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Expression and Interaction Analysis of *Arabidopsis Skp1*-Related Genes

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Specific protein degradation has been observed in several aspects of development and differentiation in many organisms. One example of such proteolysis is regulated by protein polyubiquitination that is promoted by the SCF complex consisting of Skp1, cullin, and an F-box protein. We examined the activities of the *Arabidopsis* Skp1-related proteins (ASKs). Among 19 annotated *ASK* genes, we isolated 16 of the corresponding cDNAs (*ASK1*, *2*, *3*, *4*, *7*, *8*, *9*, *10*, *11*, *12*, *13*, *14*, *16*, *17*, *18*, *19*), and examined their gene products for interactions with 24 representatives of F-box proteins carrying various classes of the C-terminal domains using the yeast two-hybrid system. As a result, we found diverse binding specificities: ASK1, ASK2, ASK11 and ASK12 interacted well with COI1, FKF1, UFO-like protein, LRR-containing F-box proteins, and other F-box proteins with unknown C-terminal motifs. We also observed specific interaction between F-box proteins and ASK3, ASK9, ASK13, ASK14, ASK16 and ASK18. In contrast, we detected no interaction between any of the 12 ASK proteins and F-box proteins containing CRFA, CRFB or CRFC domains. Both histochemical and RT-PCR analysis of eight *ASK* genes expression revealed unique expression patterns for the respective genes.

Keywords: *Arabidopsis thaliana* — *ASK* — F-box protein — GUS — Protein degradation — Yeast two-hybrid system.

Abbreviations: *ASKs*, *Arabidopsis SKP1*-related genes; GUS, β-glucuronidase; RT-PCR, reverse transcription-PCR.

Introduction

Specific protein degradation has been observed in several aspects of development and differentiation processes in multicellular organisms (Hochstrasser 1996). In plants, recent genetic analysis using *Arabidopsis* mutants elucidated that these proteolytic processes are involved in many aspects of plant development and differentiation. Specific proteolysis begins with the labeling of target proteins. Protein ubiquitination is one such labeling process and three main enzymes are involved in it. An ubiquitin-activating enzyme (E1) catalyses the ATP-dependent activation of ubiquitin and forms a thiolester bond between ubiquitin and the cysteine residue of E1. An ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3) subsequently transfer this ubiquitin moiety from E1 to the cysteine residue of E2 and then from E2 to the lysine residue of the target proteins. Polyubiquitinated proteins are specifically degraded by the 26*S* proteasome complex (Patton et al. 1998).

Skp1 is a component of the E3 ubiquitin-ligase complex termed the SCF complex consisting of Skp1, cullin, and F-box proteins, and found in many organisms. Skp1 interacts with F-box proteins through approximately 40 conserved amino acid residues termed the F-box sequence (Schulman et al. 2000). It is reported that there are 19 *Skp1*-related genes in the *Arabidopsis* genome (Farras et al. 2001). ASK1 is the first Skp1-like protein identified in *Arabidopsis* (Porat et al. 1998) and has been demonstrated to interact with F-box proteins, UFO, COI1, TIR1, EID1 and ORE9 by yeast two-hybrid analysis or by immunoprecipitation (Gray et al. 1999, Samach et al. 1999, Dieterle et al. 2001, Woo et al. 2001, Devoto et al. 2002, Xu et al. 2002). ASK2 also has been reported to interact with COI1, TIR1 and EID1 (Gray et al. 1999, Dieterle et al. 2001, Xu et al. 2002). In *Arabidopsis* several functional F-box proteins have been reported by mutant analysis (Ruegger et al. 1998, Gray et al. 1999, Samach et al. 1999, Zhao et al. 1999, Nelson et al. 2000, Somers et al. 2000, Dieterle et al. 2001, Schultz et al. 2001, Woo et al. 2001, Devoto et al. 2002, Kim and Delaney 2002, Stirnberg et al. 2002, Xu et al. 2002). In addition, we and other researchers have identified 568 and 694 *Arabidopsis* F-box protein genes by database analysis, respectively (Gagne et al. 2002, Kuroda et al. 2002). However, most of the F-box proteins identified have not been revealed to interact with ASK proteins.

As well as forming a component of the SCF complex, it has recently been suggested that Skp1 performs a function distinct from protein degradation. Skp1 of *Saccharomyces cerevisiae* forms a complex with Rav1 and Rav2 called RAVE, a reg-

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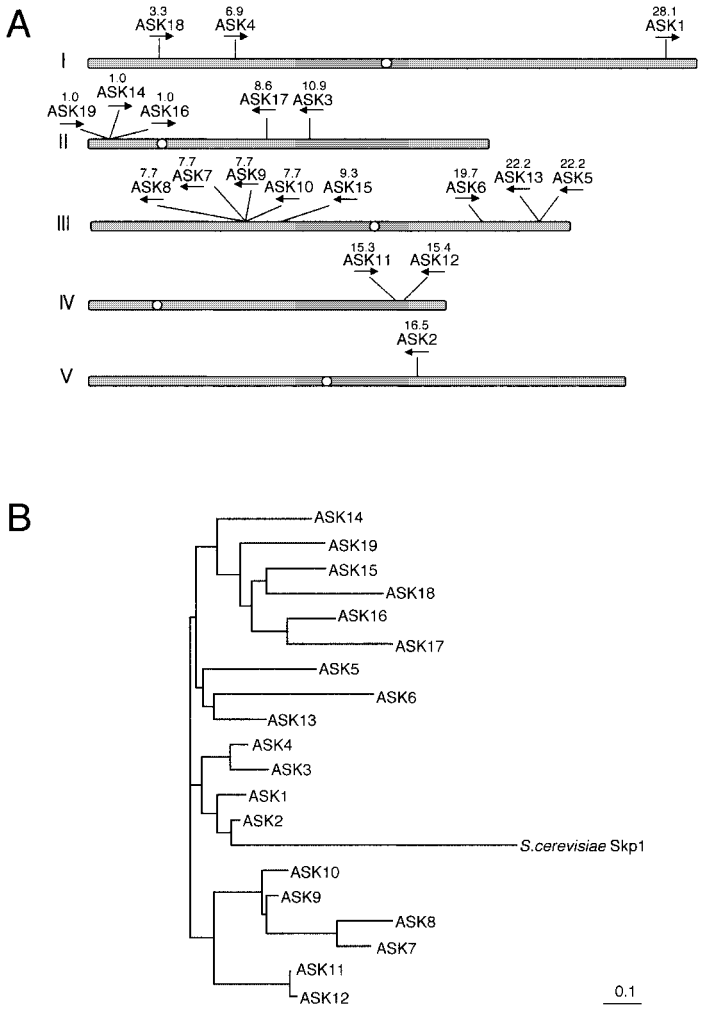


Fig. 1 Chromosomal locations and phylogenic tree of *Arabidopsis ASK* genes. (A) Bars represent the five *Arabidopsis* chromosomes. The centromeric regions are represented by circles. For each gene, its physical distance from the top of the chromosome is given in megabases. Arrows indicates the transcriptional direction of each gene. (B) A Neighbor-Joining (NJ) tree of ASK proteins and *S. cerevisiae* Skp1 generated using ClustalX and TreeView. Branch lengths are proportional to the estimated number of amino acid substitutions. Scale bar indicates the estimated amino acid substitutions per site.

ulator of (H^+)-dependent-ATPase (V-ATPase) assembly and is involved in glucose-induced assembly of the V-ATPase holoenzyme (Seol et al. 2001). Skp1 proteins in several organisms also have diverse functions. In spite of the importance of Skp1 and Skp1-related proteins in the control of development and signal transduction for specific degradation of target proteins and their formation of complexes with other proteins, very little is known about the functions of the individual proteins in *Arabidopsis*.

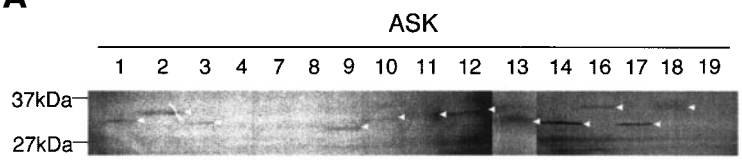
As a first step to understand the functions of the Skp1-related gene (*ASK*) family in *Arabidopsis*, we took an in vitro approach to the classification of the *ASK* gene family for their ability to bind with F-box proteins. We also examined the expression patterns of several *ASK* genes by histochemical and RT-PCR analysis, and analyzed T-DNA and transposon insertion mutant lines of the ASK genes. We will present results showing the specificity of individual ASK proteins in their binding with F-box proteins and their expression patterns in several tissues.

Results

ASK genes form clusters in Arabidopsis genome

Based on the genome sequence, nineteen *ASK* genes have been annotated in the *Arabidopsis thaliana* genome (Arabidopsis Genome Initiative 2000, Farras et al. 2001). On the basis of this annotation data, we mapped these genes on the chromosomes. We found that they were dispersed on all five chromosomes, and that some of them make clusters in the genome (Fig. 1A). We constructed a phylogenic tree based on the amino acid sequence similarity of the ASK proteins (Fig. 1B). The *S. cerevisiae* Skp1 protein is most similar to ASK2 (At5g42190) and ASK1 (At1g75950) out of the 19 ASK proteins. Some proteins that make clusters on chromosomes are included in the same clusters on the phylogenic tree. For example, *ASK7* (*At3g21840*), *ASK8* (*At3g21830*), *ASK9* (*At3g21850*) and *ASK10* (*At3g21860*) that cluster on chromosome 3 lie close together on the tree.

A



B

F-box protein		ASK protein											
Name	Domain	1	2	3	9	10	11	12	13	14	16	17	18
At2g39940	LRR	+	+	–	–	–	+	+	–	–	–	–	–
At2g25490	LRR	+	+	–	–	–	+	+	–	–	–	–	–
At5g48990	Kelch	–	–	–	–	–	–	–	–	–	–	–	–
At4g39550	Kelch	–	–	–	–	–	–	–	+	+	–	–	–
At1g68050	PAS/PAC	+	+	+	–	–	+	+	+	–	–	–	–
At3g61590	unknown	+	+	+	+	–	+	+	+	+	+	–	+
At5g49610	unknown	+	+	–	–	–	+	+	+	+	–	–	+
At2g02360	unknown	–	+	–	–	–	+	+	–	–	–	–	–
At2g34280	unknown	–	–	–	–	–	–	–	–	–	–	–	–
At5g43190	unknown	–	–	–	–	–	–	–	–	–	–	–	–
At5g14160	unknown	–	–	–	–	–	–	–	–	–	–	–	–
At1g78730	CRFA	–	–	–	–	–	–	–	–	–	–	–	–
At5g56370	CRFA	–	–	–	–	–	–	–	–	–	–	–	–
At5g38590	CRFA	–	–	–	–	–	–	–	–	–	–	–	–
At4g09920	CRFA	–	–	–	–	–	–	–	–	–	–	–	–
At2g42720	CRFA	–	–	–	–	–	–	–	–	–	–	–	–
At3g51170	CRFB	–	–	–	–	–	–	–	–	–	–	–	–
At3g16740	CRFB	–	–	–	–	–	–	–	–	–	–	–	–
At2g17830	CRFB	–	–	–	–	–	–	–	–	–	–	–	–
At3g23260	CRFB	–	–	–	–	–	–	–	–	–	–	–	–
At3g18980	CRFB	–	–	–	–	–	–	–	–	–	–	–	–
At4g38870	CRFC	–	–	–	–	–	–	–	–	–	–	–	–
At1g70390	CRFC	–	–	–	–	–	–	–	–	–	–	–	–
At3g57590	CRFC	–	–	–	–	–	–	–	–	–	–	–	–

Fig. 2 Two-hybrid interaction between ASK proteins and F-box proteins. (A) Immunoblot analysis of ASK proteins fused to the Gal4 DNA-binding domain. Arrowheads indicate the bands of fusion proteins. (B) Summary of the results of interactions of ASK preys and F-box baits, and ASK baits and F-box preys. Plus (+) and minus (–) indicate positive and negative interactions, respectively. Black boxes; interaction was detected in both combinations. Gray boxes; interaction was detected in only one combination (ASK prey and F-box bait).

Interaction between ASKs and F-box proteins

Skp1 directly binds with F-box proteins through their F-box domains (Schulman et al. 2000). We examined whether there is specificity between the 19 ASK proteins and F-box proteins in *Arabidopsis* by the mating-type yeast two-hybrid system. For the interaction study, we amplified cDNA clones of 16 *ASK* genes. We tried to amplify all of them but we did not succeed in cDNA amplifications of *ASK5* (*At3g60020*), *ASK6* (*At3g53060*) and *ASK15* (*At3g25650*), because some *ASK* genes are in a very low abundance. *ASK5* is reported to have been cloned from cDNA (Gagne et al. 2002). However, neither of *ASK6* and *ASK 15* had ESTs or full-length cDNA clones (Seki et al. 2002).

We examined the expression of ASK proteins fused to the Gal4 DNA-binding domain in yeast by Western blotting using the Gal4 monoclonal antibody. We observed expression of fusion proteins for ASK1, ASK2, ASK3 (At2g25700), ASK9, ASK10, ASK11 (At4g34210), ASK12 (At4g34470), ASK13 (At3g60010), ASK14 (At2g03170), ASK16 (At2g03190), ASK17 (At2g20160) and ASK18 (At1g10230). We confirmed the sequence of the fusion genes. However, we detect no fusion proteins for ASK4 (At1g20140), ASK7, ASK8 and ASK19 (At2g03160) (Fig. 2A). It is reported in the manual of the Gal4 two-hybrid system that fusion proteins with a Gal4 activation domain are in low abundance in yeast cells and they are difficult to detect by Western blotting (Clontech protocol #PT3024–1). As reported in the manual, we also detected no expression of any of these fusion proteins in yeast cells by Western blotting (data not shown). Our lab and other researchers have reported, respectively, that there are at least 568 and 694 F-box-related protein sequences in the *Arabidopsis* genome (Gagne et al. 2002, Kuroda et al. 2002). These proteins were classified into 19 groups based on the C-terminal domains that recruit substrates by interaction using these motifs (Kuroda et al. 2002). We selected 24 F-box proteins carrying various classes of the C-terminal domains and cloned full-length cDNAs. The

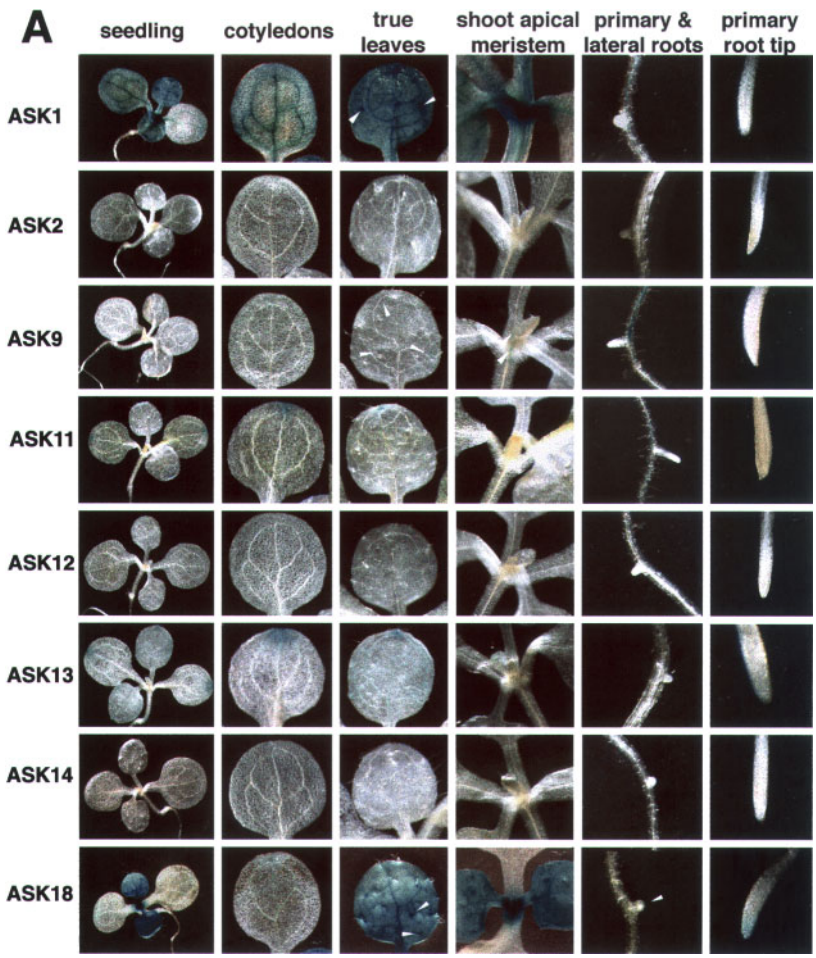


Fig. 3A

cDNAs were cloned into a Gal4 DNA-binding domain fusion vector (pGBK-RC) or a Gal4 activation domain vector (pGAD-RC).

COI1 (At2g39940) that contains a LRRs domain was used as the positive control for F-box proteins in this study, because this protein has been reported to interact with ASK1 and ASK2 (Devoto et al. 2002, Xu et al. 2002). As shown in Fig. 2B, ten out of 12 ASK proteins interacted with F-box proteins. In addition to ASK1 and ASK2, we found that ASK11 and ASK12 interacted with COI1. Interestingly some ASK proteins bound specifically to subgroups of F-box proteins. For example, four ASK proteins that interacted with COI1 bound to LRRs, PAS/PAC and unknown domain groups, but did not bind to Kelch groups. On the other hand, ASK13 and ASK14 bound to a Kelch protein. ASK9 and ASK16 interacted with an unknown domain protein. In the cases of ASK10 and ASK17, there was no interaction with any F-box protein examined. ASK18 only interacted with two F-box proteins with unknown domains. We also examined the interaction of ASK proteins with three subgroups of F-box proteins containing the newly identified C-terminal motifs, CRFA, CRFB and CRFC. In all cases we observed no interaction in our assay.

Tissue-specific expression of ASK genes

In the control of specific interactions between ASK proteins and F-box proteins, ASK proteins may have different tissue-specific expression patterns. To address this question, we performed histochemical analysis using transgenic plants containing promoter-β-glucuronidase (*GUS*) gene fusion constructs and RT-PCR analysis. We examined the tissue-specific expressions of genes that showed specific interaction with F-box proteins, i.e. *ASK1*, *ASK2*, *ASK9*, *ASK11*, *ASK12*, *ASK13*, *ASK14* and *ASK18* (Fig. 2B).

The results of the histochemical analysis are shown in Fig. 3 and summarized in Fig. 4. We detected GUS staining of all *ASK* genes tested. Each *ASK* gene showed its own staining pattern, although all the genes tested were expressed in flower tissue. *ASK1* expression was observed in almost all tissues except lateral and primary root tips in 10-day-old seedlings. In flowers, as reported previously by in situ hybridization (Porat et al. 1998), The expression was detected in the pollen. In the silique, the gene expression was also observed in mature seeds (Fig. 3, ASK1). The expression of *ASK2* and *ASK14* was detected only in pollen (Fig. 3, ASK2 and ASK14). *ASK9* gene expressed in true leaves was weak and spotted in several

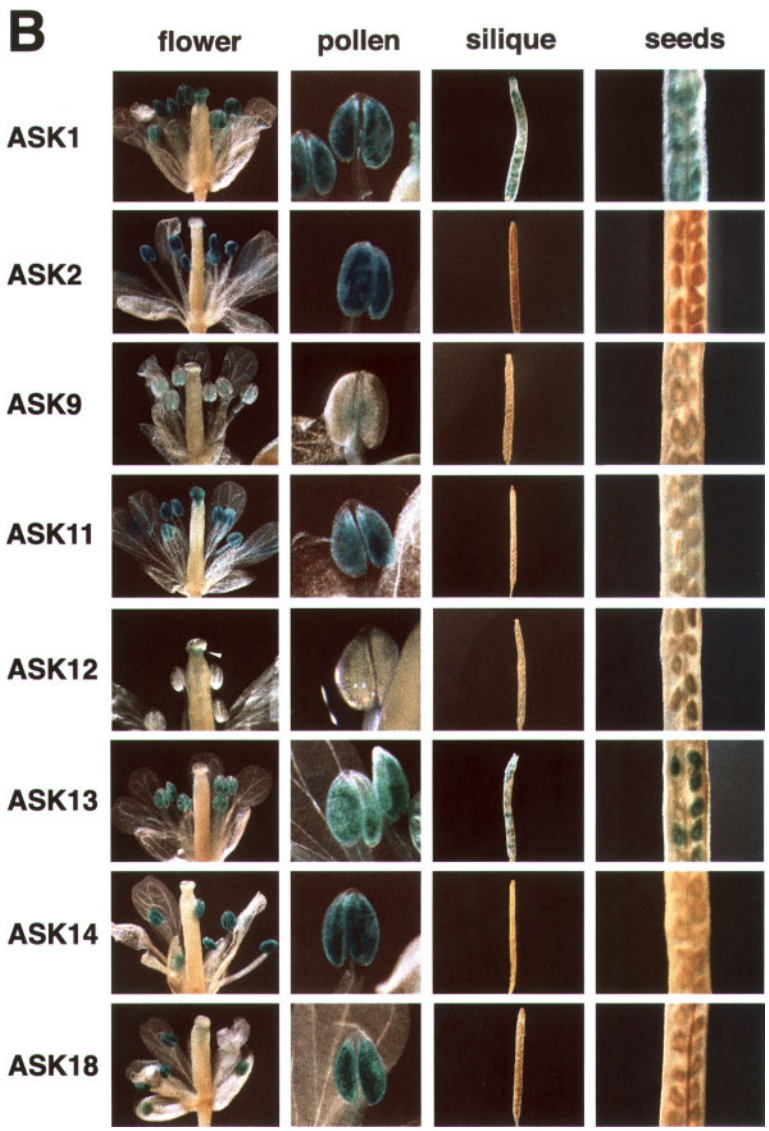


Fig. 3 Expression patterns of *ASK* genes in several tissues. (A) Ten-day-old transgenic plants grown on GM plates were examined. For each *ASK* transgenic line whole seedlings, cotyledons, true leaves, shoot apical meristems, primary and lateral roots and primary root tips were examined. Arrowheads in 'true leaves' of ASK1, ASK9 and ASK18 indicate basal cells of trichomes. Arrowheads in 'true leaves' and 'shoot apical meristems' of ASK9 indicate very localized and spotted *GUS* expression. Arrowhead in 'primary and lateral roots' of ASK18 indicates *GUS* expression at lateral root tip. (B) For each *ASK* transgenic line, flowers, pollen, siliques and seeds were examined for GUS activity. Arrowhead in 'flower' of ASK12 indicates *GUS* expression near the top (or neck) of stigma.

regions where basal cells of trichomes were present. Also, relatively strong expression was observed in primary roots (Fig. 3, ASK9). *ASK11* expression was detected in very small regions of the cotyledons and true leaves (Fig. 3, ASK11). The expression of *ASK12* was observed only near the top of the stigma (Fig. 3, ASK12). Strong expression of *ASK13* was observed in seeds. In addition, *ASK13* gene expressed in very small regions of the cotyledons and true leaves as observed with *ASK11* (Fig 3, ASK13). *ASK18* was the only gene out of the eight *ASK* genes examined with which we detected expression in lateral root tips and primary root tips (Fig. 3, ASK18). All of the *ASK* genes studied except *ASK12* are expressed in pollen but only *ASK1* and *ASK13* are expressed in siliques and seeds.

We further examined tissue-specific expression of eight *ASK* genes by RT-PCR using total RNA extracted from flowers, siliques, stems, cauline and rosette leaves of 4-week-old plants and roots from liquid culture. The expression patterns were almost the same as those obtained with the promoter-*GUS* transgenic plants (Fig. 5). For example, we observed strong expression of the *ASK1* gene in all tissues examined. *ASK13* was expressed in flowers, siliques and vegetative tissues, but not in roots. However, the expression patterns of some of the *ASK* genes were inconsistent with the results of the promoter-*GUS* assay.

Analysis of knockout mutants of ASK genes

Among the 19 *ASK* genes, only the *ask1* mutant is known and this mutation causes male sterility and formation of abnormal flowers (Yang et al. 1999, Zhao et al. 1999). To dissect the functions of other *ASK* genes, we analyzed knockout mutants. We obtained *Ds*-transposon insertion mutants for *ASK14* and *ASK18* from a database search of *Ds* transposon-tagged lines, and T-DNA insertion mutants for *ASK11* and *ASK12* from the Arabidopsis Biological Resource Center (see Material and

	ASK1	*ASK2*	*ASK9*	*ASK11*	*ASK12*	*ASK13*	*ASK14*	*ASK18*
cotyledon	+	–	–	+	–	+	–	–
true leaves	+	–	+	+	–	+	–	+
shoot apical meristem	+	–	+	–	–	–	–	+
primary & lateral roots	+	–	+	+	–	–	–	–
primary root tip	–	–	–	–	–	–	–	+
flower	+	+	+	+	+	+	+	+
pollen	+	+	+	+	–	+	+	+
silique	+	–	–	–	–	+	–	–
seeds	+	–	–	–	–	+	–	–

Fig. 4 Summary of the results of the expression patterns of various *ASK* genes. Plus (+) and minus (–) indicate whether *ASK* genes were expressed or not in tissues examined.

Methods). These mutants were null because expression of the *ASK* genes was not detected by RT-PCR but was in wild type (data not shown). These four mutants were grown to maturity and selfed to select homozygous knockout lines. A homozygous line for each mutant was selected by genomic PCR (data not shown). These homozygous lines were examined throughout their lifecycles from seedling stage to senescence. We observed no obvious differences in phenotype when compared with wild type.

Discussion

ASK proteins interact specifically with F-box proteins

We cloned 16 *ASK* cDNAs and found 10 of them interacted with F-box proteins. These results indicate that the 16 of the *ASK* genes are actually expressed and 10 of the ASK proteins act as a component of the SCF complex. We found clear specificities for the interactions between ASK proteins and F-box proteins, suggesting that the combinations of ASK proteins with F-box proteins produce diverse substrate selectivity. Also, we observed pair wise interaction patterns for F-box proteins such as ASK1 and ASK2, ASK11 and ASK12, ASK13 and ASK14 (Fig. 2B). With the exceptions of ASK13 and ASK14, these pairs of proteins lie close together on the phylogenic tree indicating that these proteins have very similar sequences. These results suggest that some *ASK* genes were generated by recent gene duplication events, and have redundant function.

We detected no interaction between the ASK proteins and any of the F-box proteins with CRFA, CRFB or CRFC domains. It is possible that the newly identified F-box proteins have a different function from the specific protein degradation demonstrated for F-box proteins forming SCF complexes.

Diverse expression patterns of ASK gene family

We observed specific *GUS* expression for individual *ASK* genes (Fig. 3, 4). *ASK11* and *ASK12* had similar amino acid sequences and were clustered on the same chromosome but were expressed differently in both flowers and seedlings (Fig. 3, 4). Thus it is likely that expression of the *ASK* genes is controlled differently when the sequence similarity is high and the genes are located in the proximal region on a chromosome, and these complex expression patterns may produce unique combinations of interactions between F-box proteins and ASK proteins.

The expression patterns detected by RT-PCR analysis were almost same as those obtained by promoter-*GUS* transgenic plants. However, the expression patterns of some *ASK* genes were inconsistent with the results from the *GUS* assays. For instance, from the promoter-*GUS* assays, there was

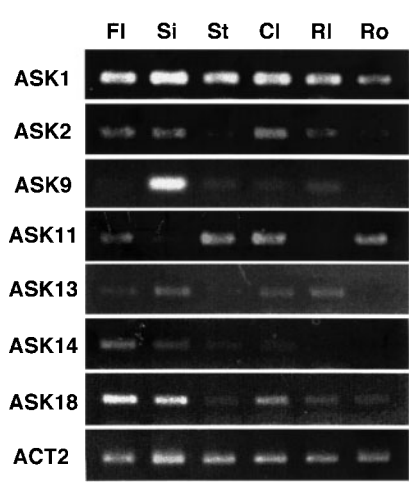


Fig. 5 Expression of *ASK* genes in various tissues of 4-week-old *Arabidopsis* and roots from liquid culture. Total RNAs from flowers (Fl), siliques (Si), stems (St), cauline leaves (Cl), rosette leaves (Rl) and roots (Ro) were used for RT-PCR analysis. The *ASK12* transcript was not detected (data not shown).

almost no expression of *ASK2* in vegetative tissues nor of *ASK9* or *ASK18* in siliques, while all were detected by RT-PCR (Fig. 4, 5). This discrepancy is probably because the sensitivity of RT-PCR is higher than that of the *GUS* assay.

ASK proteins may have overlapping functions and also have other functions besides forming components of SCF complex

We observed no phenotypic abnormalities in the T-DNA insertion lines of *ASK11* and *ASK12*, and the *Ds*-transposon inserted mutant lines of *ASK14* and *ASK18*. This observation suggests that mutations in some *ASK* genes can be compensated for by other *ASK* genes.

Two ASK proteins, ASK10 and ASK17, did not interact with any F-box proteins examined in our assay. One possibility is that these ASK proteins interact with other F-box proteins that we did not examine in this study. On the other hand it has been reported recently that the Skp1 protein has different functions apart from its involvement in the SCF complex. Skp1 protein in *S. cerevisiae* makes a complex with Rav1 and Rav2 proteins, interacts with the vacuolar membrane (H^+)-ATPase (V-ATPase) and promotes glucose-induced assembly of the V-ATPase holoenzyme (Seol et al. 2001). It has been reported that it also makes a complex named the centromere-binding factor 3 (CBF3) that is composed of multiproteins encoded by *NDC10/CTF14/CBF2*, *CEP3/CBF3b*, *CTF13* and *SKP1* (Stemmann et al. 2002). This complex binds to conserved sequences of the centromere and has a kinetochore function. Mammalian Skp1 also makes a complex with Siah-1 (a RING-finger protein), SIP and Ebi1 (a F-box protein) (Liu et al. 1999, Matsuzawa and Reed 2001). This complex is involved in poly-ubiquitination of β-catenin in response to DNA damage. Thus Skp1 protein has the potential to form various protein complexes. Judging from these observations, it is assumed that the ASK proteins have unique functions different from protein ubiquitination through the SCF complex.

Further study to establish the interaction partners for these ASK proteins will elucidate the functions of individual ASK proteins and also reveal the network for the regulation and interaction between F-box proteins and ASK proteins.

Very recently, similar experiments were reported by Zhao et al. (2003). These results will also support our argument.

Material and Methods

cDNA cloning

Total RNA extracted from whole 4-week-old *Arabidopsis* plants was used for RT-PCR to generate the full coding cDNA clones of the *ASK* and F-box protein genes. First-strand cDNA was synthesized using the SuperScript and oligo-dT primer (Invitrogen Corp., Carlsbad, CA U.S.A.). The forward and reverse primer pairs used to amplify the *ASK* cDNAs are given as supplemental data as are the primer pairs used to amplify the 24 F-box cDNAs. PCR products were cloned into the GATEWAY donor vector pDONR201 by BP reaction (Invitrogen Corp., Carlsbad, CA, U.S.A.). In total 16 out of 19 *ASK* genes and 24 F-box genes were cloned. We also used full-length cDNA in our group (Seki et al. 2002).

Plasmid construction

To produce fusion proteins with the GAL4 DNA binding domain or the GAL4 activation domain, the pGBK-RC bait plasmid and pGAD-RC prey plasmid (Ito et al. 2000) were digested with *Bam*HI and blunt ended using T4 DNA polymerase. The GATEWAY reading frame cassette (Invitrogen Corp., Carlsbad, CA, U.S.A.) was introduced into the blunt end sites to generate pGBK-RC-GATEWAY and pGAD-RC-GATEWAY. Sixteen *ASK* cDNAs and 24 F-box protein cDNAs were cloned into both these plasmids by LR reaction (Invitrogen Corp., Carlsbad, CA, U.S.A.). To construct the *ASK* promoter-*GUS* fusions, genomic DNA containing the promoter regions and the first 30 nucleotides of the individual *ASK* genes was amplified from *Arabidopsis* genomic DNA by PCR. The forward and reverse primer pairs and the lengths of the promoter fragments used are given as supplementary data. The pGWB3 binary vectors for promoter-*GUS* fusions were provided by Dr. Tuyoshi Nakagawa (Shimane University). The PCR products were cloned into the GATEWAY destination vector pGWB3 using the GATEWAY system (Invitrogen Corp., Carlsbad, CA, U.S.A.).

Immunoblotting

Protein extracts for immunoblotting were prepared according to the method by Printen and Sprague (1994). Total proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Advantec Toyo, Ltd. Tokyo, Japan) using electroblot apparatus (Model BP-312, Biocraft Laboratories, Inc. Tokyo, Japan) in transfer buffer (10 mM Tris, 192 mM glycine, 20% methanol). After electrophoresis, the membranes were blocked by incubation with 10% bovine serum albumin in TBS buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl) for 1 h, and then incubated with a GAL4 monoclonal antibody (Clontech Laboratories, Inc., CA, U.S.A.) at a dilution of 1 : 5,000 for 8 h at room temperature. Membranes were washed with TBS buffer containing 0.1% Tween-20 (TBST). The membranes were then incubated with a horseradish peroxidase conjugated with goat anti-mouse IgG antibody (KPL, Kirkegaard & Perry Laboratories, Inc., CA, U.S.A.) at a dilution of 1 : 5,000 for 2 h at room temperature. After washing with TBST, antibody locations were detected by chemiluminescence using 0.2 mg ml^{-1} diaminobenzidine supplemented with 0.06% hydroxy peroxide in phosphate buffer.

Protein interaction by mating type yeast two-hybrid analysis

All the pGBD-RC-GATEWAY constructs were introduced into the yeast haploid strain PJ69-2A (Mat a) (Ito et al. 2000), whereas all the pGAD-RC-GATEWAY constructs were introduced into the yeast haploid strain MaV204K (Matα) (Ito et al. 2000). Interactions between the fusion proteins were assayed in the PJ69-2A/MaV204K diploid strains according to the method of Ito et al. (2000).

Plant material

In all experiments unless otherwise noted the *A. thaliana* Col-0 ecotype was used. Plants were grown in soil or on GM medium (Valvekens et al. 1988) containing 0.8% agar with 16 h light and 8 h dark at 22°C. Transgenic plants were generated by *Agrobacterium*-mediated transformation using the floral dipping method (Bechtold et al. 1993). Transgenic seedlings were selected on GM agar plates containing 50 μg ml^{-1} kanamycin.

Knockout mutant analysis

Ds-transposon insertion lines in the Nossen (No-0) ecotype for *ASK14* and *ASK18* were obtained from a database search of *Ds* transposon-tagged lines that we have been generating. The *Ds* transposon is inserted 27-bp upstream of the start codon in *ASK14* and is inserted in

the middle of the coding region of *ASK18*. SALK T-DNA knockout mutant lines in the Col-0 ecotype for *ASK11* (SALK 031601) and *ASK12* (SALK 052841) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The T-DNA is inserted in the coding exons of both *ASK11* and *ASK12*. Transgenic seedlings of the *Ds* lines were selected on GM plates containing 20 μg ml^{-1} hygromycin. Homozygous lines for each mutant were selected by genomic PCR. *ASK* gene expression was investigated by RT-PCR using primers that were the same as those used for *ASK* cDNA cloning.

β-glucuronidase (GUS) assay for histochemical expression pattern

Several independent T1 and T2 transgenic lines were stained for GUS activity. Tissues were placed in staining solution [100 mM phosphate buffer (pH 7.0), 1 mM X-gluc (Nacalai, Tokyo, Japan), 10 mM EDTA, 0.1% (v/v) Triton-X] and incubated at 37°C overnight. After staining, the tissue was soaked in several changes of 95% ethanol and photographed with a Leica Plan-Apo microscope.

RT-PCR

RT-PCR amplifications were performed with 0.1 μg of total RNA from flowers, siliques, stems, cauline leaves, rosette leaves and roots. First-strand cDNA synthesis was performed using Superscript and oligo-dT primers (Invitrogen Corp., Carlsbad, CA, U.S.A.). RT-PCR primer sets were the same as those used for *ASK* cDNA cloning. For *actin2* gene amplification, forward primer 5′-AGCGCTGAGGCTGATGATATTCAAC-3′ and reverse primer 5′-TCTAGAAACATTTTCTGTGAACGATTC-3′ were used. PCR conditions were 22–31 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and finally 72°C for 5 min.

Supplementary Material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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