with a myriad of vital biological processes (Takahashi et al. 2004). Much attention has been focused on SKP1 proteins because they are one of the most important components of the SKP1/Cullin/F-box (SCF) complex, an E3 ubiquitin ligase. Through its interaction with different F-box proteins to ubiquinate different substrates, SKP1 performs various functions including control of cell cycle progression, transcriptional regulation, signal transduction, and other cellular processes (Hellmann and Estelle 2002).

Among studied SKP1-like homologues in plants, the greatest progress has centered around Arabidopsis SKP1-like proteins (ASK proteins), this is especially true for ASK1, the most studied ASK at present. ASK1 is a component of SCF^{TIR1}, SCF^{COI1}, and $SCF^{SLEEPY1}$ complexes that, respectively, regulate auxin (Gray et al. 1999), jasmonic acid (Xie et al. 1998) and gibberellic acid (McGinnis et al. 2003) signal transduction pathways. Recent studies demonstrated that ASK1, together with other SCF complex members, plays an important role in control of circadian rhythm (Somers et al. 2000), flowering timing (Imaizumi et al. 2005), light signaling (Dieterle et al. 2001), defense response (Wang et al. 2013), and cross-pollen compatibility in S-RNase-based self-incompatibility systems (Zhao et al. 2010). Genetic studies on ASK1/ASK11 have revealed its important functions in vegetative growth and male meiosis (Yang et al. 1999; Gusti et al. 2009). ASK1 also regulates B-function gene (B subfamily MADS-box gene) expression in cooperation with UFO and LEAFY to regulate flower development and floral organ identity (Zhao et al. 1999, 2003).

Apart from Arabidopsis, SKP1 functional studies of other plant species, including rice OSKs (Kahloul et al. 2013) and wild tomato SSKs (Zhang et al. 2015), have made some progress. For example, an examination of tissue-specific expression patterns revealed that some *SSK*s were expressed in a large number of tissues (e.g., fruits, petals, and sepals). In addition, expressions of *SSK*s (*SSK4*, *SSK7,* and *SSK12*) were found to be responsive to different abiotic stresses, including heat and salinity, with expression levels of nearly 50% of analyzed *SSK*s up-regulated by abscisic acid (ABA) and salicylic acid (SA) treatments (Zhang et al. 2015). The transcriptional profiles of these *SSK*s under abiotic stress treatment provided new insights to the function of *SKP1* homologous genes. In another functional study, that of wheat *TSK1*, the specific expression pattern and interaction between TaSKPs and selected F-box proteins demonstrated the possible function of these proteins in various growth and flower development processes (Hong et al. 2013). Preliminary research carried out in woody plants such as pummelo (*Citrus grandis*, *CgSKP1*), citrus (*C. reticulate*, *CrWSKP1*), and sweet cherry (*Prunus avium*, *PacSSK1*) suggests that their *SKP1-like* genes are involved in flower development and self-incompatibility reactions (Chai et al. 2010; Li et al. 2015; Matsumoto et al. 2012).

Except for the above-mentioned species, little is known about *SKP1-like* genes in plants, especially those without genomic information. Tree peony (*Paeonia suffruticosa*), a member of section Moutan in the genus *Paeonia* of Paeoniaceae (Li 1999), is a perennial deciduous shrub with excellent medicinal, ornamental, and seed oil properties (Li et al. 2010; Li et al. 2014). The root bark of tree peony is a valuable ingredient in Chinese traditional medicine. In addition, this ornamental species is world-renowned for its large showy flowers in various colors and shapes. Because of the high content of unsaturated fatty acids (92%) especially α-linolenic acid (42% of total fatty acids), as well as phenolic and monoterpene glycosides, tree peony seeds are used for food oil and pharmaceuticals (Li et al. 2014). Given the multiple uses of this species, aspects of tree peony, such as flower color, root compounds, oil content and abiotic stress response,

are of great research interest. Although *SKP1* family genes play vital roles in flower development, reproduction, seed set, hormone signaling, and other processes (Zhang et al. 2015), little is known about the function of these genes in tree peony. To explore the function of *SKP1-like* genes in tree peony, we isolated a *SKP1-like* gene homologue (denoted as *PSK1*) in this study and analyzed its expression profile in different tissues and its subcellular localization. We also carried out an ectopic expression analysis of its function in Arabidopsis. The results of this study provide a basis for future investigations of *SKP1-like* genes in tree peony and related species. In regard to functional conservation and diversification, our findings are also beneficial to help clarify the functions of *SKP1-like* genes in non-model plants.

Materials and methods

Isolation of *PSK1* gene and bioinformatic analysis

A *SKP1-like* gene homologue was previously isolated from a flower-bud cDNA library of *P. suffruticosa* and denoted as *PSK1* (GenBank accession no. FE529999) (Shu et al. 2009). Genomic DNA was prepared from young leaves according to (Han et al. 2008). The genomic DNA sequence was amplified using the primers listed in ESM_S1. *PSK*1 homologues were identified by BLASTX analysis against the NCBI database. Predicted amino acid sequences of tree peony and related plant species were aligned in Clustal X (Thompson et al. 1997) followed by manual refinements, and a phylogenetic tree was constructed in MEGA4.1 with 1,000 bootstrap replicates (Tamura et al. 2007). Conserved domains of PSK were predicted by Pfam and SMART programs (Letunic et al. 2002; Punta et al. 2012), while, subcellular localization of PSK1 was predicted using the Plant-mPLoc program (Chou and Shen 2010).

Quantitative real-time PCR (qPCR) analysis of *PSK1*

The expression pattern of PSK1 was studied in various tissues and petals at different developmental stages of *Paeonia suffruticosa* 'Gunpohden' under natural conditions, collected from the Beijing Botanical Garden, Institute of Botany, Chinese Academy of Sciences. After isolation of total RNA from various tissues with an RNAprep pure kit (Tiangen, Beijing, China), cDNA was synthesized using a Fast Quant Kit (Tiangen) according to the manufacturer's protocol. Flower petals at different developmental stages (Du et al. 2015), namely, flower-bud (S1), swollen flower-bud (S2), bloom initiation (S3), half-blooming (S4), and blooming (S5) stages, as well as leaves, stems, and sepals at the blooming stage (S5) were collected as samples in triplicate. Following treatment of 3-week-old plants of *PSK1* transgenic lines and wild-type (WT) Arabidopsis with 150 mM NaCl for 0 h, 4 h, 8 h, and 12 h, leaves were collected in triplicate. Total RNA and cDNA were obtained as described above. Quantitative assays were performed using SuperReal qPCR PreMix (SYBR Green, Tiangen, China) and a StepOnePlus^{TM} Real-Time PCR system (Applied Biosystems, Life Technologies, USA) following the manufacture's protocol. Each 20-µl qPCR mixture was subjected to the following program: 95 °C for 15 min, followed by t 40 cycles of 94 °C for 5 s, 56–60 °C for 20 s, and 72 °C for 32 s. The qPCR assay was carried out on three biological replicates with three technical replicates performed per sample. Relative quantification of mRNA transcripts was performed in triplicate with normalization to *β-tubulin* of *P. suffruticosa* (accession no. EF608942) or *ACTIN* of *Arabidopsis thaliana* (AY120779). All primers used in the qPCR analysis are listed in ESM_S1. Relative expression levels were calculated by comparing $2^{-\Delta\Delta Ct}$ values based on with threshold cycle (Ct) (Livak and Schmittgen 2001; Yoshida et al. 2003).

Transformation vector construction and transgenic plant generation

To generate *PSK1*-overexpressing transgenic plants, the full-length coding sequence of *PSK1,* was amplified using forward primer 5′-ACTCTAGAATGGTTAGGGTCGTGACTTTG-3′ (*Xba*I site underlined) and reverse primer 5′-AAGGATCCCTCAAATGCCCACTGGTTC-3′ (*Bam*HI site underlined), was inserted into the *Xba*I/*Bam*HI site of a pPZP vector (Chen et al. 2007) under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. Transformation of Arabidopsis was performed by the floral dip method using *Agrobacterium tumefaciens* (EHA105) cells (Clough and Bent 1998). Seeds from transgenic and WT Arabidopsis sown in half-strength Murashige-Skoog (MS) medium or pots were maintained at 4 °C for 3 days in the dark to break residual dormancy; the germinated seedlings were then grown in a culture room at 22 °C under a 16/8 h light/dark photoperiod and 60% relative humidity. *PSK1* expression levels in transgenic Arabidopsis lines were checked by qPCR; lines with the highest expression levels were used for subsequent analyses. For phenotypic analyses, we used T_3 homozygous lines selected from T_2 plants on half-strength MS medium with 50 µg ml^{-1} kanamycin. Three T_3 generations of Arabidopsis transgenic lines were selected for stress tolerance studies.

Analysis of transgenic plants under salinity stress

To test salinity tolerance of transgenic Arabidopsis plants, germination assays were carried out in triplicate by sowing at least 40 seeds from each transgenic lines or WT line on plates containing half-strength MS medium supplemented with various concentrations (0, 100 or 125 mM) of NaCl. After incubation at 4 °C for 3 days, the seeds were maintained in a culture room at 21–22 °C until germination. Upon germination, each seedling was transferred to 22 °C conditions and the day of germination was recorded as day 1. Fully emerged radicle tips or fully opened cotyledons were used as a reference for counting germinated seeds. After growing for 2 weeks, seeds developing green cotyledons were counted. For seedling growth measurements, 20 seedlings from each line were weighed and fresh weight was calculated as the average value. To determine the effect of NaCl on root growth, 7-day-old plants were transplanted to vertically standing half-strength MS plates containing NaCl with root length measured after 1 week. All of the above experiments were repeated at least three times. The relative expression level of stress-associated genes after NaCl treatment was analyzed using 3-week-old seedlings treated with 150 mM NaCl. The seedlings were sampled after treatment for 0–12 h and subjected to qPCR assay for *AtDREB*, *AtRAB18*, *AtSOS1-3,* and *AtP5CS*, with physiological parameters also measured. To determine the extent of lipid peroxidation, malondialdehyde (MDA) levels were assayed according to Kramer et al. (1991) with a few minor modifications, namely, 0.5 g of leaf tissue was homogenized, and 1 ml instead of supernatant rather than 2 ml was combined with 2 ml of glucosinolates barbituric acid (TBA) reagent [0.5% (w/v) TBA in 20% (w/v) trichloroacetic acid (TCA)] and heated at 100 °C for 10 min instead of 20. Proline and soluble sugar contents were determined following protocols described by Shan et al. (2007) and Bailey (1958). To further explore *PSK1* function in flowering development, we used qPCR to investigate the expression of genes involved in the flower development pathway including *AtCO*, *AtFT*, *AtLEAFY*, *AtUFO,* and *ASK1* in transgenic and control Arabidopsis plants. Primers used for the qPCR analyses are indicated in ESM_S1. Flower-bud formation and flowering time were also observed and calculated from 20 plants of three transgenic lines.

Statistical analysis

Data in this study including data from analysis of gene expression, germination, measurements of cotyledon greening root length and fresh weight, and plant physiological parameters (MDA, proline and soluble sugar contents) were subjected to analysis of variance (ANOVA) using SPSS 21.0. Differences were considered to be significant at $p < 0.05$. Figures were drawn in SigmaPlot 10.0. Each data point represented three replicates with error bars used to indicate SD.

Results

Sequence analysis of *PSK1*

A full-length gene (accession no. FE529999) isolated from a flower-bud cDNA library of tree peony (Shu et al. 2009) was revealed by BLASTX analysis against the NCBI database to be a close homologue of plant, *SKP1-like* genes. We accordingly designated this as *PSK1* (for *Paeonia suffruticosa* SKP1-like1). The cDNA of *PSK1* was 730 bp long, with a 462-bp open reading frame (ORF) encoding a predicted protein of 153 amino acids and a molecular mass of 17 kDa. The full-length gene was 1,634 bp long and included a large 904-bp intron (Fig. 1a). The predicted protein contained a SKP1-POZ domain. Alignment of *PSK1* with homologues from *Theobroma cacao*, *Gossypium hirsutum,* and *Vitis vinifera* demonstrated the presence of conserved amino acids and motifs shared by plant SKP1-like family members (Fig. 1b). A few conserved motifs were identified among the aligned amino acids. At the N-terminus (positions 40–50), for example, a PEST motif rich in proline, glutamic acid, serine, and threonine residues for protein turn over (Rogers et al. 1986) was uncovered. At the C-terminus (positions 135–140), a P-loop for binding GTP/ATP was identified: as indicated in Fig. 1b, the conserved motif was AXXXXGRT/S except in yeast, where arginine (R) was replaced by lysine (K) to give the conserved motif AXXXXGKT/S (Winkler et al. 2000). Phylogenetic analysis indicated that PSK1 is homologous to SKP1 proteins from *Amborella trichopoda*, *T. cacao,* and *G. hirsutum* (Fig. 2).

Wide expression of *PSK1* in various tissues of *P. suffruticosa* 'Gunpohden'

PSK1 expression levels were monitored in stems, sepals, leaves, and petals at the flower-blooming stage (S5). At S5, *PSK1* was expressed in all tissues. The highest level was in sepals, followed by leaves, with no significant difference ($p < 0.05$) observed between petals and stems (Fig. 3). When expression levels in petals from S1 to S5 were traced, however, the highest expression level was observed in petals at S3, followed by S1. Because expression level at S1 was nearly two times higher than that of S5, *PSK1* expression appears to be down-regulated, except during transition from S2 to S3, as floral development processes (Fig. 3).

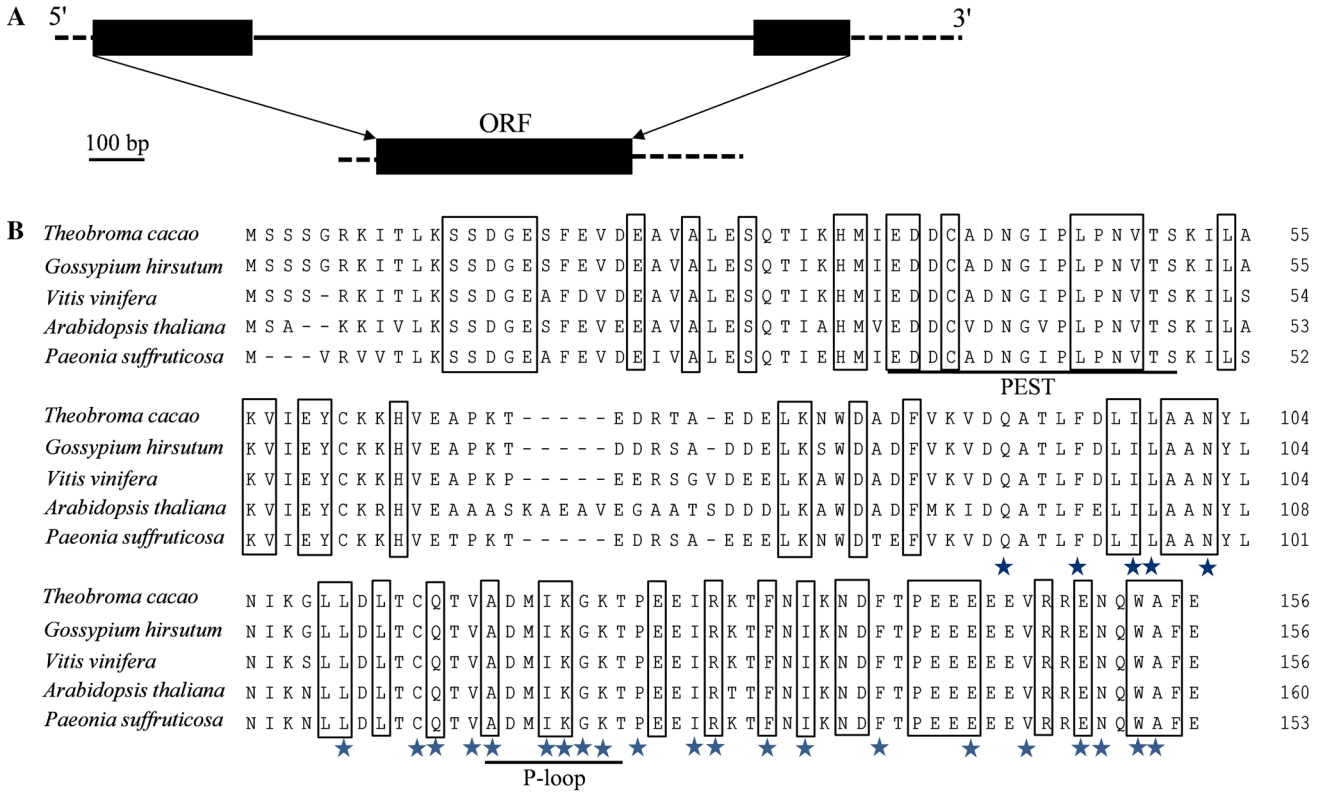

Fig. 1 Analyses of gene structure and sequence alignment of *PSK1*. **a** Genomic structure of *PSK1*, containing two exons (*black boxes*), one intron (*solid line*), and 462-bp long ORF. *Dotted lines* correspond to untranslated regions. **b** Amino acid sequence alignment of PSK1 and SKP1 sequences from *Theobroma cacao* (EOX90784.1), *Gossypium hirsutum* (AFM75900.1), *Vitis vinifera* (XP_002270061.1), and *Arabidopsis thaliana* (AEE35780.1). Identified sequence regions are indicated by *blocks*. *Blue-asterisks* below the aligned sequences indicate amino acid residues closely associated with the interaction between SKP1 and F-box proteins (color figure online)

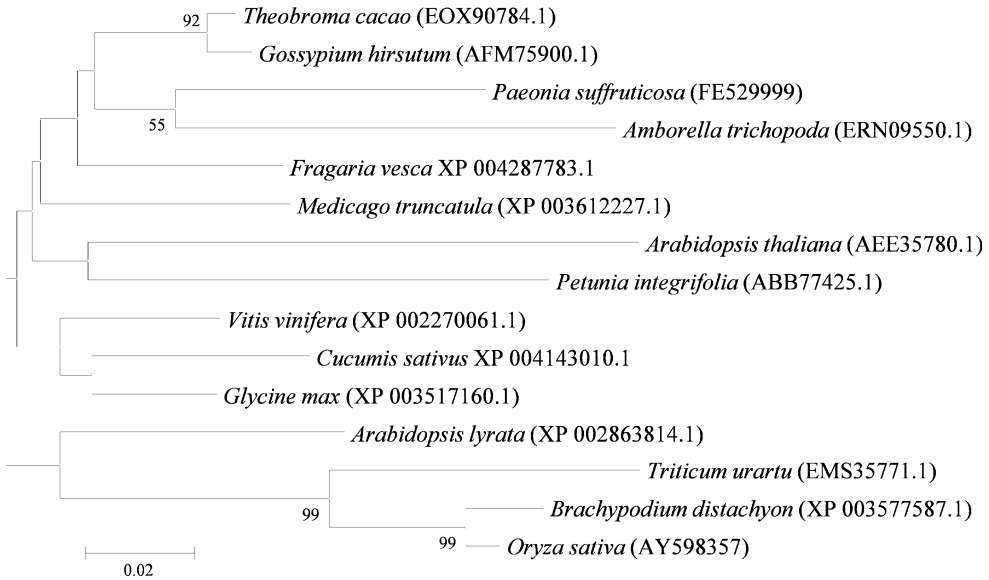

Fig. 2 Phylogenetic tree of PSK1 and SKP1 proteins from *Theobroma cacao* (EOX90784.1), *Gossypium hirsutum* (AFM75900.1), *Vitis vinifera* (XP_002270061.1), *Arabidopsis thaliana* (AEE35780.1), *Arabidopsis lyrata* subsp. *lyrata* (XP_002863814.1), *Amborella trichopoda* (ERN09550.1), *Brachypodium distachyon* (XP_003577587.1), *Cucumis sativus* (XP_004143010.1), *Fragaria vesca* subsp. vesca (XP_004287783.1), *Glycine max* (XP_003517160.1), *Medicago truncatula* (XP_003612227.1), *Oryza sativa* (AY598357), *Petunia integrifolia* subsp. *inflata* (ABB77425.1), and *Triticum urartu* (EMS35771.1). Numbers above and below branches are bootstrap percentages based on 1000 replicates using MEGA 4.1

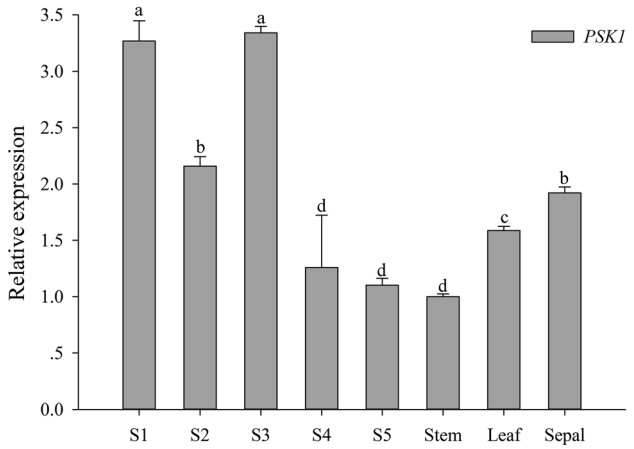

Fig. 3 Expression pattern of *PSK1* at the different developmental stages (*S1*–*S5*) and in different tissues measured by qRT-PCR with the *β-tublin* gene used as a reference. Developmental stages are as follows: *S1* flower-bud stage, *S2* swollen flower-bud stage, *S3* bloom initiation stage, *S4* half-blooming, and *S5* blooming stage; Stems, leaves, and sepals at *S5*

Increased salinity tolerance of *PSK1-overexpressing* transgenic Arabidopsis plants

In total, nine positive transgenic lines were obtained. Lines 7-9, which showed the highest PSK1 expression levels were used for further study of salinity tolerance (ESM_S2). All transgenic seeds displayed tolerance to salinity stress, and 90% of seeds germinated on the first day at 22 °C (Fig. 4a–c). In contrast, WT seeds germinated two days later than those of transgenic lines, with the transgenic lines displaying significant difference ($p < 0.05$) in germination compared with the WT at 22 °C on the first 3 days (ESM_S3-A). The transgenic lines exhibited significantly higher cotyledon greening rates (ESM_S3-B) than those of WT plants after NaCl treatment. For example, 90.3, 91.3, and 90.3% of L7, L8, and L9 seedlings displayed green cotyledons on medium with 125 mM NaCl, compared with 58.7% of the WT. In addition, a seedling growth assay was carried out to further test the phenotypes of transgenic lines under salinity stress. Significant increases in fresh weights of transgenic lines occurred in the presence of salinity. Even in the absence of salinity, transgenic lines showed a greater increase in fresh weights compared with those of WT plants, thus indicating that *PSK1* may promote the growth of transgenic Arabidopsis plants (Fig. 4d).

To further investigate the role of *PSK1* in the conferral of salinity stress tolerance, root length was measured after NaCl treatment. Although no significant differences were observed between transgenic lines and WT plants at lower concentration of salinity (0–100 mM NaCl), a marked reduction in root elongation was noted in WT plants compared with transgenic lines on medium containing 125 mM NaCl (ESM_S3-C). Therefore, owing to early germination, high germination percentage, increased cotyledon greening rates, and accelerated seedling growth, including shoot and root growth, *PSK1* overexpression was able to enhance plant tolerance to salinity stress.

Increased proline and soluble sugar content of *PSK1-overexpressing* transgenic Arabidopsis plants under salinity stress

To explore the effect of *PSK1* overexpression on salinity tolerance of Arabidopsis plants, we investigated the following physiological parameters: proline, soluble sugar, and MDA contents. In the absence of salinity stress (0 h), all transgenic lines accumulated higher levels of proline than the WT. When subjected to salinity stress for 12 h, an obvious increase in proline content was observed in transgenic lines (L7 and L9) relative to the WT (Fig. 5a). MDA, a product of lipid peroxidation in biomembranes, was measured as an indicator of salinity stress damage due to reactive oxygen species (ROS) (Niu et al. 2012), but little difference was observed between transgenic lines and the WT after salinity treatment (Fig. 5b). *PSK1-overexpressing* plants were assayed for soluble sugar content, which was found to be higher in transgenic lines than WT plants with or without salinity stress; this was especially true after salinity treatment for 8 h, when a significant difference ($p < 0.05$) was observed (Fig. 5c).

Variable expression of salinity-related genes up-regulated in *PSK1-overexpressing* transgenic Arabidopsis lines and their possible roles in salinity tolerance

To investigate the mechanism through which *PSK1* participates in enhancement of salinity stress tolerance in transgenic plants, we assayed the relative expression levels of several salinity stress-related genes after NaCl treatment (150 mM). Among several known salinity defense pathway, the salinity overly sensitive pathway (SOS) appears to be a first line of defense with *SOS1*, *SOS2,* and *SOS3* genes playing a major role in this system. In the absence of salinity stress, expression levels of these three genes were higher in transgenic lines than those in the WT; this was especially the case for L7 and L8, which displayed, respectively, 2.8- and 3.2-fold higher expression of *SOS1*, 1.3- and 2.8-fold higher expression of *SOS3*, and 2.0- and 1.9-fold higher expression of *SOS2* relative to the WT (ESM_S3). After salinity stress for 12 h, the expression levels of these three genes were higher in *PSK1-overexpressing* lines than those in WT plants (ESM_S4). Transcription factors, including *DREB* and the functional gene *RAB18,* displayed opposite patterns of expression. The *AtDREB* gene showed up-regulated in L8 and the WT

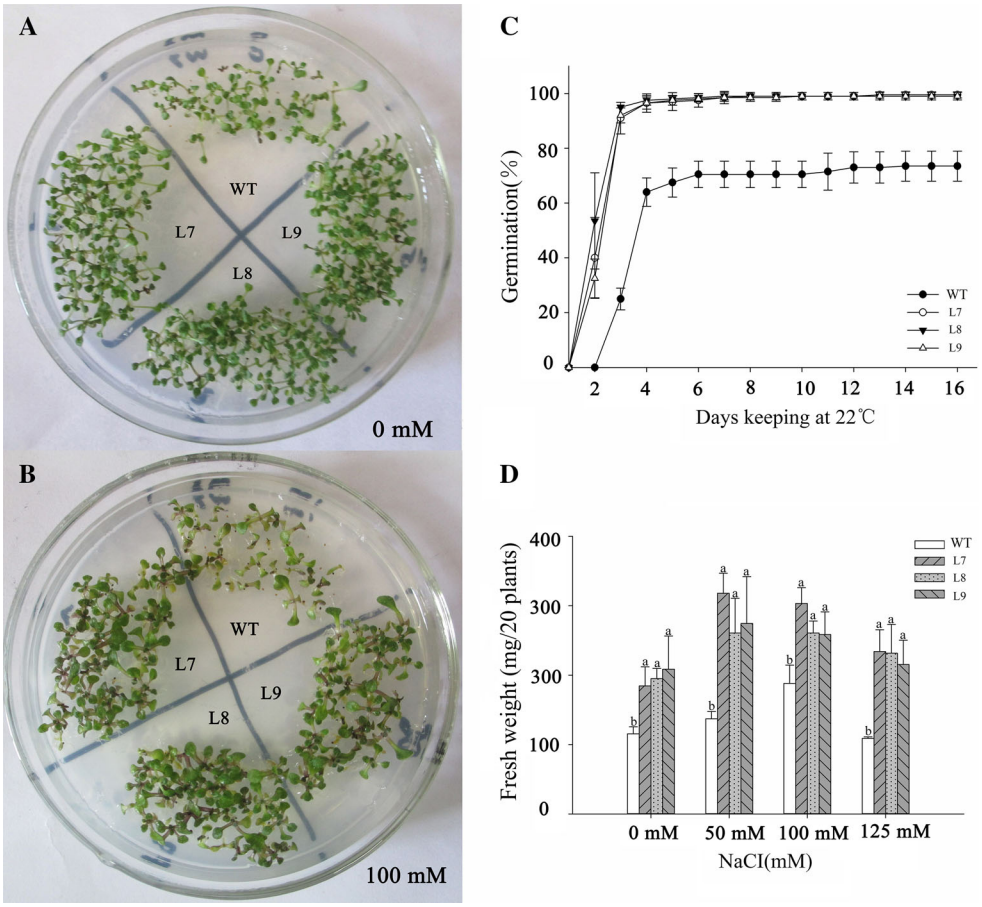

Fig. 4 Germination response of *PSK1-overexpressing* transgenic lines (*lines 7–9*) and WT plants to salinity stress. The growth was assayed on medium containing 0 mM (**a**) or 100 mM NaCl (**b**). The germination process was analyzed within 16 days after sowing in medium containing 100 mM NaCl, with the day when seedlings were transferred from 4 to 22 °C (**c**). Fresh weights of 20 plants growing in medium containing 100 mM NaCl was measured (**d**). For **c** and **d**, *each column* represents the average of three replicates, and the *bar* indicates SD. Data from each treatment were subjected to analysis of variance using SPSS 21.0. The *different characters* above *each column* represent various significant differences at $p < 0.05$

under salinity treatment, with the highest level at 8 h; in contrast, expression in L7 initially decreased and then rose to its highest level after 8 h treatment, whereas the highest level of *AtDREB* in L9 was detected after 4 h of salinity treatment (Fig. 5d). Expression levels of *AtRAB18* were higher in transgenic lines than those in the WT, with significant differences ($p < 0.05$) after salinity treatment for 12 h (Fig. 5e), while *AtP5CS* expression levels exhibited a significant increase ($p < 0.05$) in all transgenic lines compared with those of WT after salinity treatment for 8 h (Fig. 5f).

Possible promotion of flowering by higher expression levels of flowering developmental genes in *PSK1-overexpressing* transgenic Arabidopsis lines

To further explore the function of *PSK1* in flower development, the expression levels of genes involved in the flower development pathway, including *CONSTANS* (*CO*), Flowering Locus T (*FT*), *LEAFY*, and *ASK1*, were investigated in transgenic Arabidopsis and WT plants. Relative expression levels of all detected genes were higher in transgenic plants than in the WT. Average expression level were 3.5 times higher for *FT*, 6.2 times higher for *LEAFY*, 4.1 times higher for *CO,* and 5.7 times higher for *ASK1* (Fig. 6a) in line 7–9 than in WT plants. In this study, *PSK1*-overexpressing Arabidopsis plants (L7–9) exhibited early formation of flower-bud formation and flowered 1 week earlier (ESM_S5) than WT plants (Fig. 6b), indicating that increased expression of the above-mentioned genes may promote early flowering in transgenic plants.

Discussion

In this study, we obtained a *SKP1-like* gene homologue from a tree peony cDNA library. Denoted as *PSK1*, this gene was predicted to encode 153 amino acids. According

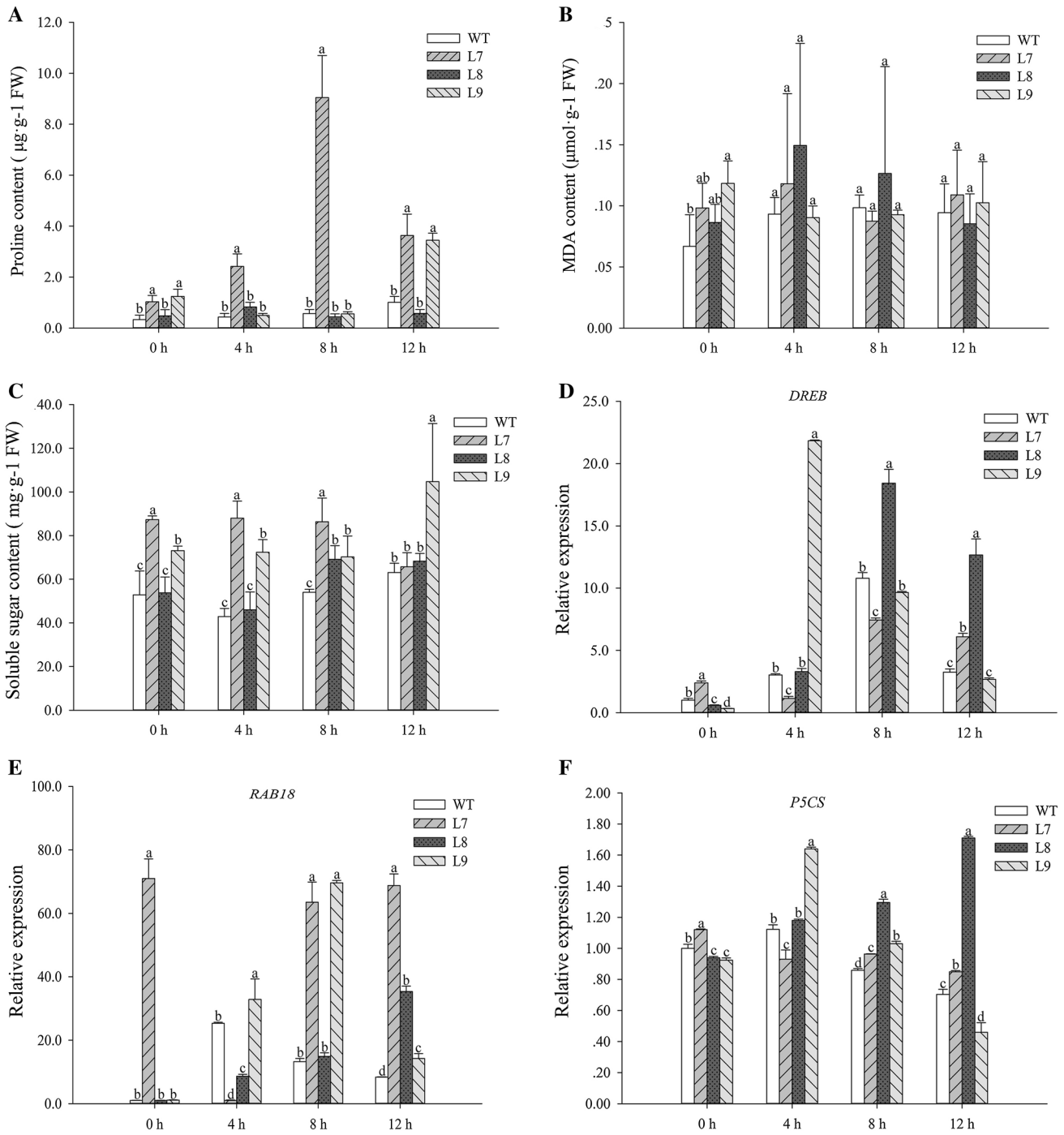

Fig. 5 Measurements of proline, soluble sugar and MDA contents and expression analysis of related genes in WT and *PSK1*-overexpressing Arabidopsis plants treated with 150 mM NaCl for 0–12 h. **a** Proline content. **b** MDA content. **c** Soluble sugar content. Expression levels of *DREB* (**d**), *RAB18* (**e**) and *P5CS* (**f**) between WT and *PSK1*-overexpressing Arabidopsis plants. *Each column* represents the average of three replicates, and the *bar* indicates SD. Data from each treatment were subjected to analysis of variance using SPSS 21.0. The *different characters* above each *column* represent various significant differences at $p < 0.05$

to phylogenetic analysis, *PSK1* is most closely related to homologues from *Amborella trichopoda*, *T. cacao,* and *G. hirsutumf* (Fig. 1c). PSK1 also possesses conserved amino acids and motifs (indicated by a blue star in Fig. 1b) that are important for the interaction of SKP1 with F-box proteins (Schulman et al. 2000). *PSK1* showed wide expression in tree peony tissues, consistent with previous studies in Arabidopsis (Zhao et al. 2003; Takahashi et al. 2004; Dezfulian et al. 2012), in addition, it displayed increased expression as flower-bud development and

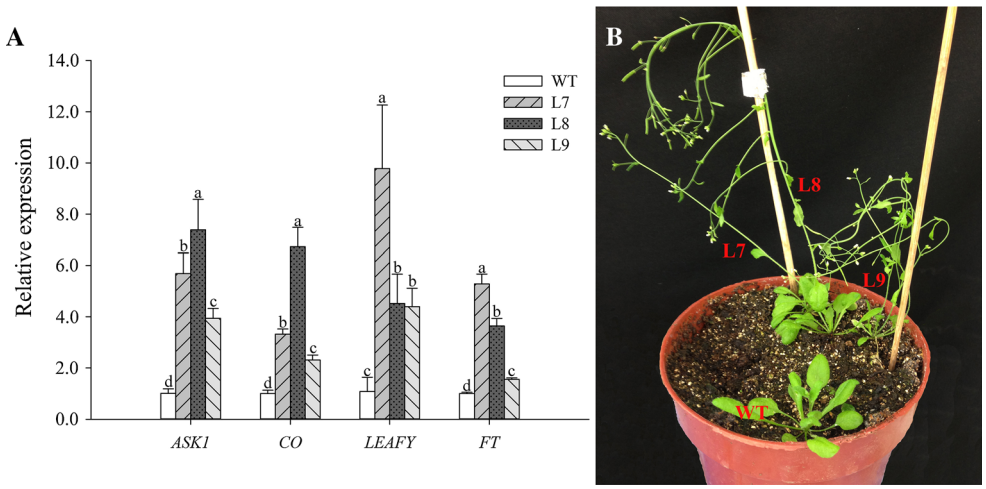

Fig. 6 Analysis of expression levels of flower-related genes and demonstration of early flowering. **a** Expression levels of genes including *ASK1*, *LEAFY*, *CO,* and *FT* in WT and *PSK1*-overexpressing Arabidopsis plants (*lines 7–9*). Each column represents the average of three replicates, and the *bar* indicates SD. Data from each treatment were subjected to analysis of variance using SPSS 21.0. The *different characters* above *each column* represent various significant differences at $p < 0.05$. **b** Flowering of WT and *PSK1*-overexpressing Arabidopsis plants (*lines 7–9*)

flowering progressed, which indicates *PSK1* may be functionally important to tree peony flower development and blooming. To confirm this possible function, additional studies are needed.

A previous phylogenetic analysis (Kong et al. 2007) has suggested that all Arabidopsis and rice *SKP1-like* genes are derived from a single ancestral gene and can be classified into three types, namely, type Ia with one intron and two exons, type Ib lacking introns, and type II containing several introns. As revealed by analysis of its genomic structure, *PSK1* contains only a single long intron at a conserved position. In the phylogenetic tree of Kong et al. (2007), *ASKs* and *OSKs* with one conserved intron occupied the same basal position, whereas intronless genes generally formed terminal clades. *OSK1* and *OSK20* both have one intron at a conserved position, thereby demonstrating their orthologous relationship. A detailed analysis of plant, animal, and insect genomes has suggested that genuine orthologous genes have more conserved intron positions than those of non-orthologous genes (Henricson et al. 2010). In contrast, nearly 50% (144/288) of plant *SKP1-like* genes are intronless, which indicates an active retroposition phenomenon (Kahloul et al. 2013). Despite these findings, investigation of the evolution and genomic structure of all *SKP1-like* genes from tree peony is still needed to understand their functional conservation and diversification.

Plant displayed various responses to salinity stress, including early germination, vigorous growth, and early development and rapid completion of the life cycle. At the cellular level, concentrations of many metabolites are increased when plant confronts salinity stress. In this study, we found that *PSK1-overexpressing* transgenic Arabidopsis plants increased salinity tolerance as a consequence of elevated accumulation of proline and soluble sugars (Fig. 5a, c). In contrast, MDA content, an indicator of salinity stress damage (Cheng et al. 2013), did not change significantly. We deduce that the conferral of salinity stress on Arabidopsis plants by overexpression of *PSK1* due to increased proline and soluble sugar accumulation, as soluble sugar would prevent cell dehydration (Niu et al. 2012), while, free proline has multiple functions in cells, such as ROS scavenging and osmoprotection (Zsigmond et al. 2012; Niu et al. 2012). Furthermore, transgenic lines demonstrated vigorous growth as reflected by increased fresh weight (Fig. 4d). As *ASK1* has been confirmed to play a vital role in early development (Liu et al. 2004), vigorous growth may help plants overcome the disadvantage of salinity stress, thereby aiding their reproduction and benefiting future generations (Kim et al. 2013).

Expression profiles of genes responsible for salinity stress were also analyzed. This analysis revealed that expression levels of detected genes, including *AtSOS1-3*, *AtRAB18*, *AtDREB,* and *AtP5CS,* were up-regulated in *PSK1-overexpressing* transgenic lines compared with those in WT after NaCl treatment (Fig. 5d–f; ESM_S4), but with slight discrepancy displayed by the three transgenic lines. Previous studies have demonstrated that the above-mentioned genes can be induced under saline conditions and may play important roles in salinity tolerance (Yamaguchi-Shinozaki 2006; Yang et al. 2009). The expression levels of three genes were higher in *PSK1-overexpressing* lines than those in WT plants after salinity stress for 12 h (ESM_S4). A *SKP1-like* gene from *Trticum aestivum*

(*TSK1*) has been confirmed to be involved in ABA signaling and may positively regulate this process (Li et al. 2012), while *AtRAB18* and *AtP5CS* have been verified to be active in an ABA-dependent regulatory pathway and also induced by abiotic stress (Szabados and Savouré 2010; Cheng et al. 2013). *P5CS* expression, which has been shown to be induced by salinity stress, is mainly responsible for proline accumulation during salinity or drought stress (Ziaf et al. 2011; Szabados and Savouré 2010). Lines 7–9, especially L7, displayed extremely high *AtRAB18* expression levels, even in the absence of salinity stress (Fig. 5e). Although this observation implies that this gene may be involved in ABA signaling or another salt tolerance pathway, further studies are needed to uncover its contribution to salinity tolerance. These results suggest the existence of transcriptional regulatory mechanism for stress tolerance in *PSK1*-overexpressing Arabidopsis operated by elevating the expression of transcriptional factors and enzymatic genes, including *DREB*, *RAB18*, *SOS1-3,* and *P5CS*. In Arabidopsis *ask1* mutant flowers, increased expression of two proteins has been detected. One of these proteins is a class I heat shock protein, which is stress induced and confers cytoprotection against oxidative injury; the other is an oxygen-evolving complex-related protein, which indicates that mutation of ASK1 may directly or indirectly cause oxidative stress in flower organs (Wang et al. 2006). This finding suggests that overexpression of *PSK1* in Arabidopsis increases salinity tolerance through up-regulation of salt-stress-related genes as well as accumulation of some functional proteins. Previous research on plant *SKP1-like* genes has mainly focused on their function in male fertility and flower development addressed their role in the hormone signaling pathway via SCF-mediated protein degradation in model plants (rice and Arabidopsis). Little information has been made available concerning the function of these genes in abiotic stress. Our study represents the first attempt to uncover additional functions of *SKP1-like* gene, but much work is still required to fully understand all possible functions in plants.

Plant *SKP1* genes in eudicots and monocots have evolved by multiple duplication events from a single ancestral gene. Such gene duplication events are important for gene family evolution with duplicate genes providing a good material for the evolution of new genes as well as physiological and morphological novelties (Kong et al. 2007). In regard to ancestral genes, birth rate much higher than the death rate has aided the expansion of the SKP1 family in plants (Kong et al. 2007). A total of 21, 30 and 19 *SKP1-like* gene exhibiting a variety of expression patterns are found in Arabidopsis (*ASK1-21*), rice (*OSK1-30*), and wild tomato (*SSK1-19*), respectively, but not all of them can interact with the SCF complex to perform a function. In particular, ASK1 and ASK2 and possibly ASK11 have been found to participate in most positive interactions with F-box proteins in *Arabidopsis thaliana*, which suggests that ASKs play diverse roles extending beyond serving as components of the SCF complex (Zhao et al. 2003; Takahashi et al. 2004; Kuroda et al. 2012; Kahloul et al. 2013; Zhang et al. 2015). Tissue-specific expression patterns of wild tomato SSKs have revealed some to additionally be responsive to heat stress and salicylic acid treatment (Zhang et al. 2015). Despite these findings, not all *SKP1-like* genes have been thoroughly studied in a given plant species, even in Arabidopsis; future functional characterization of each of these genes would be beneficial to understand the functional diversification of duplicated genes in plants. The number of *SKP1* family members in tree peony is unknown; to gain a comprehensive knowledge on their diverse function and evolution of *SKP1-like* genes in this woody species, aspects such as their structures, expression patterns, localization, and interacting proteins remain to be further characterized.

In Arabidopsis, ASKs are essential for flower development (Liu et al. 2004; Zhao et al. 2010) and male gametophyte sterility (Marrocco et al. 2003), while SSKs may be involved in tomato fruit development (Zhang et al. 2015) and TSKs regulate male meiosis in wheat (Li et al. 2006). These functions indicate that *SKP1-like* genes play an important role in reproductive development. In this study, we found that overexpression of *PSK1* increased the expression of flowering-related genes including *CO*, *LEAFY,* and *FT* (Fig. 6a), and promoted flower formation and early flowering (Fig. 6b; ESM_S5). This effect was similar to that of ASK1 which interacts with B function proteins (B subfamily *MADS-box* gene products) and cooperates with *UFO* and *LEAFY* involved in flower development and floral organ identity (Zhao et al. 1999, 2010). A similar involvement of *SKP1-like* genes in flower development has been reported in woody plants such as pummelo (*CgSKP1*), citrus (*CrWSKP1*), and sweet cherry (*PacSSK1*) (Chai et al. 2010; Matsumoto et al. 2012; Li et al. 2015). Taking into account the current progress on *SKP1-like* genes, *PSK1* may possess a conserved function in regulation of flower development and flowering. To fully elucidate the evolution and functional diversification of *SKP1-like* genes in tree peony, a future study focused on their cloning and functional characterization is planned. Tree peony is a woody shrub, with 3 -5 years required for the transition from vegetative to reproductive growth. *PSK1* overexpression increased the transcription of genes involved in the flower development pathway. To shorten its juvenile phase, overexpression of *PSK1* in tree peony may promote early flowering. Functional characterization of *PSK1* may not only illuminate the molecular mechanism underlying salinity tolerance and its importance, but may

also provide global knowledge concerning the multiple functions of *SKP1-like* family genes under different physiological conditions and developmental stage.

Conclusions

In this study, a *SKP1-like* gene homologue was cloned from tree peony and functionally analyzed in transgenic Arabidopsis. Overexpression of *PSK1* improved their salinity tolerance of the transgenic Arabidopsis plants, which indicated a novel function of PSK1. In *PSK1-overexpressing* transgenic plants, genes positively related to flower development and flowering time were up-regulated thereby promoting flower formation and early flowering. This study has yielded new information on the function of PSK1 and its possible molecular mechanism during tree peony flower development and conferral of salinity tolerance, thus providing a theoretical basis for the regulation of flowering and abiotic stress tolerance. Because tree peony is a globally popular ornamental flowering plant, our gained insights into flowering will also be beneficial for future breeding of cultivars and new germplasm resources.

Author contribution statement HQ and RHX performed salinity stress analyses; ZJ and HSC conducted the qRT-PCR analysis; WLS and LZA carried out plants germination and growth analysis; GZM and SQY performed PSK1 cloning, vector construction, and transformation of Arabidopsis, and wrote the article.

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Compliance with ethical standards

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

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