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GsSKP21, a *Glycine soja* S-phase kinase-associated protein, mediates the regulation of plant alkaline tolerance and ABA sensitivity

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Abstract Plant SKP1-like family proteins, components of the SCF complex E3 ligases, are involved in the regulation of plant development and stress responses. Little is known about the precise function of *SKP* genes in plant responses to environmental stresses. *GsSKP21* was initially identified as a potential stress-responsive gene based on the transcriptome sequencing of *Glycine soja*. In this study, we found that GsSKP21 protein contains highly conserved SKP domains in its N terminus and an extra unidentified domain in its C terminus. The transcript abundance of *GsSKP21*, detected by quantitative real-time PCR, was induced under the treatment of alkali and salt stresses. Overexpression of *GsSKP21* in *Arabidopsis* dramatically increased plant tolerance to alkali stress. Furthermore, we found that overexpression of *GsSKP21* resulted in decreased ABA sensitivity during both the seed germination and early seedling growth stages. *GsSKP21* mediated ABA signaling by altering the expression levels of the ABA signaling-related and ABA-induced genes. We also investigated the tissue expression specificity and subcellular localization of GsSKP21. These results suggest that *GsSKP21* is important for plant tolerance to alkali stress and plays a critical regulatory role in the ABA-mediated stress response.

Keywords *Glycine soja* · Alkali stress · S-phase kinase-associated protein · ABA · Functional analysis

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Introduction

Salinization and alkalization in soils have detrimental effects on the growth, development and differentiation of plants and cause lower productivity of agricultural crops and grasses (Clark and Zeto 1996). Due to the existence of alkaline salts (Na_2CO_3 and $NaHCO_3$), in various plant species, plants growing at high soil pH (>8.0) were more vulnerable than those with soil salinization (Yang et al. 2007, 2008a, b). The high pH environment surrounding the roots can have serious influences on root cells such as direct damage of the structure and function (e.g., membrane selectivity), precipitation of Ca^{2+}, Mg^{2+} and $H_2PO_4^-$, and disruption of the ion homeostasis (Takahashi et al. 2001; Yang et al. 2007). Plants have evolved complex mechanisms of signal perception and transduction that allow them to perceive the incoming stresses and rapidly regulate their physiology and metabolism to cope with them. In recent years, tremendous progress was made in understanding plant response to external stimuli, such as the MAP kinase signal transduction

pathway of osmotic stress and SOS signal transduction pathway of ionic stress (Nakagami et al. 2005; Zhu 2002). Research was mainly focused on salt stress, and limited progress was made with regards to alkali stress.

Ubiquitination plays an important role in various cellular responses in plants, such as regulation of the cell division cycle, hormonal signaling and stress responses (Seo et al. 2012). S-phase kinase-associated protein 1 (SKP1) is a component of a SKP1-Cullin1-F-box (SCF) complex that facilitates ubiquitin-mediated protein degradation in eukaryotes (Hotton and Callis 2008). The SCF-type E3 ligase is composed of four major subunits: Cullin (CDC53 in yeast), SKP1, a RING finger protein (RBX1/HRT1/ROC1) and an F-box protein. Among them, SKP1 functions as an adaptor between F-box and Cullin1 (CUL1). SKP1 containing an N-terminal structural motif interacts with multiple F-box subunits, which specifically recognize different target proteins via a variable C-terminal domain (Bai et al. 1996; Connelly and Hieter 1996). In *Arabidopsis,* 21 *SKP1* homologs have been identified. They are collectively called *Arabidopsis-SKP1-like* (*ASK*) (Kong et al. 2007). *Arabidopsis SKP1-like 1* (*ASK1*) was the first *SKP1* homolog to be isolated, and its interaction with several kinds of F-box proteins such as TIR1 and COI1 has been reported (Gray et al. 1999; Ji et al. 2006; Li et al. 2012). The *ASK1* gene is required for homolog separation in male meiosis and is also involved in auxin response. The ASK2 protein, which has the most similar sequence to ASK1, was able to substitute for ASK1 during male meiosis (Li et al. 2012). Moreover, the *ask1 ask2* double mutant showed a developmental retardation during embryogenesis and lethality at the seedling stage (Shu et al. 2011). T-DNA-insertion mutants of *ASK11* and *ASK12* showed no obvious phenotypic changes. The phenotypes of *ASK14* and *ASK18* Ds transposon-insertion mutants were not obvious either. Unlike typical ASKs, ASK20 and ASK21 are distinguishable from other ASKs and clustered into another clade according to phylogenetic analysis of ASK proteins (Ji et al. 2006). It has been reported that ASK20A, ASK20B protein cannot interact with CUL1 in yeast two or three hybrid systems, and may prevent SCF complexes from forming by competing with other ASK proteins in their role of adaptors in the SCF complex (Ogura et al. 2008). In spite of the progress made in characterizing *SKPs*, their biochemical and physiological functions in plants are limited. Some reports investigating the role of *SKPs* in stress showed that the overexpression of *Triticum aestivum SKP1* (*TSK1*) in *Arabidopsis* resulted in enhanced tolerance to drought stress (Li et al. 2006). The effects of overexpressing *SKPs* on alkali stress tolerance have to be further explored.

The phytohormone abscisic acid (ABA) is a vital plant hormone and a central regulator that protects plants against abiotic stresses such as drought and salinity (Bhalerao et al. 1999; Hu et al. 2013; Santiago et al. 2009). In the presence of ABA, they bind to protein phosphatase 2C (PP2C), release the PP2C-mediated inhibition of SNF1-related kinase 2 (SnRK2) and subsequently activate downstream transcription factors (Komatsu et al. 2009). RCARs/PYLs were identified as the ABA receptors that can bind to ABA and interact with group A protein PP2C to inhibit the activity of the phosphatase (Gong et al. 2013; Wang et al. 2009). *ASK1* and *ASK2* were recently reported to be involved in ABA signaling. The *ask1/ask1 ASK2/ask2* seedlings exhibited reduced ABA sensitivity; overexpression of *ASK1* and *ASK2* in the *abi5-1* mutant can rescue or partially rescue the ABA insensitivity of the *abi5-1* mutant, respectively (Farrás et al. 2001). Although it was reported that ASK1/SKP1 interacted with the PRL1-binding C-terminal domains of SnRKs and that PRL1 reduced the interaction of ASK1/SKP1 with SnRKs (Farrás et al. 2001), direct evidence for *SKPs* in ABA response is limited.

Glycine soja (*G. soja*), the wild ancestor of cultivated soybean *Glycine max* (*G.max*), belongs to the same *Subgenus Soja* as the cultivated soybean. Because of the close genetic relationship and strong abiotic resistance, *G. soja* supplies valuable genetic resources for the cultivation of transgenic plants with improved abiotic tolerance (Phang et al. 2008). *G. soja* 07256 is an ideal plant candidate for isolating alkali-tolerance-related genes, as the seeds of this wild soybean can germinate in sodic soils of pH 9.02 and continue to survive in nutrient solutions containing 50 mM $NaHCO_3$ (Ge et al. 2010, 2009). In our laboratory, the sequencing transcriptome of *G. soja* 07256 under alkali and salt stresses showed that *GsSKP21* was a putative alkali-response gene. Compared with thesalt stress profile, *GsSKP21* exhibited more abundant expression under alkali stress by screening protein expression in *G. soja*. Here, we characterized *GsSKP21,* a putative novel *SKP1-like* family gene with a conserved SKP domain bearing the highly conserved amino acid pattern of *ASK21*, which is involved in plant response to alkali stress and ABA treatment. Expression levels of *GsSKP21* were greatly and rapidly induced by high alkali levels as evidenced by quantitative real-time PCR (qRT-PCR). Overexpression of *GsSKP21* in *Arabidopsis* conferred enhanced tolerance to alkali stress and downregulated expression levels of stress-responsive marker genes. Furthermore, *GsSKP21* overexpression decreased plant sensitivity to ABA and altered expression levels of ABA signal-related genes. Subcellular localization studies using an enhanced green fluorescent protein (eGFP) fusion protein showed that GsSKP21 is localized in the nucleus. All the data presented illustrate the important role of *GsSKP21* as a regulator of alkali stress tolerance and ABA signaling in plants.

Materials and methods

Plant material, growing conditions and stress treatments

Seeds of *G. soja* 07256 and *G. soja* 50109 were obtained from the Jilin Academy of Agricultural Sciences (Changchun, China). *G. max* cultivar Suinong 28 and *G. max* cultivar Hefeng 55 were obtained from the Chinese Crop Germplasm Information System. For gene expression analysis, seedlings of *G. soja* 07256 were grown in a culture room with the following settings: 60 % relative humidity, 24 °C and a light regime of 16 h light/8 h dark. The light source SON-T ARGO 400 W generated constant illumination of 30,000 lx. Before sowing, seeds of *G. soja* 07256 were shaken for 10 min in 98 % sulfuric acid. Subsequently, seeds were washed five times with sterile water. Nineteen days after sowing, seedlings in the stress treatment group were transferred into 1/4 strength Hoagland's solution with 200 mM NaCl for salt stress and 50 mM $NaHCO_3$ for alkali stress, respectively. Equal amounts of leaves and roots were sampled at eight time points, 0, 1, 3, 6, 9, 12, 24 and 48 h.

For *GsSKP21* expression analysis in different soybean varieties, 20 seeds of *G. soja* 07256, *G. soja* 50109, *G. max* Suinong 28 and *G. max* Hefeng 55 were placed on each petri dish to accelerate germination for 2 days. Germinated seedlings were then transferred into 1/4 strength Hoagland's solution. Nineteen days after sowing, seedlings in the stress treatment group were transferred into 1/4 strength Hoagland's solution with 50 mM $NaHCO_3$. Equal amounts of roots were sampled at 0 and 1 h.

Arabidopsis thaliana ecotype Columbia (Col-0), used for transformation, was grown in a greenhouse under controlled environmental conditions (21–23 °C, 100 μmol photons/m^2 s, 60 % relative humidity, 16 h light/8 h dark cycles). For the expression analysis of alkali stress and ABA response marker genes, seeds from wild-type (WT) and *GsSKP21* overexpression (OX) lines were sown on filter paper saturated with 1/2 MS solution. After 14 days of growth, seedlings were saturated with water (control), $NaHCO_3$ (50 mM) or ABA (100 μM). Rosette leaf samples were collected from three biological replicates at 0 and 6 h after treatment.

Quantitative real-time PCR

Total RNA was extracted from *G. soja* or *Arabidopsis* seedlings, and cDNA synthesis was performed using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) with the Oligo(dT)18 reverse primer. Prior to qRT-PCR assays, cDNA quality was assessed by PCR using specific primers for *GADPH* (glyceraldehyde-3-phosphate dehydrogenase, accession no. DQ355800) to exclude genomic DNA contamination. The qRT-PCR was performed using the SYBR Premix ExTaq™ II Mix (TaKaRa, Shiga, Japan) on an ABI 7500 sequence detection system (Applied Biosystems, USA). One microliter of each synthesized cDNA (diluted 1:5) was used as template. Amplifications of *GAPDH* in *G. soja* and *ACTIN2* in *Arabidopsis* were used as controls. Relative intensities were calculated and normalized as described previously (Willems et al. 2008).

Transformation of *Arabidopsis*

The coding regions of *GsSKP21* were amplified from pGEM-T-*GsSKP21*. The sequence was inserted into the pCAMBIA2300 under the control of the strong constitutive CaMV35S promoter with *Sal*I and *Bam*HI linker using primers 5′-GCGTCGACATGTCAGAAATTGACATG GCAGTTAT-3′ and 5′-CGGGATCCTCAAGCGTTTCGC CTCAGAAAACTAT-3′. The construct was introduced into *Agrobaterium tumefaciens* strain LBA4404, and transgenic *Arabidopsis* plants were generated by floral dip (Clough and Bent 1998). Transformants were selected on 1/2 MS medium containing 50 mg/l kanamycin. Seeds from each T_1 plant were individually collected. Selected T_2 plants were propagated, and homozygous overexpression lines were confirmed by qRT-PCR analysis.

Phenotypic analysis of transgenic *Arabidopsis* plants

All *Arabidopsis* seeds were sterilized by 5 % NaClO and stored at 4 °C in the dark for 3–7 days before use. All phenotypic experiments were performed in a controlled environmental chamber under the following conditions: 21–23 °C, 100 μmol photons/m^2 s^1, 60 % relative humidity and 16/8 h day-night cycles.

For germination assays, the seeds of WT and T_3 transgenic *Arabidopsis* were sown on 1/2 MS agar medium supplemented with 7, 8 and 9 mM $NaHCO_3$ or 0.8 and 0.9 μM ABA (Lee et al. 2004). The germination rate was recorded for 6 consecutive days after sowing. On the 10th day, pictures were taken to show the growth performance of each line, followed by measurement of the opening/greening of the leaves. Ninety seeds were used for each experiment, and all experiments were repeated three times.

To characterize stress tolerance at the early seedling stage, WT and OX *Arabidopsis* seeds were germinated and grown on 1/2 MS medium for 7 days, followed by a transfer to fresh medium (in the absence or presence of 8 mM $NaHCO_3$ or 20, 30 μM ABA) for 14 days of vertical growth before being photographed, and the root length was measured (Zhu et al. 2011).

The alkaline tolerance assay at the adult stage was performed using 4-week-old plants grown in pots filled with a 1:1:1 mixture of vermiculite:peat moss:nutrition soil under

controlled environmental conditions (21–23 °C, 100 μmol photons/m^2 s, 60 % relative humidity, 16/8 h day-night cycles). The plants were irrigated with 150 mM $NaHCO_3$ solution every 4 days for a total of 12 days. The photos were taken when the phenotype showed distinct differences.

Protein subcellular localization assay

For subcellular localization assays, the *GsSKP21* gene was amplified with primer pairs: 5′-GCGTCGACATGTCAGAAATTGACATGGCAG-3′ and 5′-CGGGATCCAGCGTTTCGCCTCAGAAAACTA-3′. The product was then cloned into *Sal*I/*Bam*HI-digested pBSKII-eGFP to generate pBSKII-*GsSKP21*-eGFP, in which the *GsSKP21* coding sequence was fused to the N-terminal of eGFP. The plasmids pBSKII-*eGFP* and pBSK II-*GsSKP21-eGFP* were then precipitated onto gold beads and transformed into onion (*Allium cepa*) epidermis as described. Fluorescent protein expression in the epidermis was observed using a confocal laser-scanning microscope (SP5, Leica, Germany).

Statistical analysis

All experiments with each group were performed at least in triplicate. Data were reported as mean ± SD. Data were analyzed statistically by Student's *t* test. Results were considered statistically significant when $P < 0.05$.

Results

Cloning and sequence analysis of the gene *GsSKP21*

GsSKP21 was identified as a putative stress-response gene based on transcriptome sequencing of *G. soja* under alkali stress in our laboratory. The full-length coding region of *GsSKP21* was obtained by homologous cloning with gene-specific primers designed according to the *G. max* cDNA sequence (Glyma09g39480).

A homology search against the Phytozome database showed that *GsSKP21* was homologous to *ASK21*. The similarity of GsSKP21 protein and *Arabidopsis* ASK21 protein is 79 %. Secondary structure prediction of GsSKP21 protein revealed that the N-terminus (amino acid 1–164) of GsSKP21 shared a highly conserved SKP1 domain (PF01466) and a putative POZ domain (PF03931), which are characterized by four helical regions that are conserved among SKP1 and ASK proteins, but in its C terminus (165–364 aa), no typical domain was identified. In addition, the extended C-terminal region of the GsSKP21 protein exhibited little similarity with other SKP proteins listed in the National Center for Biotechnology Information database. We also found that like ASK21, GsSKP21 differs in those well-conserved sites in ASK1-6, 9, 11-16, 18 and 19, which were demonstrated to be important for interaction with CUL1.

Phylogenetic analysis demonstrated that members of the *SKP1-like* family in angiosperms evolved at highly heterogeneous rates and were derived from a single ancestral gene (Kong et al. 2004). To determine the evolutionary relationship of GsSKP21 and SKP plant proteins, we built the phylogenetic tree of the *SKP1-like* gene family of 6 angiosperm species (*Arabidopsis, G. soja, Populus trichocarpa, Brachypodium distachyon, Oryza sativa* and *Zea mays*). Our results showed that members of the *SKP1-like* familywere divided into two groups. Those SKP1-like proteins with an extended C-terminal region such as GsSKP21, ASK20 and ASK21 were clustered into group II, whereas other SKP1-like proteins without an extended C-terminal region were clustered into group I (Fig. 1).

Expression of *GsSKP21* transcripts is induced by alkali and salinity stress in *G. soja*

To obtain further information on the role of *GsSKP21* in response to alkali and salinity stress, qRT-PCR analysis was performed to quantify its expression levels in both leaves and roots after $NaHCO_3$ and NaCl stress treatments. Results are shown in Fig. 2a. The tendencies of alkaline treatment on leaves and roots were similar to salt treatment. Under alkaline treatment, the relative transcript abundance of *GsSKP21* in roots started immediately after treatment and reached a peak at 1 h (2.5-fold); in alkaline-treated leaves, transcript abundance increased slightly at the 1-h point, but peaked at 12 h with a threefold increase. Under salt treatment, the expression of *GsSKP21* in leaves also peaked at 12 h, while in roots it rose and peaked at 3 h. These results indicate that *GsSKP21* is involved in responses to a wide variety of abiotic stresses. Moreover, $NaHCO_3$ and NaCl stress induced *GsSKP21* transcript abundance with different expression patterns from roots to leaves (Fig. 2a). In leaves, the *GsSKP21* transcript levels were fairly stable until 12 h after NaCl and $NaHCO_3$ treatment, where they showed an obvious increase. In roots, we noticed that *GsSKP21* transcripts showed the highest expression earlier than that in leaves for both treatments. This suggests that in the leaves of *G. soja*, *GsSKP21* may have similar but delayed stress-response expression profiles. This is consistent with the roots being the first point of contact with the stress treatments.

Since we screened *GsSKP21* as a putative gene response to alkali stress from *G. soja* 07265, we want to further investigate the *GsSKP21* response to alkali stress in other soybean varieties by using *G. soja* 07265, *G. soja* 50109,

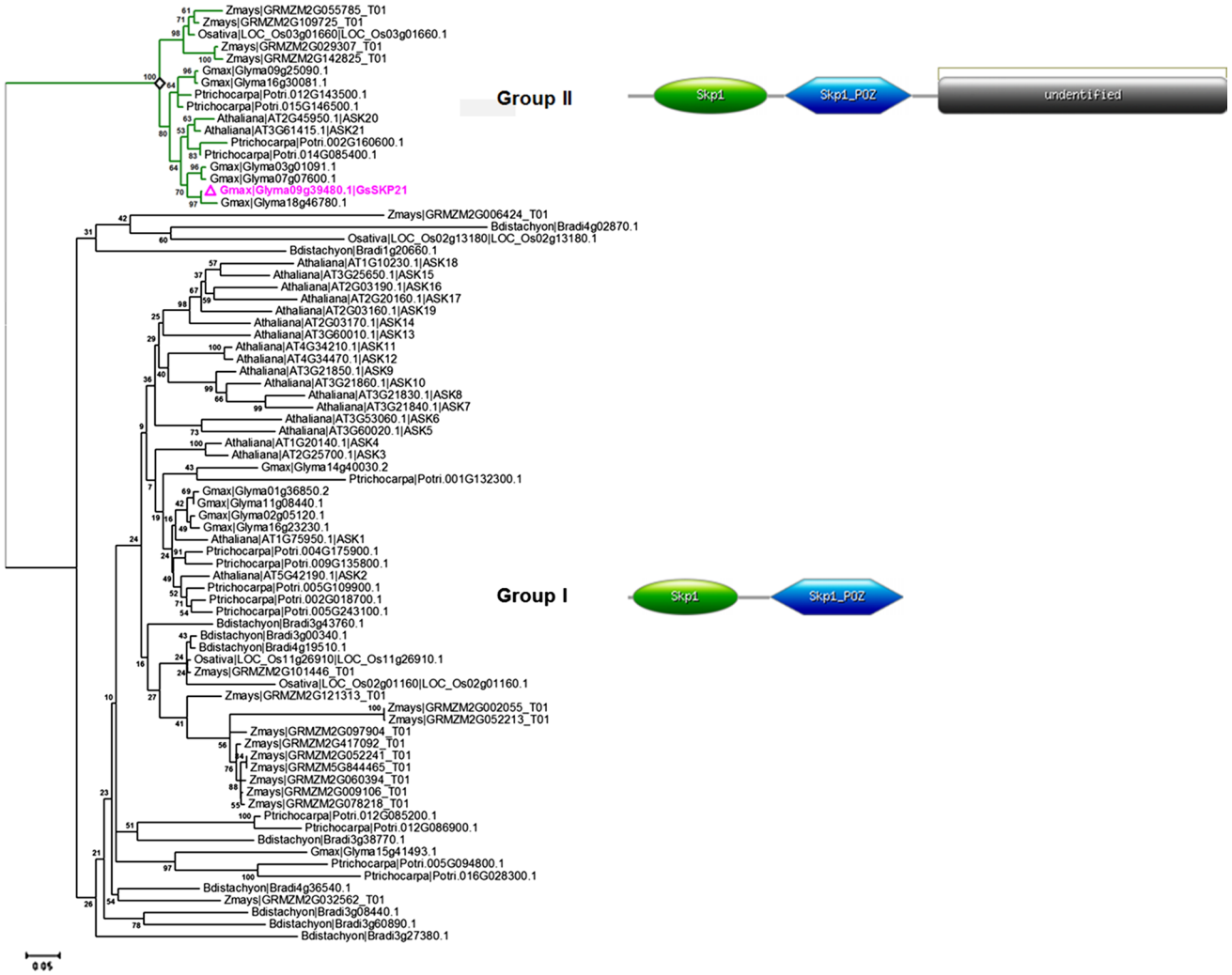

Fig. 1 Phylogenetic tree of the *SKP1-like* family of six species. The phylogenetic tree was constructed using full-length sequences of *SKP1* homologs of *Arabidopsis*, *G. soja*, *Populus*, *B. distachyon*, *O. sativa* and *Z. mays* by the maximum-likelihood method with MEGA 5.0 and a bootstrap value of 1,000. The two major phylogenetic clades are designated as groups I and II. The bar represents the branch length equivalent to 0.05 amino acid changes per residue. Shown on the right are diagrams of representative SKP1-like proteins with information on the structure and position of the SKP domain

G. max Suinong 28 and *G.max* Hefeng 55. As we know, *G. soja* 50109 was one of the highly salt-tolerant species found to tolerate up to 0.9 % of salt during the germination stage (Ji et al. 2006). Comparing with *G. soja* 50109, *G. soja* 07256 showed stronger alkaline tolerance as evidenced by relatively less change in chlorophyll content, relative conductivity and malondialdehyde (MDA) content (Supplementary Table 1). *G. max* Suinong 28 and *G.max* Hefeng 55 were Chinese soybean cultivars that exhibited much lower adaptability to a suboptimal (i.e., stressful) natural environment compared to the wild soybean (*G. soja*) (Ge et al. 2009). In the four varieties, only the germination rate of *G. soja* 07256 can reached 100 % under 100 mM $NaHCO_3$ treatment (Supplementary Figure 2). We determined the transcript expression level of *GsSKP21* using roots of four soybean variety seedlings under $NaHCO_3$ treatment by qRT-PCR (Fig. 2b); the results showed that, under alkali stress, the relative transcript abundance of *GsSKP21* in *G. soja* 07256 increased the highest with threefold, *G. soja* 50109 increased slightly by 1.7 fold, while the expression of *G. max* Suinong 28 and *G. max* Heheng 55, which showed lower tolerance to alkali stress, were relatively unchanged. This result indicates that *GsSKP21* might be an important regulating gene in alkaline-resistant soybean varieties.

Tissue expression pattern of *GsSKP21* in *G. soja*

In *Arabidopsis*, the diverse expression patterns of *ASK* genes suggest that they may regulate different developmental and

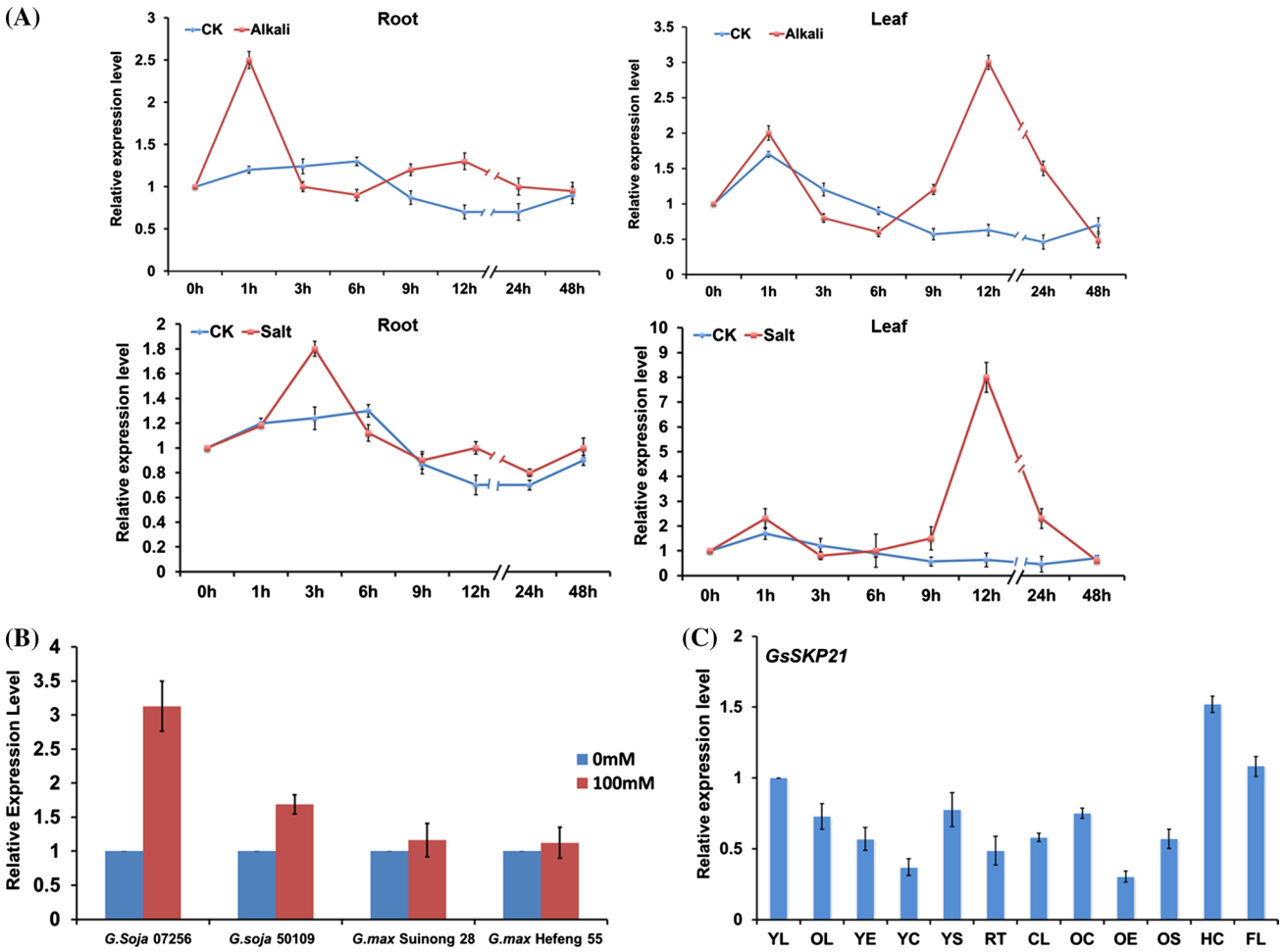

Fig. 2 Expression patterns of *GsSKP21* in *G. soja*. **a** Expression levels of *GsSKP21* were upregulated by salt-alkali stress in roots and leaves. Total RNA was extracted at the indicated time points from leaves and roots of 3-week-old *G. soja* seedlings whose roots were submerged in nutrient solution with 50 mM $NaHCO_3$ and 200 mM NaCl, respectively. Untreated plants were used as controls. Relative transcript levels were determined by qRT-PCR using *GAPDH* as an internal control. The mean value from three fully independent biological repeats and three technical repeats is shown. **b** Expression levels of *GsSKP21* in soybean varieties. Total RNA was extracted from roots of 3-week-old seedlings of four soybean varieties whose roots were submerged into nutrient solution with 50 mM $NaHCO_3$. **c** Tissue-specific expression patterns of *GsSKP21* in *G. soja*. *YL* young leaf, *OL* old leaf, *YE* young embryo, *YC* young seed coat, *YS:* young stem, *RT* root, *CL* epicotyl, *O* Old seed coat, *OE*: old embryo, *OS* old stem, *HC* hypocotyl, *FL* flower. The mean value from three fully independent biological repeats and three technical repeats is shown

physiological processes (Gao et al. 2011; Ge et al. 2010). In order to explore tissue-specific expression patterns of *GsSKP21* in *G. soja*, the expression levels in different tissues were determined by qRT-PCR analysis. As shown in Fig. 2c, *GsSKP21* was expressed in most vegetative tissues and reproductive organs. The *GsSKP21* expression levels were high in the flower, young leaf, young stem and hypocotyl. Among all tissues, the highest expression was observed in the hypocotyl, and the lowest level was detected in the old embryo. These results were consist with the expression pattern of *ASK21*, which also showed high expression levels in the flower, young leaf and young stem (Dezfulian et al. 2012).

Overexpression of *GsSKP21* resulted in enhanced tolerance to alkali stress

To further characterize the function of *GsSKP21* in alkali stress, we investigated whether *GsSKP21* overexpression affects plant tolerance to alkali stress. We compared the germination and growth of OX lines with WT by the $NaHCO_3$ gradient concentration. In the absence of $NaHCO_3$, *GsSKP21* overexpression does not affect plant germination and development under normal conditions, as shown by the similar performance of the WT and OX lines. However, in the presence of $NaHCO_3$, seeds from the *GsSKP21* OX lines were able to develop healthy cotyledons

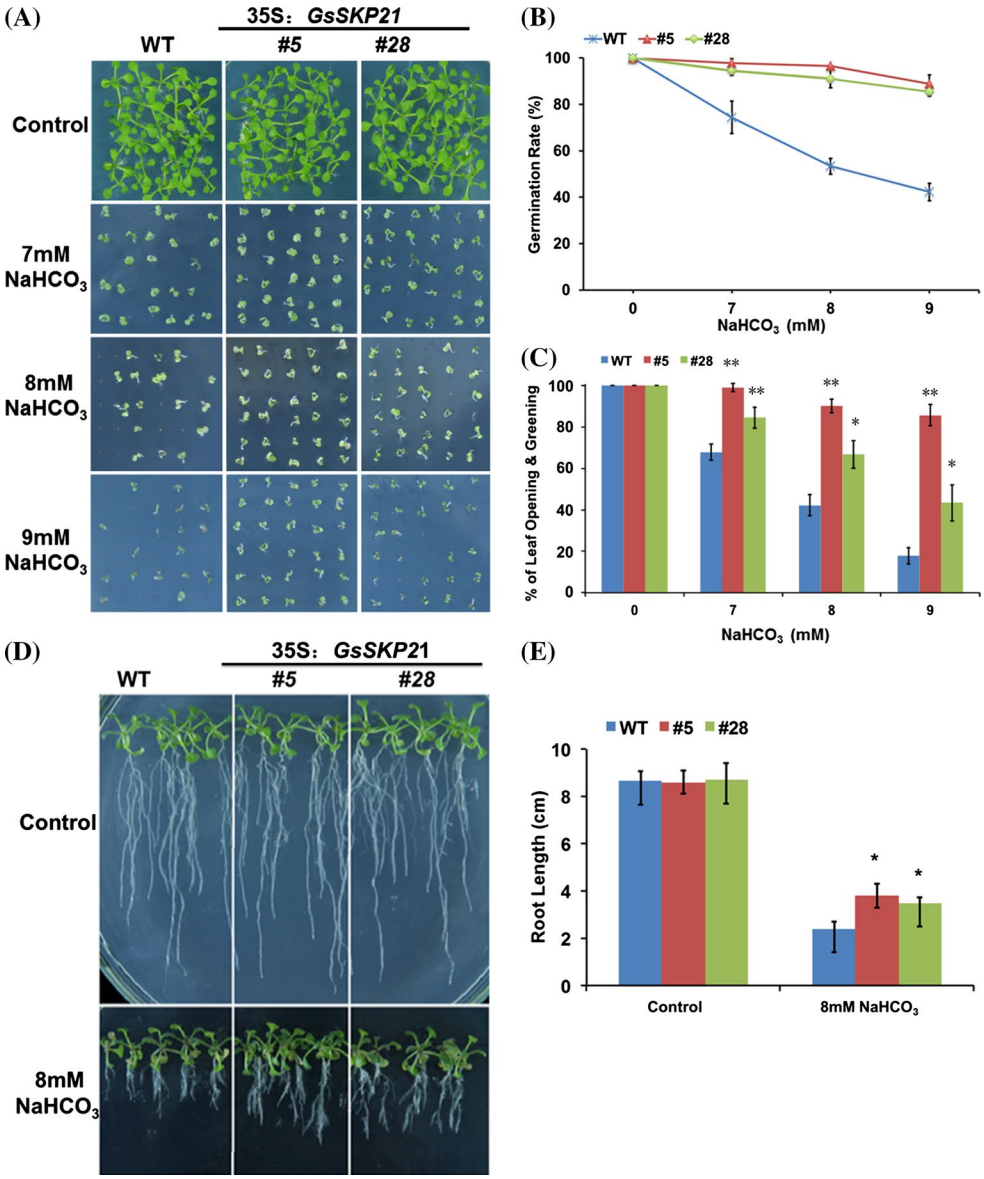

Fig. 3 The improved alkaline tolerance due to overexpression of *GsSKP21*. **a** Growth performance of WT and OX seedlings on 1/2 MS medium with 7, 8 and 9 $NaHCO_3$. Photographs were taken 10 days after stratification. **b** $NaHCO_3$ dose response of germination. Experiments were performed at least three times. The *bars* represent standard errors. The data show means (±SE) of three replicates. $*P < 0.05$, $**P < 0.01$ by Student's *t* test. **c** Quantitative analysis of the leaf opening and greening rate from the WT and OX lines. **d** Phenotypes of WT and OX seedlings grown in 1/2 MS medium with and without 8 mM $NaHCO_3$. **e** Measurements of primary root lengths of seedlings under normal and alkali stress. The data show means (±SE) of three replicates. $*P < 0.05$, $**P < 0.01$ by Student's *t* test

subsequent to seed coat breakage and radicle emergence with higher germination rates (Fig. 3a, b). In addition, on the 10th day after germination, OX lines had a much higher percentage of seedlings with open and green leaves and seedlings with at least four leaves than WT (Fig. 3c). For example, in the presence of 9 mM $NaHCO_3$, the percentage of WT seedlings with fully opened cotyledons was <20 % compared with approximately 40 % for the *GsSKP21* OX seedlings.

GsSKP21 OX also improved plant tolerance to alkali stress at the seedling stage. Seven-day-old seedlings of WT and OX were transferred to 1/2 MS medium supplemented with 8 mM $NaHCO_3$. As shown in Fig. 3d, the growth and development were significantly inhibited in WT plants compared with those of *GsSKP21* overexpression under $NaHCO_3$ treatment. OX seedlings had longer primary roots than WT under alkali stress (Fig. 3e).

Similarly, when 3-week-old soil-grown plants were irrigated with 150 mM $NaHCO_3$ for 2 weeks, the transgenic plants displayed greater alkali stress tolerance than the WT (Fig. 4a). Without $NaHCO_3$ treatment, there was almost no difference in growth and total chlorophyll content among the WT and two transgenic lines. However, after exposure to 150 mM $NaHCO_3$ stress, the total chlorophyll content decreased in both the transgenic and WT plants, and the extent of this decline in transgenic plants was less than that observed for WT (Fig. 4b). These results indicate that *GsSKP21* OX reduces the effects of high alkali stress on chlorophyll formation and enhances the alkaline tolerance of transgenic plants. It is generally accepted that under

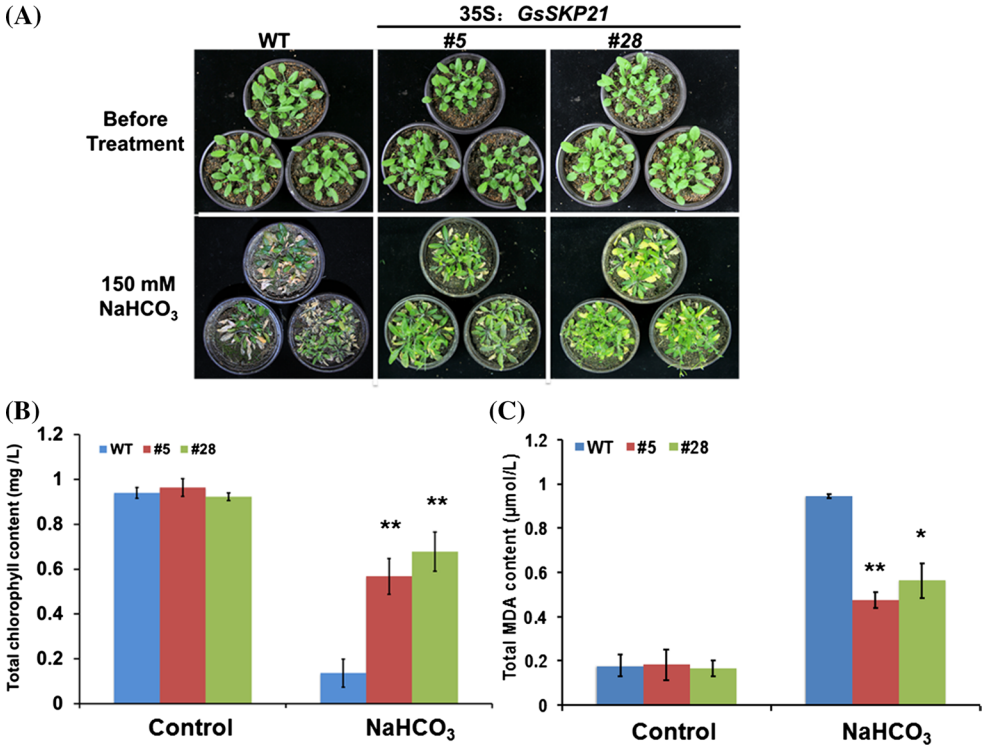

Fig. 4 Enhanced tolerance of transgenic plants to alkaline stress at the adult stage. **a** Phenotypes of WT and OX plants in response to alkali stress. **b** The total chlorophyll content of WT and OX plants. **c** The total MDA content of WT and OX plants. For the alkaline tolerance test at the adult stage, 3-week-old plants were irrigated with 150 mM $NaHCO_3$ solution every 4 days for a total of 12 days. Photos were taken on the 12th day after initial alkali treatment

conditions of abiotic stress, the level of MDA produced during peroxidation of membrane lipids is often used as an indicator of oxidative damage (Kotchoni et al. 2006; Weber et al. 2004). Therefore, we measured the MDA content in the transgenic and WT plants under alkali stress conditions and found that the WT accumulated significantly higher levels of MDA than *GsSKP21* OX lines (Fig. 4c). Together, the data indicate that overexpression of *GsSKP21* enhances alkali stress tolerance in *Arabidopsis*.

GsSKP21 overexpression decreased plant ABA sensitivity at both the seed germination and early seedling stages

It has been reported that overexpression of a homologous gene *TSK1* from wheat can change the *Arabidopsis* response to ABA (Shu et al. 2011). To gain insights into the possible roles of *GsSKP21* in ABA signaling, we examined the responses of *GsSKP21* OX lines to exogenous 0.8 and 0.9 μM ABA. In the absence of ABA, both *GsSKP21* OX lines and WT seeds showed identical germination behavior; however, in the presence of ABA, seed germination was significantly inhibited in WT compared with that in transgenic lines (Fig. 5a). Likewise, in the presence of 0.8 μM ABA, the seed germination rate was 70 % in transgenic lines compared to 40 % in the WT (Fig. 5b). Transgenic lines had more open and green leaves than WT after 12 days (Fig. 5c). These results suggest that *GsSKP21* overexpression interferes with the response to ABA.

Different concentrations of ABA can inhibit both seed germination and seedling growth of *Arabidopsis*. The ABA sensitivity of root growth was further investigated. Seven-day-old seedlings grown on 1/2 MS medium were transferred onto vertical agar plates containing 1/2 MS medium supplemented with 20 μM and 30 μM ABA. When grown under the influence of 20 μM ABA, WT root growth was more inhibited than in *GsSKP21* OX lines (Fig. 5d). The roots of the *GsSKP21* OX lines were significantly longer than those of WT plants (Fig. 5e). These results indicate that *GsSKP21* OX transgenic *Arabidopsis* was less sensitive to exogenous ABA treatment during seed germination and root growth.

GsSKP21 overexpression altered expression patterns of stress responsive and ABA signal-related genes

The induction of numerous stress-inducible marker genes is a hallmark of stress adaptation in plants (Zhu et al. 1997). The alkali phenotype of the *GsSKP21* OX lines

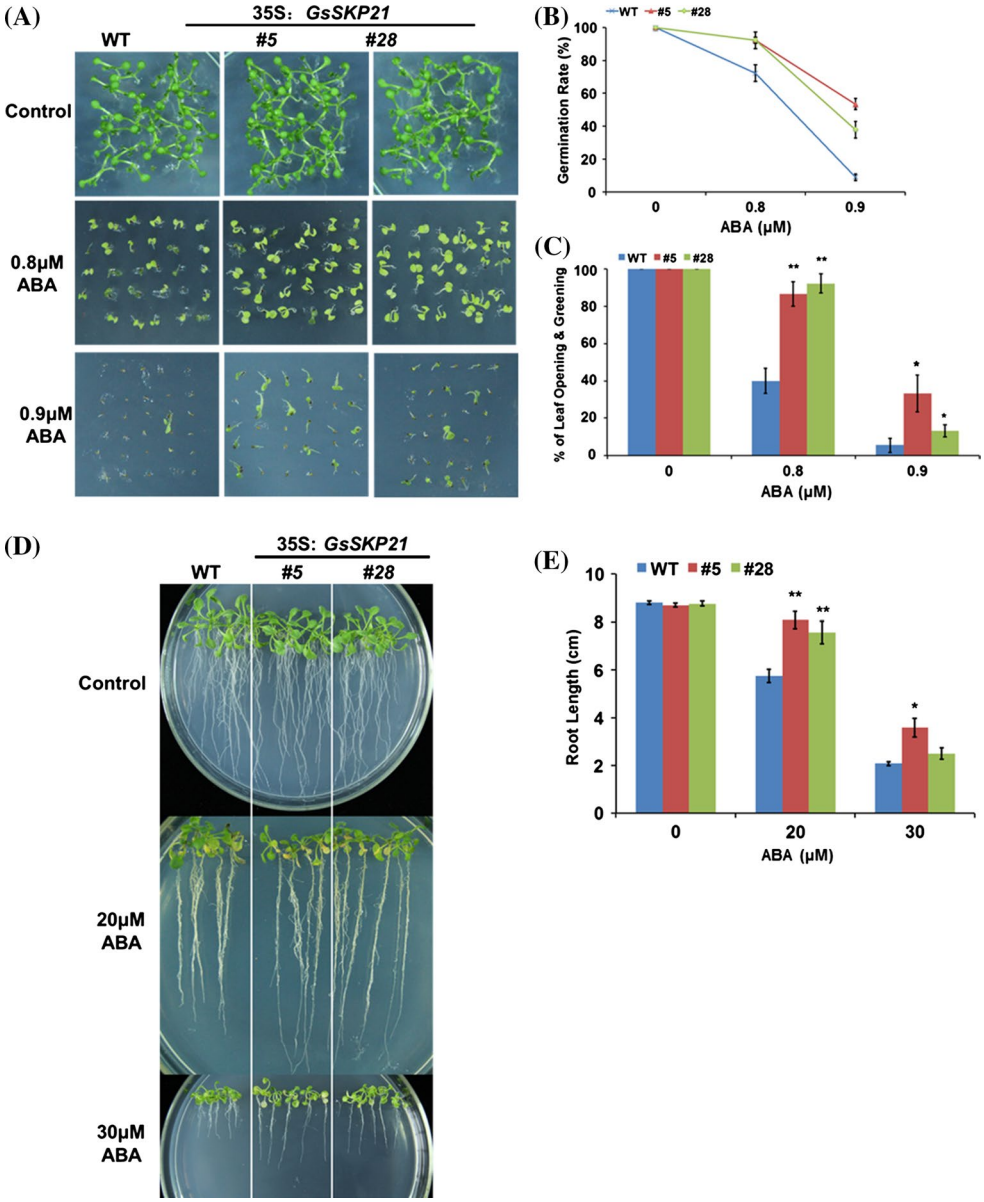

Fig. 5 *GsSKP21* overexpression in *Arabidopsis* resulted in decreased ABA sensitivity. **a** Growth performance of WT and OX seedlings on 1/2 MS medium without or with 0.8 and 0.9 μM ABA. Photographs were taken 10 days after stratification. **b** ABA dose response of germination. Experiments were performed at least three times. The *bars* represent standard errors. **c** Quantitative evaluation of the leaf opening and greening rate. **d** Phenotypes of WT and OX seedlings grown in 1/2 MS medium with and without 20 and 30 μM ABA. Ten-day-old seedlings grown on normal 1/2 MS medium were transferred to new solid agar plates supplemented with 20 and 30 μM ABA. Photographs were taken after 7 days. **e** Measurements of primary root lengths of seedlings under normal and ABA treatment. The data show means (±SE) of three replicates. $*P < 0.05$, $**P < 0.01$ by Student's *t* test

suggests that the expression of some stress-responsive genes might be altered in the *GsSKP21* OX lines. To verify this possibility, expression of some stress inducible genes (*RD29A*, *RAB18*, *COR15A*, *COR47*, *KIN1* and *NCED3*) was examined (Hu et al. 2013; Kong et al. 2004; Santiago et al. 2009; Shinozaki et al. 2003). In the presence of alkali stress treatment, the expression of all of these stress-inducible genes was upregulated in both *GsSKP21* OX lines and WT plants. *RD29A*, *RAB18*, *COR15A* and *KIN1* were upregulated in transgenic lines as compared with WT plants (Fig. 6). *COR47* and *NCED3* of *GsSKP21* OX lines showed higher transcript levels than the WT; however, they exhibited no statistically significant differences. These indicated that the overexpression of *GsSKP21* in *Arabidopsis* resulted in an alteration in the expression of stress-responsive genes.

To further investigate the effect of *GsSKP21* in ABA signaling, we used qRT-PCR to determine the expression level of various ABA-responsive genes in two independent homozygous T_3 transgenic lines, line 5 (L5) and line 28 (L28) (Fig. 7). *ABI1* and *ABI2* encode *PP2Cs* and negatively regulate ABA signaling (Hu et al. 2013; Weber et al. 2004). The qRT-PCR assay showed that the relative expression of *ABI1* was increased by 5.7- and 2.3-fold in L5 and L28, respectively, compared with that in WT. *ABI2* gene expression was increased by more than 15-fold in both L5 and L28 after ABA treatment. SnRK2s are positive regulators of ABA signaling, and their activities are

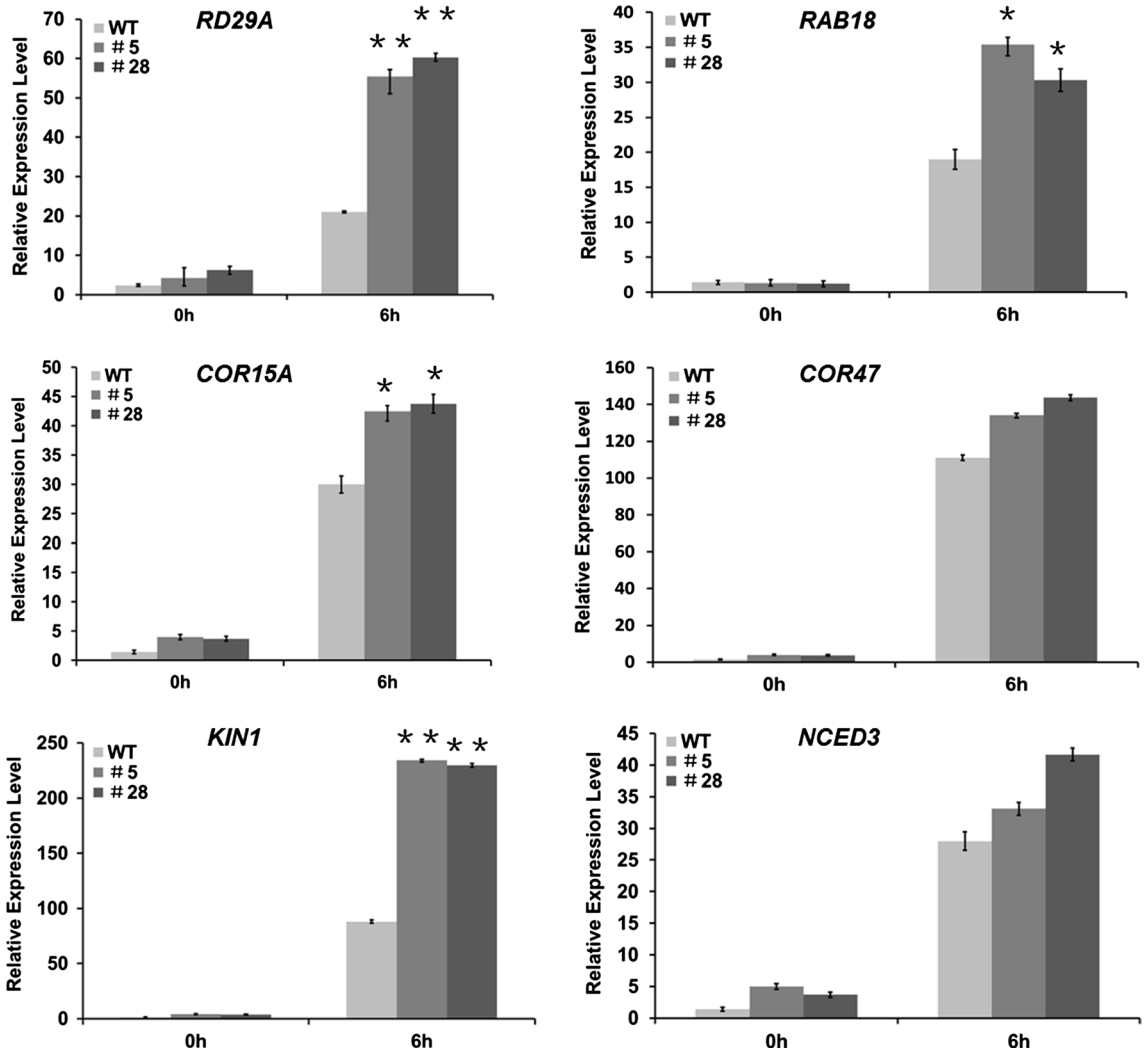

Fig. 6 Expression patterns of stress-induced marker genes in WT and *GsSKP21* transgenic *Arabidopsis* seedlings in response to alkali stress. The induction of stress-responsive genes *RD29A*, *RAB18*, *COR47*, *COR15A*, *KIN1* and *NCED3* were measured by qRT-PCR analysis. Expression of *ACTIN2* was used as an internal control. Values represent the means of three fully independent biological replicates and three technology replicates for each. $*P < 0.05$, $**P < 0.01$ by Student's *t* test

inhibited by PP2C under normal conditions. *SnRK2* genes showed stable expression under ABA stress. Our results showed that the expression levels of *SnRK2.2* and *SnRK2.3* were stable in both WT and OX plants, which is consistent with the results of previous studies. We also detected *ABF4* and *ABI5*, key transcription factors in the ABA signaling pathway (Bhalerao et al. 1999). *ABF4* and *ABI5* in *GsSKP21* OX lines were significantly lower than those in WT, suggesting that the expression of *ABI5* and *ABF4* was negatively regulated by *GsSKP21* in response to ABA signaling. Furthermore, *Pyrabactin Resistance 1* (*PYR1*), a membrane-localized ABA receptor, can be strongly down-regulated by ABA treatment (Park et al. 2009), and the expression of *PYR1* detected in OX plants was lower than in WT. Taken together, the findings consistently show that *GsSKP21* regulates the expression of some key ABA signaling regulators.

GsSKP21 protein targeted to the nucleus in the onion epidermal cells

To better understand the functions of *GsSKP21*, we examine the subcellular distribution of the GsSKP21 protein. The *GsSKP21*-coding sequence was fused in-frame at the 5′ end to eGFP. Confocal imaging showed that the GsSKP21-eGFP fusion protein was localized exclusively in the nuclei of onion (*A. cepa*) epidermal cells in a transient expression assay (Fig. 8). As a control, the eGFP protein alone was found in both the nucleus and cytoplasm.

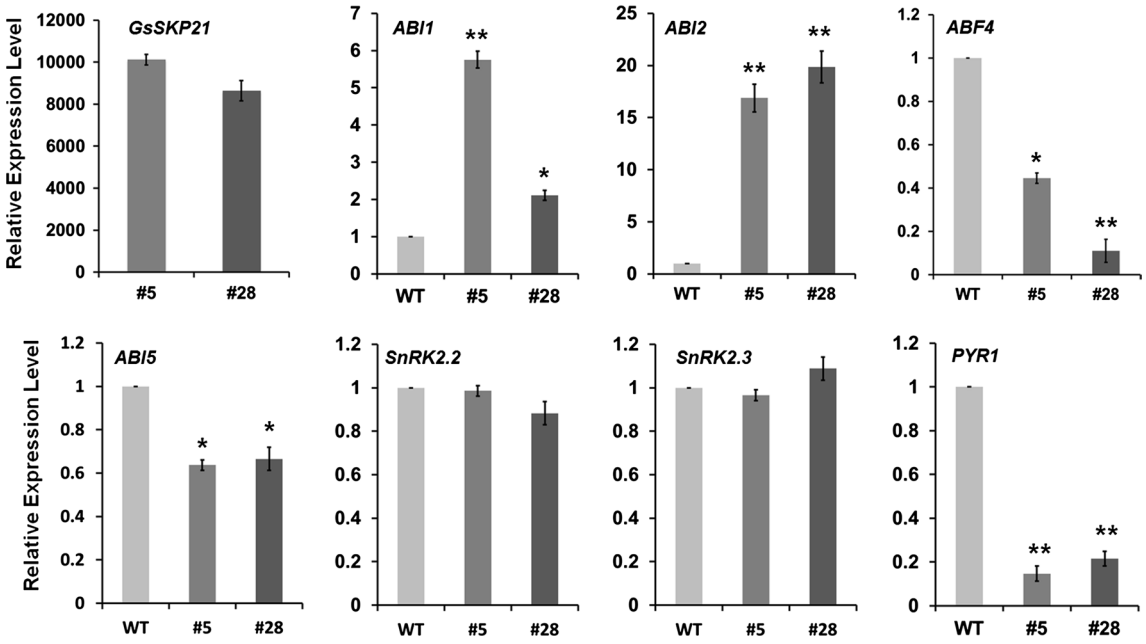

Fig. 7 Expression of ABA-responsive genes in *GsSKP21* overexpressing transgenic lines. Transcription levels of ABA-responsive genes were determined by qRT-PCR with total RNA from 2-week-old seedlings. *ACTIN2* was used as an internal control. Each value represents the means of three fully independent biological replicates, three technology replicates for each. $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t* test

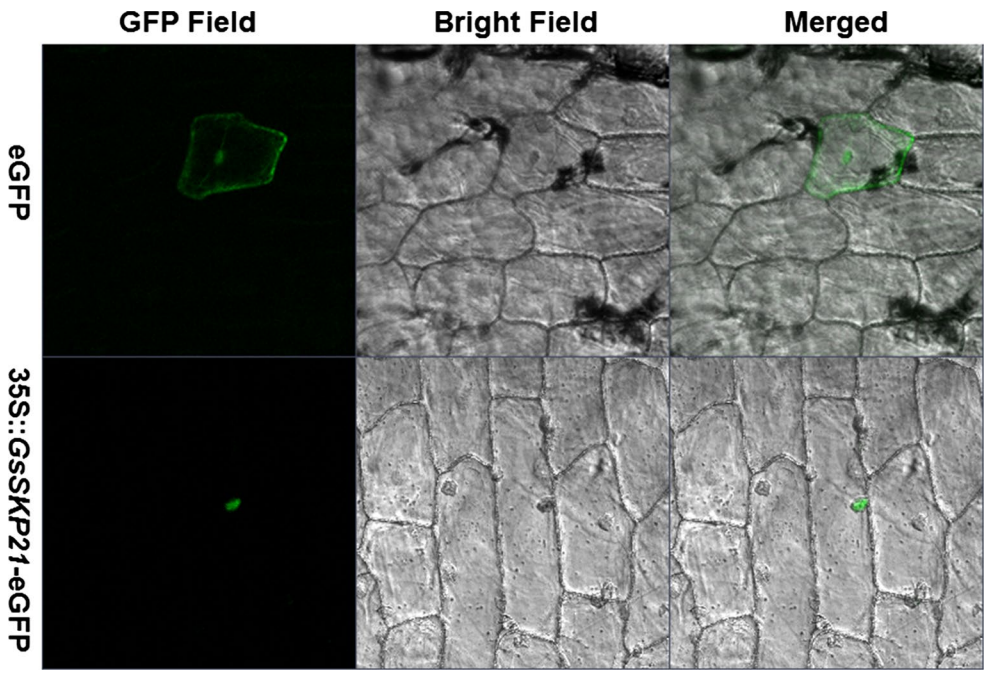

Fig. 8 Subcellular localization of GsSKP21. Subcellular localization assay of the GsSKP21 protein. Images showing onion epidermis cells expressing the eGFP (*upper lane*) or the GsSKP21-eGFP fusion protein (*bottom lane*) examined under fluorescent-field illumination to examine GFP fluorescence (*left*); under bright-field illumination (*middle*); by confocal microscopy (*right*) for an overlay of bright and fluorescent illumination

Discussion

The Western Songnen Plain of China is severely influenced by the effects of alkalization and has become one of the three largest sodic-saline areas in the world (Jin et al. 2006; Wang et al. 2009, 2010). Alkalized soil is one of the major environmental challenges limiting crop growth and productivity globally (Takahashi et al. 2001). Mining alkali-resistant genes and understanding the molecular basis of the plant response to alkali stress will therefore facilitate biotechnological efforts to breed crop plants with enhanced tolerance to high levels of alkali. The wild soybean used in this study can survive in nutrient solutions that contain 50 mM $NaHCO_3$ (pH 8.5) and is an ideal organism for the identification of carbonate stress-response genes (Ge et al. 2010; Zhu et al. 2011). Up to now, only a few of the alkali stress-response genes have been functionally analyzed (Kotchoni et al. 2006). In our previous study, *GsSKP21* was identified as a putative alkaline tolerance gene and was isolated from *G. soja,* providing us with very useful clues for the functional characterization of plant alkali stress adaptation and signal transduction.

Arabidopsis-SKP1-like genes are expressed in a variety of tissues, and they may regulate different developmental and physiological processes (Komatsu et al. 2009; Li et al. 2012; Marrocco et al. 2003). In our study, we observed ubiquitous expression of *GsSKP21* in both vegetative tissues and reproductive organs using qRT-PCR (Fig. 2c). The tissue expression pattern of *GsSKP21* is similar to *ASK20* and *ASK21*, which were found to be expressed in a large number of tissues (Farrás et al. 2001; Gao et al. 2011). *GsSKP21* was identified as a putative alkali response gene in *G. soja* 07256. Therefore, we investigated the expression pattern of *GsSKP21* under alkali treatment. The qRT-PCR results showed that *GsSKP21* was strongly induced by alkali stress (Fig. 2a). The response of *GsSKP21* to alkali stress was further confirmed in different soybean varieties. As we speculated, the highest expression change was observed in *G. soja* 07256. These results suggested *GsSKP21* was involved in the plant response to alkali stress.

It has been concluded from only a handful of reports that *SKP* genes are involved in plant tolerance to abiotic stresses. The overexpression of a wheat *SKP1* homolog resulted in an enhanced stomatal closure response to ABA and enhanced drought tolerance in transgenic plants (Li et al. 2006). As a component of E3 ubiquitin ligases, *SKP1* has been reported to regulate abiotic stress signal transduction in *Arabidopsis* (Lee and Kim 2011); however, little is known about the function of *SKPs* in alkali stress. In our study, we demonstrated that overexpression of *GsSKP21* improved plant tolerance to alkali stress by comparing the growth performance. The results of plate germination assays demonstrated that transgenic plants exhibited much better growth performance than WT with much higher germination rates and more green, open leaves (Fig. 3a–c); *GsSKP21* transgenic lines showed longer primary roots at the seedling stage (Fig. 3d, e); *GsSKP21* overexpression enhanced plant alkali resistance at the adult seedling stage with higher chlorophyll content (Fig. 4a, b). In addition, the ability of *GsSKP21* to upregulate *RD29A*, *RDAB18*, *COR15A* and *KIN1* (Fig. 6) suggested that it may be an important regulator of alkali resistance and play a key role in coordinating the expression of many aspects of plant stress-related marker genes.

ABA plays a pivotal role in coordinating the adaptive response to abiotic stress (Hartung et al. 2005). It has been reported that *ASK1* and *ASK2* genes participate in the ABA signaling pathway. *ASK1/2* overexpression rescued ABA sensitivity in *abi5-1* mutants (Shu et al. 2011). In this study, we found that *GsSKP21* overexpression also led to a decrease in plant sensitivity to ABA regarding the seed germination and root growth (Fig. 5). Moreover, *GsSKP21* overexpression altered the expression patterns of ABA-responsive genes. The expression levels of the basic leucine zipper (bZIP) transcription factor genes *ABI5* and *ABF4* are decreased in *GsSKP21* overexpressing *Arabidopsis*. Meanwhile, the expression of *ABI1* and *ABI2*, two negative regulators of the ABA signaling pathway, is induced. Moreover, ABA treatment significantly decreased the expression level of *PYR1*, which is a membrane-localized ABA receptor and can be strongly downregulated by ABA treatment (Santiago et al. 2009); the expression of *PYR1* in *GsSKP21* OX plants was obviously decreased compared to WT. The expression patterns of these genes are consistent with the ABA decreased sensitivity phenotype of *GsSKP21* transgenic *Arabidopsis* lines. It has been reported that SKP1/ASK1 recognized homologous C-terminal segments of *Arabidopsis* SnRKs that are also implicated in the binding of the SnRK inhibitor PYR1/PRL1 WD protein. Moreover, PYR1/PRL1 reduced the interaction of SKP1/ASK1 with SnRKs, and SKP1-SnRK protein kinase interactions mediate the proteasomal binding of a plant SCF ubiquitin ligase. It can be speculated that *GsSKP21* might mediate the ABA pathway by protein ubiquitination and degradation (Bhalerao et al. 1999; Farrás et al. 2001). The overexpression of *GsSKP21* results in a constitutively enhanced tolerance to alkali stress, and decreased sensitivity to ABA implies that *GsSKP21* may regulate plant alkali stress tolerance by promoting the expression of alkali defense genes within the ABA signaling pathway.

In addition, we noticed that the effect of *GsSKP21* on the ABA pathway differs from that of *ASK1* and *ASK2*, possibly because *GsSKP21* contains changed conserved amino acid sites and extra C terminal regions (Fig. 1). In *Arabidopsis*, the N-terminal regions of these ASK

proteins and SKP1 are postulated to be CUL1-binding sites, and the N-terminal regions of the GsSKP21 proteins showed low levels of similarity with those of other ASK proteins, with the exception of ASK20, 21. Crystal structural analysis of the human SCF complex revealed that the last four α-helices in the C-terminal domain of SKP1 were directly attached to the F-box protein (Schulman et al. 2000). The GsSKP21 protein, with extra C terminus regions, may prevent certain F-box proteins from forming SCF complexes by competing with other ASK proteins that are able to act as components of the SCF complex (Ogura et al. 2008). It may be speculated that GsSKP21 and ASK1 compete with certain F-box proteins because of their difference in amino acids. The end effect would be the different sensitivities to ABA between *GsSKP21*- and *ASK1*-overexpressing lines.

In summary, we have cloned and characterized an *SKP1-like* homolog, *SKP21,* from *G. soja*. We found that it can modify plant alkali stress tolerance and ABA signaling responses. It will be interesting to discover in detail how *GsSKP21* regulates plant stress tolerance and ABA signal transduction. Further investigations should elucidate the relationship between alkali stress and the ABA signaling pathway as well as the role of *GsSKP21* in the crosstalk of ABA and ABA-mediated signaling systems during plant stress responses.

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