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SKP1 is involved in abscisic acid signalling to regulate seed germination, stomatal opening and root growth in *Arabidopsis thaliana*

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ABSTRACT

Abscisic acid (ABA) regulates many aspects of plant development, including seed dormancy and germination, root growth and stomatal closure. Plant SKP1 proteins are subunits of the SCF complex E3 ligases, which regulate several phytohormone signalling pathways through protein degradation. However, little is known about SKP1 proteins participating in ABA signalling. Here, we report that the overexpression of *Triticum aestivum SKP1-like 1* (*TSK1*) in *Arabidopsis thaliana* (Arabidopsis) resulted in delayed seed germination and hypersensitivity to ABA. The opening of stomatal guard cells and the transcription of several ABA-responsive genes were affected in transgenic plants. In contrast, Arabidopsis *skp1-like 1* (*ask1*)/*ask1 ASK2*/*ask2* seedlings exhibited reduced ABA sensitivity. Furthermore, the transcription of *ASK1* and *ASK2* was down-regulated in *abi1-1* and *abi5-1* mutants compared with that in wild type. *ASK1* or *ASK2* overexpression could rescue or partially rescue the ABA insensitivity of *abi5-1* mutants, respectively. Our work demonstrates that SKP1 is involved in ABA signalling and that *SKP1-like* genes may positively regulate ABA signalling by SCF-mediated protein degradation.

Key-words: ABA; Arabidopsis; *ASK1*; *ASK2*; *TSK1*.

INTRODUCTION

S-phase kinase-associated protein 1 (SKP1) is a component of a Skp1-Cullin1-F-box (SCF) complex (Hotton & Callis 2008) that facilitates ubiquitin-mediated protein degradation in eukaryotes. 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 acts as an adaptor to link Cullin1 (Cul1) to F-box protein, which specifically recognizes different target proteins via a variable C-terminal domain and interacts with SKP1 through an N-terminal structural motif (Bai *et al*. 1996; Connelly & Hieter 1996).

SKP1 homologues have been identified in eukaryotes since the discovery of *P19*, the first *SKP1*, in humans (Zhang *et al*. 1995). In yeast, analysis of *skp1* mutants indicated that SKP1 is vital for both the transition from G1 to S and from G2 to M phases (Bai *et al*. 1996; Connelly & Hieter 1996). In plants, *Arabidopsis Skp1-Like 1* (*ASK1*) was the first *SKP1* homologue isolated. Mutation of *ASK1* resulted in male sterility and some defects in organ development (Yang *et al*. 1999). ASK1 interacts with unusual floral organs (UFO) to regulate the expression of the floral organ identity genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Zhao *et al*. 1999) and with reduced male fertility (RMF) to regulate tapetum degeneration (Kim, Jung & Park 2010). The root growth of the *ask1-1* mutant was insensitive to exogenous auxin treatment, suggesting that ASK1 is involved in auxin response (del Pozo *et al*. 2002). *ASK2*, another *SKP1* homologue in Arabidopsis, was able to substitute for *ASK1* during male meiosis (Zhao *et al*. 2003a). Interestingly, *ASK1* and *ASK2* together were essential for normal embryo development and seedling viability according to the analysis of *ask1*/*ask2* double mutants (Liu *et al*. 2004). There were 21 predicted *SKP1* homologues in the Arabidopsis genome; *SKP1-like* genes were found in other plant species (Kong *et al*. 2007), including wheat (Li *et al*. 2006) and rice (Sasaki *et al*. 2003).

Many F-box proteins, such as UFO and RMF, have been found to interact with ASK1 or ASK2, suggesting that they could form various SCF complexes (Gagne *et al*. 2002; Zhao *et al*. 2003a). SCF complexes have been demonstrated to be involved in various hormone signalling pathways, such as the auxin, gibberellin, jasmonate, ethylene and brassinosteroid pathways (Dreher & Callis 2007; Somers & Fujiwara 2009). Among the four components of the SCF complex, the F-box proteins *Arabidopsis thaliana* TUBBY-like protein 9 (AtTLP9) and drought tolerance repressor (DOR) were found to be involved in abscisic acid (ABA) signalling and

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interact with ASK1 (Lai *et al*. 2004; Dreher & Callis 2007) and ASK14 (Zhang *et al*. 2008), respectively. However, no direct evidence indicated that SKP1 protein is involved in ABA signalling.

ABA is a key plant hormone that regulates many physiological processes, including the maintenance of seed dormancy, inhibition of seed germination and seedling growth, control of stomatal closure and protection against various environmental stresses. Recent data demonstrated that ABA is transported into the cytosol by ATP-binding cassette (ABC) transporters localized at the plasma membrane (Kang *et al*. 2010; Kuromori *et al*. 2010). Significant progress was made by the discovery of a soluble ABA receptor in 2009 (for review, see Raghavendra *et al*. 2010). RCARs/ PYLs were identified as the ABA receptor that can bind to ABA and interact with group A protein phosphatase 2C (PP2C) to inhibit the activity of the phosphatase (Ma *et al*. 2009; Park *et al*. 2009). Several group A PP2Cs have been found to be the negative regulators of early ABA signalling including ABI1 and ABI2 (Leung, Merlot & Giraudat 1997), HAB1 and HAB2 (Leonhardt *et al*. 2004; Saez *et al*. 2004), and AHG1 and AHG3 (Yoshida *et al*. 2006; Nishimura *et al*. 2007).

In the presence of ABA, the inactivation of PP2Cs allows the release of the *SNF1*-related kinase 2 (SnRK2), which phosphorylates the downstream targets, including the basic leucine zipper (bZIP) transcription factors and ion channels (Geiger *et al*. 2009; Sato *et al*. 2009). The regulation of SnRK2 activity by PP2Cs is the upstream step in ABA signal transduction (Nakashima *et al*. 2009). The transcription factors playing a positive role include ABI3 of B3-containing proteins (Giraudat *et al*. 1992), bZIP-type transcription factors (ABI5, ABF1, ABF2, ABF3 and ABF4) (Finkelstein & Lynch 2000; Kang *et al*. 2002; Choi *et al*. 2005; Finkelstein *et al*. 2005; Fujita *et al*. 2005), the AP2-type transcription factor ABI4 (Finkelstein *et al*. 1998), the bHLH-type member AtMYC2 and the MYB family member AtMYB2 (Abe *et al*. 2003). Constitutive expression of ABI3, ABI4 and ABI5 confers hypersensitivity to ABA (Finkelstein *et al*. 1998; Lopez-Molina, Mongrand & Chua 2001; Lopez-Molina *et al*. 2002). These transcription factors regulate downstream ABA-regulated genes such as *RD29B* containing ABRE *cis* elements (Himmelbach, Yang & Grill 2003; Nakashima *et al*. 2006) and *SOMONS* containing RY *cis* elements (Park *et al*. 2011).

Another ABA receptor, ABAR/CHLH, directly interacts with the transcription factor WRKY40 at high level of ABA to recruit WRKY40 from the nucleus to the cytosol and induce *ABI5* expression to trigger downstream physiological processes (Shen *et al*. 2006; Pandey, Nelson & Assmann 2009). ATHB6 from the homeodomain-leucine zipper transcription factor family (Himmelbach *et al*. 2002) and several WRKY family transcription factors (Shang *et al*. 2010) are negative regulators of ABA signalling.

Although *ASK1* and *ASK2* are required for the signal pathways of several plant hormones, the role of *SKP1* in ABA signalling remains unclear. In this study, we investigated the relationship between SKP1 and ABA signalling based on the ABA-related phenotypes, taking a clue from the transgenic Arabidopsis lines overexpressing *Triticum aestivum SKP1-like 1* (*TSK1*), an *SKP1* homologue from wheat. We also examined the functions of *ASK1* and *ASK2* in the ABA signalling pathway using the Arabidopsis mutants.

MATERIALS AND METHODS

Plant materials

The *TSK1*-overexpressing transgenic lines and *ask1-1* mutant were in the *A. thaliana* C24 and Landsberg *erecta* ecotypes, respectively. The *ASK1*- and *ASK2*-overexpressing transgenic lines were in the Col-0 ecotype. The *ASK1/ask1 ask2/ask2* plants and *TSK1*-overexpressing transgenic Arabidopsis were previously described (Liu *et al*. 2004; Li *et al*. 2006). The *ASK1/ask1 ask2/ask2* plant was crossed with the wild type, and seeds from the *ASK1/ask1 ASK2/ask2* plants were collected for further analysis. The *abi1-1* and *abi5-1* mutants were described previously (Leung *et al*. 1997; Finkelstein & Lynch 2000). Arabidopsis seeds were maintained at 4 °C for 3 d in the dark to break residual dormancy, and were grown in a greenhouse at 22 °C under a 16 h light/8 h dark cycle.

PCR-based genotyping in mutants

Primers were designed to amplify *ASK1*, *ask1-1*, *ASK2* and *ask2-1* alleles according to previous reports (Xu *et al*. 2002; Liu *et al*. 2004; Ni *et al*. 2004) with slight modifications. The wild-type *ASK1* allele was confirmed by A1 (5′-ATG TCT GCG AAG AAG ATT GTG-3′) and oMC383 (5′-GAA GAT AGT CAT GAT TCA TGA AG-3′) primers. The Ds insertion in the *ask1-1* mutant was determined by A1 and oMC490 (5′-CGT TCC GTT TTC GTT TTT TAC C-3′). The *ASK2* wild-type allele was amplified by B1 (5′-ATG TCG ACG GTG AGA AAA ATC-3′) and oMC593 (5′-AAA TGG GTC GAG GAC ATG AC-3′). The T-DNA insertion in *ask2-1* mutant was confirmed by B1 and 320T (5′-CAT TTT ATA ATA ACG CTG CGG ACA TCT AC-3′). The *abi5-1* mutant was genotyped by dCAPS (Neff *et al*. 1998) with the primers for bZIPFOR (5′-CAA TCA ACA ACA AGC AGC AG-3′) and bZIPREV (5′-TCT CTC CAC TAC TTT CTC CAC-3′) (Supporting Information Fig. S2). The wild-type allele produces three DNA fragments of 686, 114 and 26 bp after *Ava*II digestion of the PCR products, whereas the *abi5-1* allele only produces two bands of 800 and 26 bp.

Transformation vectors and construction of transgenic plants

To generate *ASK1* and *ASK2* overexpressing transgenic plants, the full-length cDNAs of *ASK1* (At1g75950) and *ASK2* (At5g42190) were amplified, and the fragments were cloned to the *Bam*HI-*Kpn*I site of the pSN1301 vector (Li *et al*. 2006), in which the transgene expression is under the

control of the CaMV 35S promoter. Transformation of the Arabidopsis ecotype Col-0 was performed by the floral dip method using *Agrobacterium tumefaciens* strain C58 (Clough & Bent 1998). For the phenotypic analysis, the T3 or T4 homozygous lines were used. T3 homozygous transgenic lines were selected through screening seeds harvested from T2 plants on the 1/2 MS medium containing 20 μg mL^{-1} hygromycin. To generate the *35S::ASK1*/*abi5-1* and *35S::ASK2*/*abi5-1* plants, T2 *ASK1* and *ASK2* overexpressing transgenic lines were crossed with the *abi5-1* mutant, respectively, and F2 plants were selected by hygromycin to obtain homozygous plants of *35S::ASK1*/*abi5-1* and *35S::ASK2*/*abi5-1*. The *abi5-1* mutant was identified mentioned above. Homozygous plants of *35S::ASK1*/*abi5-1* and *35S::ASK2*/*abi5-1* were used for phenotypic analysis.

Germination assay and the response of the root to ABA

Seeds were collected at the same time. For aseptic growth, seeds were treated with 0.2% Triton X-100 and 10% sodium hypochlorite for 10 min, washed six times with sterile water and put on plates with 1/2 MS medium solidified with 0.8% agar. After incubation at 4 °C for 3 d, the plates were transferred to the greenhouse for germination. The fully emerged radicle tip or the fully opened cotyledon and turning green was used as a standard for identifying the germinated seeds.

Seeds germinated on 1/2 MS plates for 4 d in the greenhouse. The surviving seedlings were transferred to the MS medium containing different concentrations of ABA, and the position of each primary root tip was marked. After 3 or 4 d, the root length was measured. The relative root length of each genotype was the root elongation on 1/2 MS medium containing ABA divided by that without ABA.

Measurement of stomatal aperture

The stomatal aperture was measured on epidermal strips isolated from the abaxial side of leaves of 4–5-week-old plants. Methods for incubation and ABA treatment were described previously (Ephritikhine *et al*. 1999). Photographs were taken under a Zeiss microscope, and the stomatal aperture was measured by use of ImageJ 1.34 s (http://rsb.info.nih.gov/ij/). Leaves of 10 different plants of the wild-type and transgenic lines were used in each experiment.

Measurement of ABA content

The ABA content in 3-week-old whole plants was measured by high-performance liquid chromatography (HPLC). Fresh materials were carefully weighed, frozen in liquid nitrogen and dried by speed vacuum. The freeze-dried samples were homogenized in 80% methanol in the dark and incubated at 4 °C for 15 h. Samples were centrifuged at 3000 *g* for 20 min, and the supernatant was collected. The pellet was re-suspended in 2 mL of 80% methanol, stirred for 5 min and centrifuged again at 3000 *g* for 20 min. The two supernatants were combined, lyophilized, dissolved in 8 mL of 0.1 M ammonium acetate (pH 9.0) and centrifuged at 27 000 *g* for 20 min. The ABA content in the supernatant was purified by a PVP, a DEAE Sephadex A-25 and a C18 Sep-pak column (Classical; Waters, Milford, MA, USA), and then washed with 50% methanol. The extract was subjected to HPLC (Waters Spherisorb, 4.6 × 25 cm) analysis. The fraction was eluted at a speed of 1 mL min^{-1} and detected at 262 nm.

Quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For real-time PCR, 2 μg of total RNA was used for reverse transcription with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). The cDNA samples were diluted 50–100-fold. For each RT-PCR reaction, 15 μL of total volume with gene-specific primers was used with 7.5 μL of SYBR GreenMaster mix (Applied Biosystems, Foster City, CA, USA), 0.25 μL of primers (10 μM) and 5 μL of diluted cDNA sample. Triplicate quantitative assays were performed under an ABI7900HT Fast-Real Time system according to the manufacturer's protocol (Applied Biosystems). The delta-delta Ct method (Yoshida *et al*. 2003) was used to evaluate quantitative variation among replicates. The amplification of *Tubulin* was used as an internal control to normalize all data. Primers are listed in Table 1.

RESULTS

Overexpressing *TSK1* Arabidopsis altered ABA sensitivity regarding germination and root growth

Our previous results indicated that *TSK1*, an *SKP1* homologous gene from wheat, partially rescued the meiotic defect in the *ask1-1* mutant, and *TSK1* overexpressing transgenic Arabidopsis seedlings exhibited an altered response to auxin (Li *et al*. 2006).

As known, ABA controls seed dormancy and germination. The inhibition of seed germination assay demonstrated that the germination of *TSK1*-overexpressing transgenic lines was delayed compared with that of the wild type. Within 16 h, 80% of the wild-type seeds had germinated, whereas less than 20% of the transgenic seeds of five independent transgenic lines had germinated. At the extremeness, few germinated seeds were observed in lines 21 (L21) and 82 (L82) (Fig. 1a,b). This deficient germination suggested that *TSK1* overexpression interferes with the response to ABA. To gain more insights into the possible roles of *TSK1* in ABA signalling, we examined the responses of *TSK1*-overexpressing lines to exogenous ABA. Seed germination was significantly inhibited in transgenic lines compared with that in the wild type (Fig. 1c,d). For example, in the presence of 0.3 μM ABA, less than 30%

Table 1. Primers for qRT-PCR

Gene name	Sequence (5′ to 3′)
TSK1	F: CCAGACTGTTGCTGACATGA
	R: GGCGTAAAGTCGTTCTTGATG
ABF3	F: CTTTGTTGATGGTGTGAGTGAG
	R: GTGTTTCCACTATTACCATTGC
ABF4	F: AGAAAACGAATCAAGAACTGCA
	R: GCTTCGACGTTTCTTTCAGC
ABI1	F: CGGTTCTCAGGTAGCGAAC
	R: TCACCATCGCAGAGCATC
ABI2	F: CACCGGCAGCTTCTATCCTC
	R: ATCACAAACCGGATAGGGATGA
ABI3	F: CATGGAGATTCCATTAGACAG
	R: GGTGTCAAAGAACTCGTTGCTATC
ABI5	F: GTGGAATTGGAAGCTGAACT
	R: TCAAAATACTGTTGCTTCCTC
ADH1	F: TCGATGCAAAGCTGCTGT
	R: TGTTTCTGCGGTGGAGC
ATHB6	F: TCTTTATGTCCTACAACTTCCACA
	R: ATCCTTCAAGCATCGACTGA
RAB18	F: GAACATGGCGTCTTACCAGA
	R: TCGTCATACTGCTGCTGGAT
ERA1	F: TTTCGGTTGCAAGCATCCTA
	R: CCCTCCAATGCCACCTTC
RD29B	F: CTTTGGAAAATGGAGTCACAGT
	R: TTCTGGATGGTGAATTCTGATT
ASK1	F: GAACTCATTCTGGCTGCTAATT
	R: TCTTCTGGAGTCTTTCCTTTGA
ASK2	F: GCTGCGTCGGGTTCTAG
	R: GCAGCCAGGATAAGATCG
Tubulin	F: ATCCGTGAAGAGTACCCAGAT
	R: AAGAACCATGTACTCATCAGC

F, forward primer; R, reverse primer.

of transgenic seedlings had fully opened cotyledons after 4 d. In contrast, 97% of wild-type seedlings had fully opened cotyledons after 4 d. In the presence of 0.9 μM ABA, the percentage of transgenic seedlings with fully opened cotyledons was less than 10%, compared with approximately 40% for the wild type (Fig. 1d).

Likewise, the root growth of *TSK1* transgenic seedlings was also more inhibited by exogenous ABA than the wild type (Fig. 1e). When the 4-day-old seedlings were transferred to a medium containing 0.3 μM ABA, the relative root length of wild-type seedlings was not affected by growth for 4 d, whereas that of both L21 and L82 was suppressed to less than 30% of the control (Fig. 1f). These results indicated that *TSK1*-overexpressing transgenic Arabidopsis was hypersensitive to exogenous ABA treatment during seed germination and root growth.

Endogenous ABA biosynthesis was not affected in *TSK1*-overexpressing lines

To test whether the biosynthesis of ABA was affected in *TSK1*-overexpressing lines, 10 or 30 μM abamine, an inhibitor of ABA biosynthesis (Han *et al*. 2004), was added to the medium for seed germination. As shown in Fig. 2a, the seed germination rate of the wild type increased approximately sevenfold within 12 h under abamine treatment. Germination was also promoted in L21 and L82, but was still lower than that of the wild type. For example, in the presence of 30 μM abamine, the germination rate was about 100% in wild type within 18 h. In contrast, less than 30% of seeds germinated in the transgenic lines. Likewise, when 30 μM abamine and 0.6 μM exogenous ABA were added together in the medium, approximately 70% of wild-type seeds had germinated after sowing for 4 d, compared with approximately 31% in L21 and 25% in L82 (Fig. 2b). The wild type, L21 and L82 exhibited similar levels of ABA (0.680 ± 0.049, 0.780 ± 0.044 and 0.745 ± 0.015 ng g^{-1} fresh weight, respectively) (Fig. 2c), which was not reached to significant difference level in statistics between wild type and the *TSK1* overexpression lines. The data suggested that the overexpression of *TSK1* affects the ABA signalling pathway rather than the endogenous ABA biosynthesis.

Stomatal aperture was hypersensitive to ABA treatment in *TSK1* transgenic lines

It is well known that ABA can induce stomatal closure. To test for the possible effects of *TSK1* overexpression on the ABA response of stomatal cell, epidermal strips were first floated on incubation solution for 2 h under light to fully open the stomata before adding ABA. As shown in Fig. 3a, the mean stomatal width in the transgenic lines (3.86 ± 0.24 μm in L21 and 3.75 ± 0.17 μm in L82) was smaller than that of the wild type (5.12 ± 0.38 μm) in the light. The stomatal aperture of the wild type and transgenic plants decreased with increasing exogenous ABA concentrations, and stomatal aperture response in the transgenic plants was more sensitive to the ABA treatment (Fig. 3b). In addition, transgenic Arabidopsis lines displayed enhanced tolerance to drought stress (Fig. 3c). These data suggested that the overexpression of *TSK1* resulted in an enhanced stomatal closure response to ABA and enhanced drought tolerance in transgenic plants, and that *TSK1* transgenic Arabidopsis was hypersensitive to ABA.

Overexpression of *TSK1* altered expression patterns of ABA-responsive genes in transgenic lines

To further investigate the effect of *TSK1* in ABA signalling, the expression of various ABA/stress-responsive genes in L21 and L82 was determined by quantitative real-time PCR (qPCR) (Fig. 4). *ABI1* and *ABI2* encode PP2Cs and negatively regulate ABA signalling (Leung *et al*. 1997). The qPCR assay indicated that the relative expression level of *ABI1* was increased by 2.6- and 8.5-fold in L21 and L82, respectively, compared with that in wild-type C24. The expression level of *ABI2* was increased by more than 10-fold in both L21 and L82. The homeodomain protein ATHB6, a negative regulator of the ABA signalling

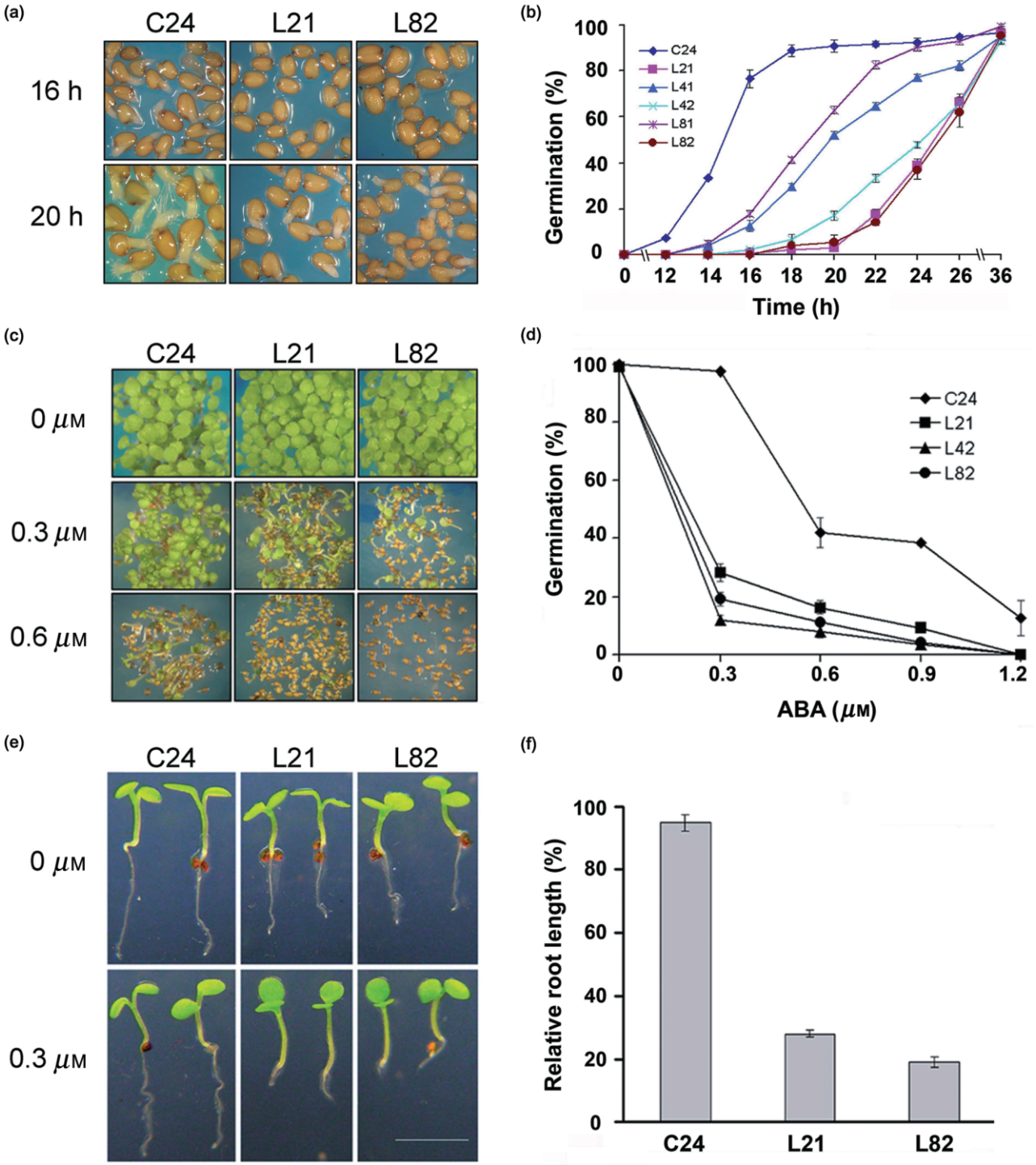

Figure 1. Abscisic acid (ABA) sensitivity of *TSK1*-overexpressing lines. (a) Representative germination at 16 and 20 h. (b) Germination rates of wild type and *TSK1*-overexpressing lines. Seeds of C24 wild type and lines L21, L41, L42, L81 and L82 were plated on 1/2 MS medium and incubated at 4 °C for 3 d, after which the germinated seeds were counted (fully emerged radicle tip) at various times. Each data point represents the mean of triplicate experiments ($n = 50$–200 each). Some standard errors are smaller than the symbols. (c) Germination of representative plants on media containing 0, 0.3 or 0.6 μM ABA. (d) ABA dose–response of germination. Seeds were incubated at 4 °C for 3 d and germinated on 1/2 MS medium containing various concentrations of ABA, and seedlings with fully expanded cotyledons were counted after 4 d. Experiments were performed at least three times ($n > 50$ each). The bars represent standard errors. (e) Growth of transgenic plants on MS medium containing 0 or 0.3 μM ABA. The representative seeds were germinated and grown for 5 d. Bar = 0.5 cm. (f) Response of root growth to ABA. Seeds were germinated on 1/2 MS without ABA for 4 d, and then seedlings ($n > 20$) were transferred to 1/2 MS containing 0.3 μM ABA. The relative root length was measured 4 d later. The experiments were repeated at least three times. Bars represent standard errors.

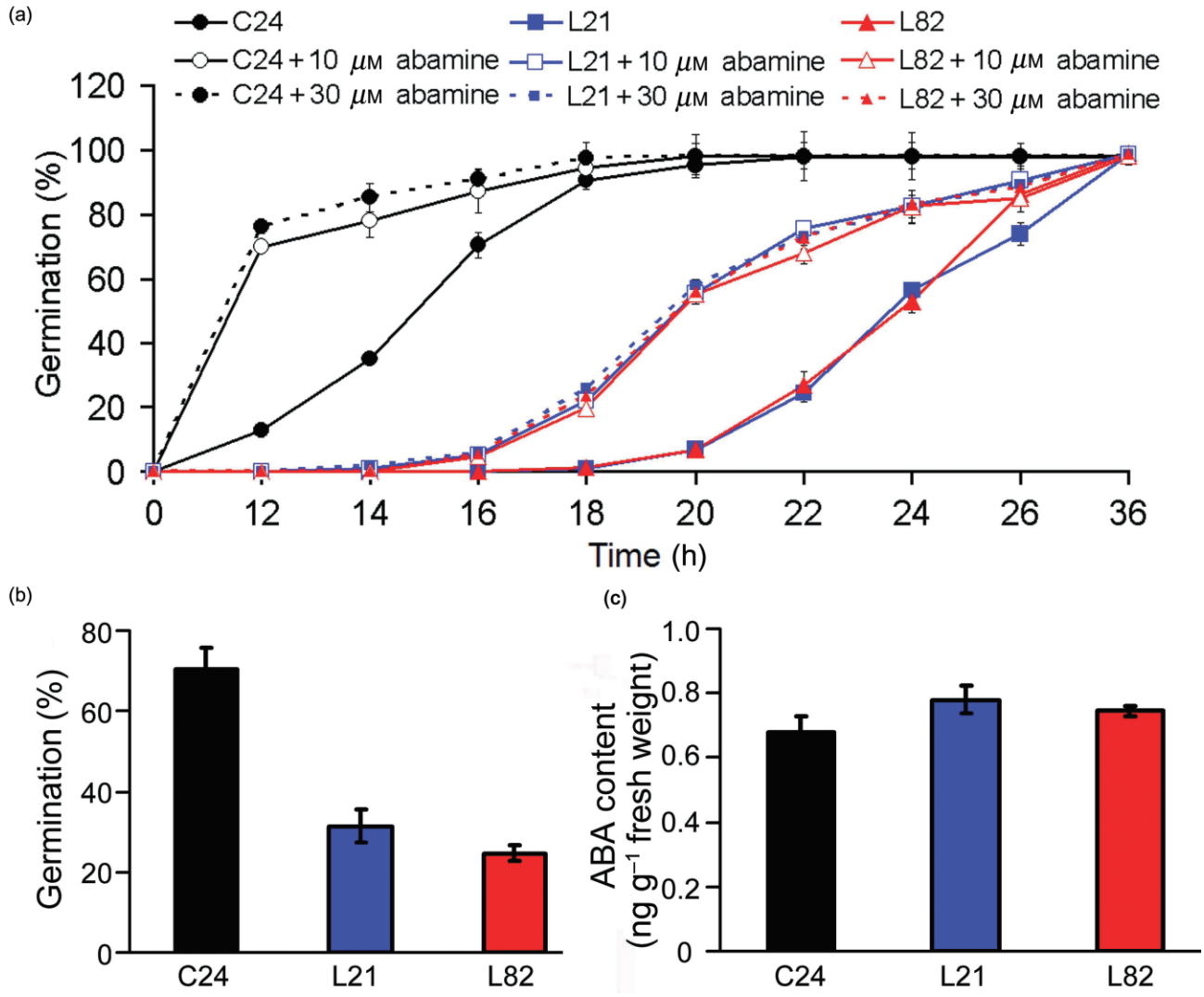

Figure 2. Abscisic acid (ABA) synthesis in *TSK1*-overexpressing transgenic lines. (a) Abamine treatment in transgenic lines. Seeds of the wild type and transgenic lines L21 and L82 were germinated on 1/2 MS plates containing 10 or 30 μM abamine. After 3 d of cold treatment, the germination was scored (fully emerged radicle tip) at various times. Each data point represents the mean of triplicate experiments ($n = 50$–100 each). (b) Seeds were germinated on 1/2 MS plates containing 0.6 μM ABA and 30 μM abamine. Seeds germinated for 4 d after incubation at 4 °C for 3 d. The seedlings with fully emerged cotyledons were counted after 4 d. Experiments were performed at least three times ($n = 100$ each). Bars represent standard errors. (c) Seeds from wild type and transgenic plants were geminated on MS medium. The ABA content of 3-week-old seedlings was measured (ng g^{-1} fresh weight). Each data point represents mean ± SE. Experiments were performed three times.

pathway, is a target of ABI1 and acts downstream of *ABI1* (Himmelbach *et al.* 2002). In L21 and L82, the relative expression levels of *ATHB6* were reduced to 0.07- and 0.22-fold, respectively, of that in wild type. *ERA1* is another negative regulator of ABA signal transduction, and its mutation resulted in ABA hypersensitivity regarding stomatal closing and seed germination (Brady *et al.* 2003). The qPCR result demonstrated that the *ERA1* transcription level was decreased to 0.5- and 0.25-fold in L21 and L82, respectively, of that in the wild type.

We also detected several key transcription factors in the ABA signalling pathway, including *ABI3, ABI5*, *ABF3* and *ABF4*, the constitutive expression of which in Arabidopsis results in hypersensitivity to ABA (Lopez-Molina *et al.* 2001, 2002; Kang *et al.* 2002). Compared with the expression in the wild type, the relative expression level of the four genes was increased by more than twofold in the transgenic lines. Our results suggested that *TSK1* affected the transcription of ABA signalling components.

To investigate the changes of known ABA up-regulated genes in transgenic lines, *RD29B*, *RAB18* and *ADH1* were selected. *RD29B* is induced by ABA and abiotic stress (Yamaguchi-Shinozaki & Shinozaki 1994). *RAB18* and *ADH1* encode a dehydrin and an alcohol dehydrogenase, respectively (Lang & Palva 1992; de Bruxelles *et al.* 1996). Compared with the expression in the wild type, expression

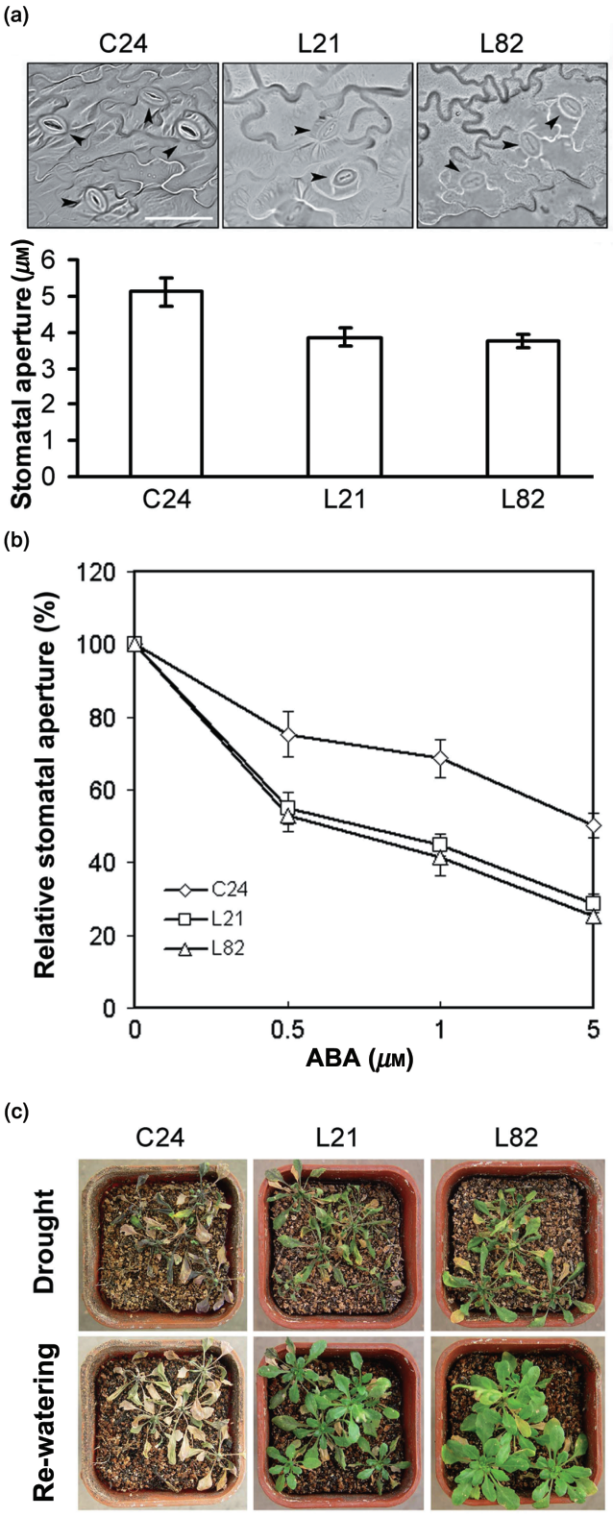

Figure 3. The stomata opening in wild type and *TSK1*-overexpressing transgenic lines. (a) Stomatal guard cells of the wild type and transgenic lines (L21 and L82). Arrows indicate the guard cells. Bar = 50 μm. (b) Effect of ABA on the closure of stomata in the wild type and transgenic lines. Epidermal strips were treated with different ABA concentrations under light. The results are expressed as relative stomatal aperture ± SE of 100 stomata. The experiments were repeated three times. The mean aperture width before adding ABA (taken as 100%) was 5.12 ± 0.38 μm in wild-type plants, 3.86 ± 0.24 μm in L21 plants and 3.75 ± 0.17 μm in L82 plants. (c) Drought tolerance of the wild type and transgenic lines. Top, water was withheld for 2 weeks; bottom, after a 2 week drought treatment, the seedlings were re-watered, and photographs were taken after 7 d.

level of *RD29B* was increased by 26.7- and 107.6-fold in L21 and L82, respectively. The transcription levels of *RAB18* and *ADH1* were also increased, as expected, in the *TSK*-overexpressing lines L21 and L82. Thus, the overexpression of *TSK1* in Arabidopsis resulted in an alteration in the expression of ABA/stress-responsive genes.

Knockdown of *SKP1* led to reduced ABA sensitivity in the *ask1* and *ask2* double mutant

Previous studies demonstrated that both *ASK1* and *ASK2* are required for Arabidopsis floral organ and embryo development, and ASK2 can partly substitute for ASK1 in male meiosis (Zhao *et al*. 2003a; Liu *et al*. 2004). These results indicated a functional overlap and redundancy of ASK1 and ASK2.

To further elucidate a possible role of *SKP1* in ABA signalling, we investigated whether the loss of function of *ASK1* and *ASK2* affected the sensitivity to ABA. Because of the possible functional redundancy between *ASK1* and *ASK2* and the lethality of the loss of function of both *ASK1* and *ASK2*, the seeds harvested from *ASK1/ask1 ASK2/ask2* plants (Ni *et al*. 2004) were used for ABA response analysis (Fig. 5). As shown in Fig. 5a,b, the genotype of each seedling was determined by PCR with four sets of primers as previously reported (Liu *et al*. 2004; Ni *et al*. 2004). Approximately 6% of the mutant seeds did not germinate or produce roots after 4 d of growth on MS medium without ABA. These seeds were genotyped as *ask1/ask1 ask2/ask2* double mutants by PCR. Four-day-old seedlings were transferred to MS medium containing 10 μM ABA, and root elongation was measured after 3 d. The seedlings of wild type, *ASK1/ask1*, *ask2/ask2*, *ASK2/ask2* and *ASK1/ask1 ASK2/ask2* exhibited no significant difference in root elongation after ABA treatment. However, the double mutant *ask1/ask1 ASK2/ask2* exhibited less ABA sensitivity in relative root length (~56%) than did the wild type (~33%) (Fig. 5c). These data suggested that homozygous mutations of *ASK1* and heterozygous mutations of *ASK2* result in reduced ABA sensitivity in Arabidopsis.

ASK1 and *ASK2* act downstream of *ABI5*

The Arabidopsis *ABI5*, which encodes a bZIP transcription factor, is involved in ABA and stress responses from the late embryonic stage through early seedling development (Finkelstein & Lynch 2000). The decreased ABA sensitivity of the *ask1/ask1 ASK2/ask2* double mutant allowed us to address whether *ASK1* and *ASK2* expression is regulated

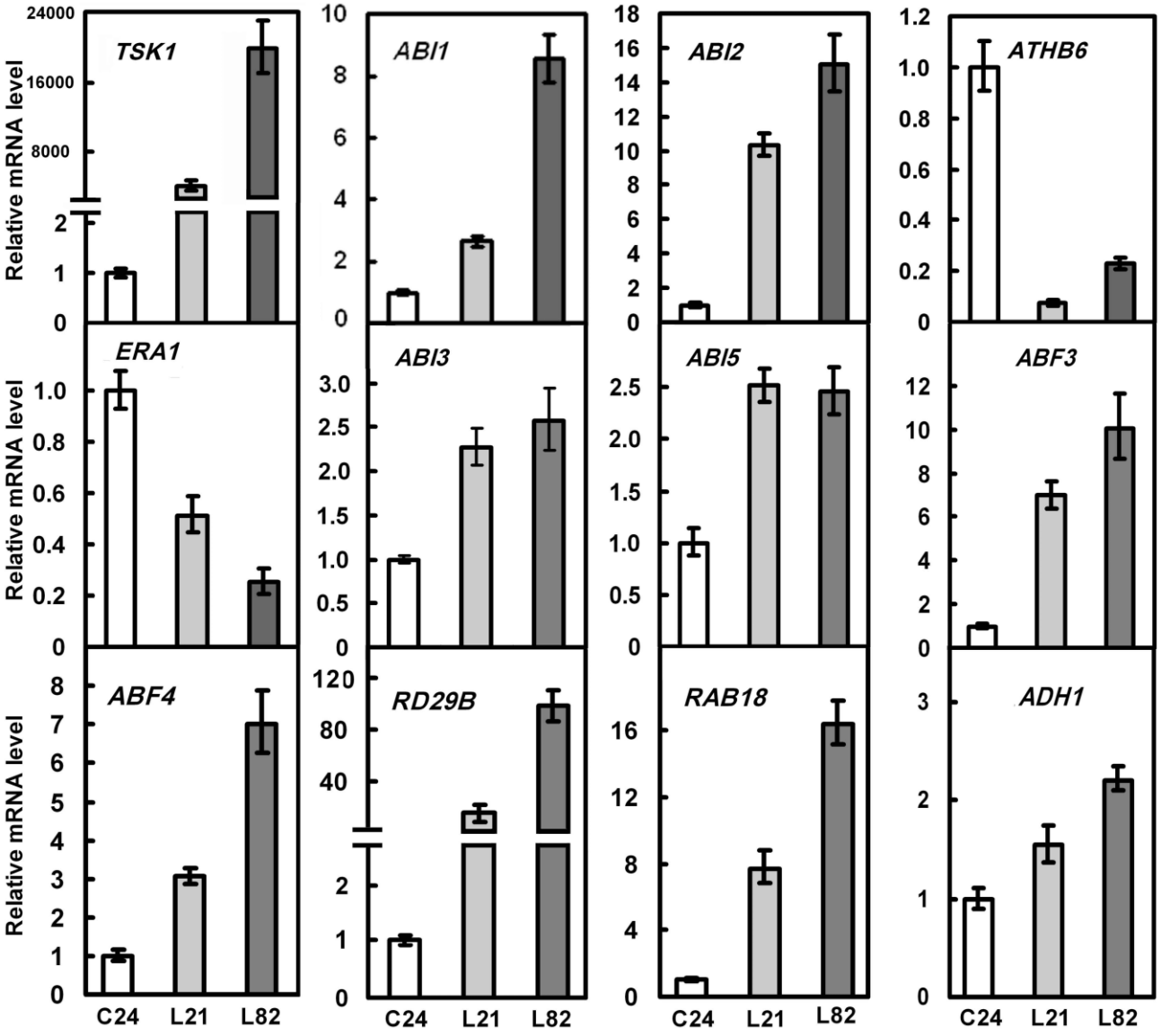

Figure 4. Expression of abscisic acid (ABA)-responsive genes in *TSK1*-overexpressing transgenic lines. Transcription levels of ABA-responsive genes were determined by quantitative RT-PCR with total RNA from 2-week-old seedlings growing in soil. *Tubulin* was used as an internal control. The data represent results of experiments performed in triplicate. Error bars represent ± SE.

by ABA signalling. The expression of *ASK1* and *ASK2* in the ABA-insensitive mutants *abi1-1* and *abi5-1* was detected by qPCR. Compared with the expression in the wild type, the expression levels of *ASK1* and *ASK2* were decreased to 0.6- and 0.4-fold, respectively, in the *abi1-1* mutant, and 0.1- and 0.08-fold, respectively, in the *abi5-1* mutant (Fig. 6a). This result suggested that *ASK1* and *ASK2* acts downstream of *ABI5* to mediate ABA signalling.

It can be expected that the overexpression of *ASK1* or *ASK2* may rescue the ABA-insensitive phenotype of the *abi5-1* mutant. The *ASK1*- and *ASK2*-overexpressing Arabidopsis were generated in both the wild-type and *abi5-1* backgrounds. However, unlike *TSK1* transgenic Arabidopsis with ABA hypersensitivity, *35S::ASK1* and *35S::ASK2* transgenic Arabidopsis exhibited similar ABA sensitivity as the wild type regarding germination (Fig. 6b), cotyledon expanding, and turning green (Fig. 6c,d), as well as root growth (Fig. 6e). In addition, the expression pattern of ABA-responsive genes was not changed significantly in *35S::ASK1* transgenic lines (Supporting Information Fig. S3). The ABA sensitivity of *35S::ASK1/abi5-1* and *35S::ASK2/abi5-1* was also examined. In the presence of 3 μM ABA, the germination rates of *abi5-1*, *35S::ASK1/abi5-1*, *35S::ASK2/abi5-1* and wild type were 92, 33, 51 and 31%, respectively (Fig. 6b). In the presence of 0.3 μM ABA, the cotyledon expanding and turning green percentages of *abi5-1*, *35S::ASK1/abi5-1*, *35S::ASK2/abi5-1* and wild type were 95, 67, 78 and 65%, respectively (Fig. 6c,d). The relative root lengths of *abi5-1*, *35S::ASK1/abi5-1*, *35S::ASK2/abi5-1* and wild type were 77, 56, 65 and 53%, respectively

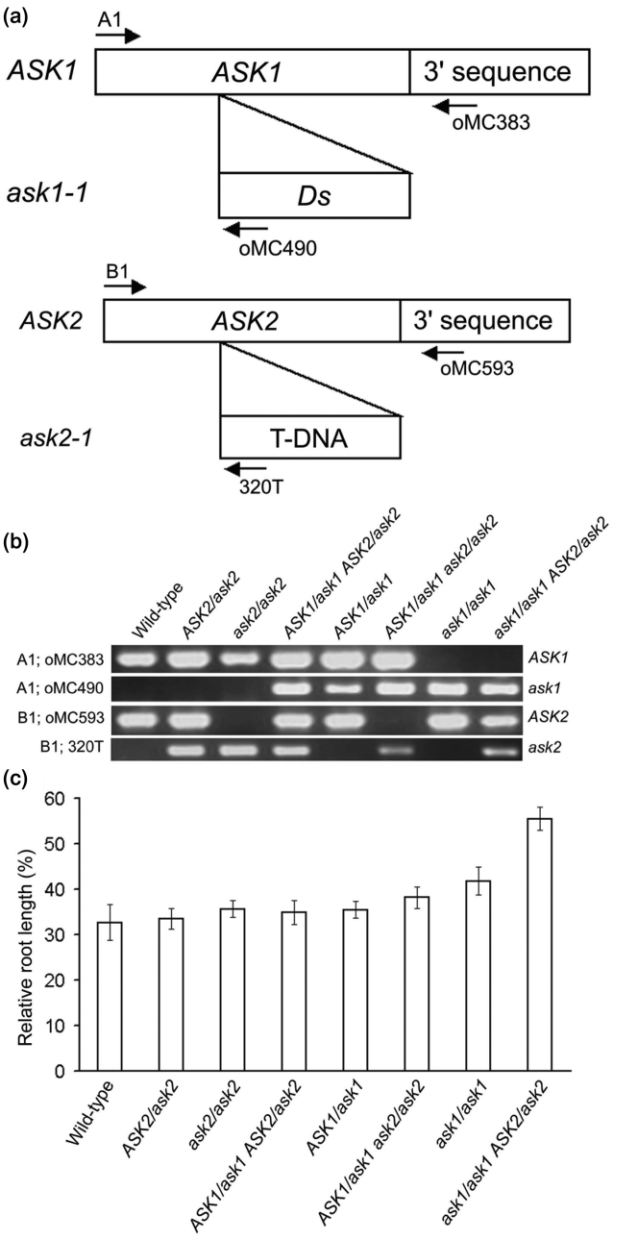

Figure 5. Sensitivity of progeny from *ASK1/ask1 ASK2/ask2* plants to ABA. (a) The schematic map of mutations in *ask1-1* and *ask2-1*, and the primers for genotyping *ask1-1* and *ask2-1* alleles. (b) PCR identification of *ASK1*, *ask1-1*, *ASK2* and *ask2-1* alleles in seedlings. (c) Response of root elongation to ABA. Seeds obtained from wild-type and *ASK1/ask1 ASK2/ask2* plants were germinated on 1/2 MS plates for 4 d. The surviving seedlings were transferred to MS containing 10 μM ABA. After 3 d, the relative root length was measured. Each data point represents the mean of experiments performed in triplicate (n = 150–200 each).

(Fig. 6e). These results suggested that overexpression of *ASK1* can rescue the ABA insensitivity of the *abi5-1* mutant, and the overexpression of *ASK2* can partially rescue the ABA insensitivity of the *abi5-1* mutant. Our results indicated that ABI5 is required for the expression of *ASK1* and *ASK2*, and *ASK1* and *ASK2* act downstream of *ABI5* to mediate ABA signalling for biological processes.

To determine whether *ASK1* and *ASK2* were acting in the same pathway with *ABI5*, the *ASK1/ask1 ASK2/ask2 abi5/abi5* genotype was generated, and its progeny seeds were used to examine the ABA response (Fig. 6f). The genotypes for the double mutant *ask1/ask1 abi5/abi5* and *ask2/ask2 abi5/abi5* and the triple mutant *ask1/ask1 ASK2/ask2 abi5/abi5*, as well as the wild type, were identified by PCR (Fig. 5b and Supporting Information Fig. S2). When treated with ABA, the relative root length of the *abi5-1* mutant was 86%, compared with 47% in the wild type (Fig. 6f), suggestive of stronger resistance of *abi5-1* to ABA. In contrast, the relative root lengths of the double mutant *ask1/ask1 abi5/abi5* and *ask2/ask2 abi5/abi5* were 50% and 50%, respectively, under the same treatment, and this was similar to the length of the wild type. The triple mutant *ask1/ask1 ASK2/ask2 abi5/abi5* exhibited increased ABA resistance regarding relative root length (71%) compared with the wild type, but the relative root length did not equal that of the *abi5-1* mutant. The relative root length of the *ask1/ask1 ASK2/ask2* double mutant was 23% more than that of the wild type (Fig. 5c), and that of the *ask1/ask1 ASK2/ask2 abi5/abi5* triple mutants was 24% more than that of the wild type (Fig. 6f). Therefore, introducing *ABI5* mutations into the *ask1/ask1 ASK2/ask2* background did not significantly affect the root elongation under ABA treatment compared with that in *ask1/ask1 ASK2/ask2* plants. This result indicated that the relationship between *ASK1 (ASK2)* and *ABI5* was complicated, and that *SKP1* and *ABI5* were not in the parallel pathway to regulate ABA signalling pathway.

DISCUSSION

SKP1-like genes encoding one of the components of SCF complexes have been found to regulate many hormone signalling pathways in plants via protein degradation, such as auxin, gibberellin, ethylene and jasmonic signalling pathways (del Pozo & Estelle 1999; Xu *et al*. 2002; Guo & Ecker 2003; Sasaki *et al*. 2003; Dharmasiri, Dharmasiri & Estelle 2005; Kepinski & Leyser 2005). SDIR1, a RING finger E3 ligase, was found to positively regulate ABA signalling (Zhang *et al*. 2007). Our data suggested that SKP1, a component of the SCF complex, participates in ABA signalling to regulate a series of biological processes including germination, stomatal aperture closure and root growth.

Our results demonstrated that *TSK1* overexpression in Arabidopsis can lead to ABA hypersensitivity regarding seed germination, root growth and stomatal closure. The expression levels of transcription factors, including the B3 domain gene *ABI3* and the bZIP transcription factor genes *ABI5*, *ABF3* and *ABF4*, are increased in *TSK1*-overexpressing Arabidopsis. The ABA up-regulated genes *RD29B*, *RAB18* and *ADH1* are also up-regulated in transgenic Arabidopsis. Meanwhile, the expression of *ERA1* and *ATHB6*, two negative regulators of the ABA signalling pathway, is reduced. The expression patterns of these

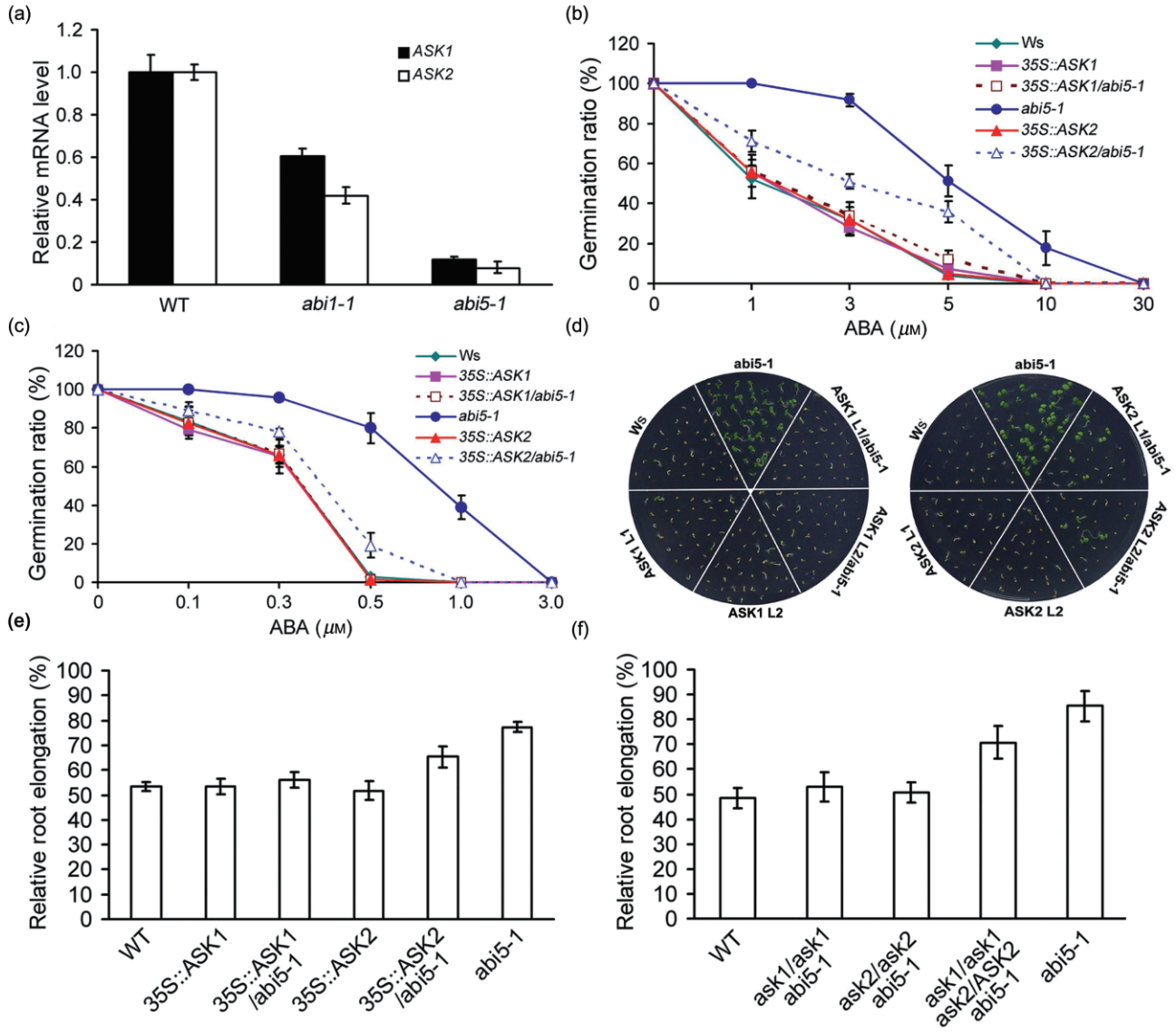

Figure 6. *ASK1* and *ASK2* act downstream of *ABI5*. (a) The transcription levels of *ASK1* and *ASK2* are down-regulated in *abi1-1* and *abi5-1* mutants. *Tubulin* was used as an internal control. The data represent results of experiments performed in triplicate. Error bars represent ± SE. (b) Germination rates of *ASK1*- or *ASK2*-overexpressing lines in the wild-type or *abi5-1* background. Seeds with emerged radicles were counted after growing 5 d in plates containing different concentrations of abscisic acid (ABA); data are expressed as the means of three independent experiments with $n \approx 100$ seeds for each genotype. (c) Cotyledon turning green rates of *ASK1*- or *ASK2*-overexpressing lines in the wild-type or *abi5-1* background. Seeds with fully expanded and greened cotyledons were counted after growing 5 d in plates containing different concentrations of ABA; data are expressed as the means of three independent experiments with $n \approx 100$ seeds for each genotype. (d) Growth of *ASK1*- or *ASK2*-overexpressing lines in either the wild-type or *abi5-1* background on 1/2 MS plates containing 0.5 μM ABA. The picture was taken after seeds were germinated and allowed to grow for 5 d. (e) Root elongation response of *ASK1*- or *ASK2*-overexpressing lines in the wild-type or *abi5-1* background to ABA. Seeds were germinated on 1/2 MS plates for 4 d. The seedlings were then transferred to 1/2 MS containing 1% sucrose and 10 μM ABA. After 3 d, the relative root length was measured. Each data point represents the mean of experiments performed in triplicate ($n = 30$–50 each). (f) Response of root elongation to ABA. Seeds obtained from wild-type and *ASK1/ask1 ASK2/ask2 abi5/abi5* plants were germinated on 1/2 MS plates for 4 d. The surviving seedlings were transferred to 1/2 MS containing 1% sucrose and 10 μM ABA. After 3 d, the relative root length was measured. Each data point represents the mean of experiments performed in triplicate ($n = 30$–50 each).

aforementioned genes are consistent with the ABA hypersensitivity phenotype of *TSK1*-overexpressing transgenic Arabidopsis lines. The endogenous ABA levels are not significantly changed in *TSK1*-overexpressing Arabidopsis lines. These results indicate that the overexpression of *TSK1* enhanced ABA signalling and might function as a positive regulator of ABA signalling.

The expression levels of two members of the PP2C family, *ABI1* and *ABI2*, are increased in *TSK1*-overexpressing transgenic Arabidopsis lines. *ABI1* and

ABI2 are the negative regulators of the early step of the ABA signalling pathway (Leung *et al*. 1997). The transcription level of PP2Cs was down-regulated in both the ABA-insensitive mutant *abi5-1* and the triple mutant *snrk2.2/snrk2.3/snrk2.6* of the SnRK2s protein kinase, a positive regulator of ABA signalling (Nakashima *et al*. 2009). Furthermore, the expression of *ABI1* and *ABI2* was up-regulated in *ABF3*- and *ABF4*-overexpressing Arabidopsis (Kang *et al*. 2002). In other words, enhancing ABA signalling up-regulates PP2C gene transcription, whereas blocking ABA signalling down-regulates PP2C gene transcription. This hypothesis is supported by the increased transcription levels of *ABI1* and *ABI2* in *TSK1*-overexpressing lines. These data indicate that the negative regulator PP2C is a key switch that can be regulated by downstream ABA signalling to maintain the ABA signal steady state.

How dose the SKP1 regulate ABA signalling in Arabidopsis? The SCF complex functions in ubiquitin-mediated proteolysis to degrade the specific substrates. In fact, SCF complex was identified to participate in early hormone response (Dreher & Callis 2007; Somers & Fujiwara 2009). For example, the F-box protein TIR1 was identified as the auxin receptor, which can interact with ASK1 or ASK2 to form a SCF complex. Aux/IAA protein is the substrate of TIR1. Aux/IAA inhibits the transcription activity of the ARFs transcription factors in auxin signalling transduction pathway. DELLA proteins act as transcription repressors of GA signalling pathway and are recognized by the F-box protein SLY1, which can interact with ASK1 or ASK2. Is there a similar regulation in ABA signalling pathway? Based on the positive effects of ASK1 and ASK2 on ABA signalling, it can be speculated that the negative regulators of ABA pathway might be candidates of the SCF-mediated targeting substrate. The group A PP2Cs were identified as negative regulators in the early step of ABA signalling pathway, including ABI1 and ABI2 (Leung *et al*. 1997), HAB1 and HAB2 (Leonhardt *et al*. 2004; Saez *et al*. 2004), and AHG1 and AHG3 (Yoshida *et al*. 2006; Nishimura *et al*. 2007). It is possible that one member of the group A PP2Cs is the substrate. ABI5 is unlikely to be the substrate because ABI5 is a positive regulator of ABA signalling pathway and ABA insensitivity phenotype of *abi5-1* mutant can be rescued by overexpression of *ASK1*. Based on present results, it is difficult to determine which member of PP2C is the real target protein of ASK1- or ASK2-participated SCF complex and further biochemical tests are needed to confirm the target.

Phylogenetic studies demonstrated that members of the *SKP1* family in angiosperms have evolved at highly heterogeneous rates (Kong *et al*. 2004). *SKP1* homologues from eudicots and monocots formed two separate clades, suggesting that they are derived from a single ancestral gene in the most recent common ancestor. The very low d_N/d_S (ratios of non-synonymous to synonymous nucleotide substitutions) for *ASK1/2* in Arabidopsis and *OSK1* in rice evolved slowly during plant evolution, suggesting that they are highly similar in sequence and critical functions (Kong *et al*. 2004, 2007). Sequence alignments of SKP1 homologues indicate that some amino acids are highly conserved in humans, plants and other species, and these conserved amino acids are identical in ASK1, ASK2, OSK1 and human SKP1 proteins (Schulman *et al*. 2000; Kong *et al*. 2004). Analysis of ASK1/2, TSK1 and OSK1 sequences (Supporting Information Fig. S1) indicates that excluding Leu 114 in ASK1 or Leu 125 in ASK2, which is replaced by Pro 129 in TSK1, all of the other conserved residues in TSK1 are identical to those in ASK1 and ASK2, and thus TSK1 may have functions similar to those of ASK1 and ASK2. Our previous study also revealed some conserved functions between TSK1 and ASK1 in male meiosis and auxin signalling (Li *et al*. 2006). Because the *ask1-1* mutant is male sterile (Yang *et al*. 1999), the *ask1/ask1 ask2/ask2* double mutant is lethal in embryogenesis (Liu *et al*. 2004), and because of the functional redundancy of ASK1 and ASK2 (Yang *et al*. 1999; Zhao *et al*. 1999, 2001, 2003a), *ask1/ask1 ASK2/ask2* plants were used to study their functions in ABA signalling. Our results indicated that the *ask1/ask1 ASK2/ask2* plant was ABA insensitive. Furthermore, the transcription levels of *ASK1* and *ASK2* are reduced in *abi5-1* mutant compared with the wild-type levels, and the overexpression of *ASK1* and *ASK2* in the *abi5-1* mutant can rescue or partially rescue the ABA insensitivity of the *abi5-1* mutant, indicating that *ASK1* and *ASK2* act downstream of *ABI5*. Therefore, *ASK1* and *ASK2* are involved in the ABA response and are consistent with ABA-hypersensitive phenotypes in *TSK1*-overexpressing Arabidopsis lines.

Our results also reveal some functional divergence between *TSK1* and *ASK1* or *ASK2*. We generated *ASK1*- and *ASK2*-overexpressing lines in a Col-0 background and investigated the ABA response, including the seed germination and post-germination growth of *ASK1*- and *ASK2*-overexpressing transgenic lines. In contrast to the ABA-hypersensitive phenotypes in *TSK1*-overexpressing Arabidopsis lines, we found no significant differences between *35S::ASK1* and *35S::ASK2* lines, and wild type (Fig. 6b–e). The transcription levels of the ABA-responsive genes were not changed compared with those of the wild type (Supporting Information Fig. S3). We propose that the endogenous ASK1 and ASK2 levels under normal conditions are necessary to maintain the ABA response, but excess ASK1 and ASK2 proteins may not be sufficient for ABA-hypersensitive responses in Arabidopsis, which differs in the effect of *TSK1* overexpression. In Arabidopsis, approximately 700 F-box genes (Gagne *et al*. 2002) and 21 *SKP1* genes (Initiative 2000; Risseeuw *et al*. 2003; Zhao *et al*. 2003b) were identified. It is possible that one SKP1 protein can interact with more F-box proteins in different tissues or developmental stages. Some of the amino acid residues in SKP1 are important for its interaction with F-box proteins, and some slight differences in amino acid sequence in SKP1 might lead to different F-box protein-binding affinities (Schulman *et al*. 2000; Zheng *et al*. 2002). Crystal structure analysis of 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; Zheng *et al*. 2002). It may be speculated that TSK1 and ASK1 might interact with different F-box proteins as a result of difference of amino acid between TSK1 and ASK1 and result in different sensitivity to ABA between *TSK1*- and *ASK1*-overexpressing lines. Gene duplication, followed by functional diversification, is a mechanism of gene evolution. Homologous sequences might share redundancy but they could diverge in functions through changes in expression patterns and accumulating variations in the coding regions. Analysis of evolution in *SKP1* gene family demonstrated that the low-evolving genes may possess the conserved function and express widely at high levels, whereas the rapid evolving members may acquire new functions and express at low levels or in specific tissue (Kong *et al*. 2004). Our previous report revealed the *TSK1* gene was mainly expressed in young roots and spikes and at a very low level in leaves (Li *et al*. 2006). *ASK1* and *ASK2* were expressed ubiquitously and at a very high level in leaves (Zhao *et al*. 2003b). The tissue specific expression pattern suggested *TSK1* may evolve rapidly and obtain new function compared with *ASK1* and *ASK2*. Taking together, our results provide evidence for the conservation of the ancestral function of *SKP1* genes in regulating ABA signalling pathway since the separation of eudicots and monocots about 150 million years ago. However, they also indicated the monocot-specific neo- or sub-functionalization during evolution.

In conclusion, constitutive overexpression of *TSK1* in Arabidopsis results in an ABA-hypersensitive phenotype. The double mutant *ask1/ask1 ASK2/ask2* exhibits reduced ABA sensitivity. Additionally, *ASK1/2* overexpression rescued ABA sensitivity in the *abi5-1* mutant. The expression level of *ASK1/2* was reduced in the *abi5-1* mutant. These data suggest that SKP1 is involved in ABA signalling. This study supports the hypothesis that ABA signalling is co-regulated by *SKP1*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Sequence alignments of ASK1, ASK2, TSK1, OSK1 and P19. The arrow shows the Leu 116 of P19 (human SKP1), which is replaced by Pro in TSK1. Accession numbers: TSK1 (AY316293), ASK1 (AEE35780), ASK2 (AED94777), P19 (NP_733779) and OSK1 (AAQ01198).

Figure S2. PCR-based assay for determining the genotype of the *abi5-1* mutant. PCR products were amplified by the primers bZIPFOR (CAA TCA ACA ACA AGC AGC AG) and bZIPRVE (TCT CTC CAC TAC TTT CTC CAC). The PCR products were digested by *Ava* II, and the fragments were separated on 1.5% agarose gel and stained with ethidium bromide.

Figure S3. The expression of ABA-responsive genes in *35S::ASK1* transgenic lines as measured by RT-PCR. ASK1-OE1 and ASK1-OE2 were two independent *35S::ASK1* transgenic lines; *Actin* was used as an internal control.

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