Members of the *Arabidopsis-SKP1-like* Gene Family Exhibit a Variety of Expression Patterns and May Play Diverse Roles in Arabidopsis[1]

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Ubiquitin-mediated proteolysis by the proteasome is a critical regulatory mechanism controlling many biological processes. In particular, SKP1, cullin/CDC53, F-box protein (SCF) complexes play important roles in selecting substrates for proteolysis by facilitating the ligation of ubiquitin to specific proteins. In plants, SCF complexes have been found to regulate auxin responses and jasmonate signaling and may be involved in several other processes, such as flower development, circadian clock, and gibberellin signaling. Although 21 Skp1-related genes, called *Arabidopsis-SKP1-like* (*ASK*), have been uncovered in the Arabidopsis genome, *ASK1* is the only gene that has been analyzed genetically. As a first step toward understanding their functions, we tested for expression of 20 *ASK* genes using reverse transcription-polymerase chain reaction experiments. Also, we examined the expression patterns of 11 *ASK* genes by in situ hybridizations. The *ASK* genes exhibit a spectrum of expression levels and patterns, with a large subset showing expression in the flower and/or fruit. In addition, the *ASK* genes that have similar sequences tend to have similar expression patterns. On the basis of the expression results, we selectively suppressed the expression of a few *ASK* genes using RNA interference. Compared with the *ask1* mutant, the strong *ASK1* RNA interference (RNAi) line exhibited similar or enhanced phenotypes in both vegetative and floral development, whereas *ASK11* RNAi plants had normal vegetative growth but mild defects in flower development. The diverse expression patterns and distinct defects observed in RNAi plants suggest that the *ASK* gene family may collectively perform a range of functions and may regulate different developmental and physiological processes.

Selective proteolysis of proteins has been recognized as a very important mechanism for regulating many cellular events (Hershko and Ciechanover, 1998; Zheng et al., 2002). A major pathway for controlled protein destruction is the ubiquitin-mediated proteolysis by the proteasome (Hershko and Ciechanover, 1998; del Pozo and Estelle, 2000). Three types of enzymes (E1, E2, and E3) are responsible for ubiquitination of proteins. E1 is known as the ubiquitin-activating enzyme, E2 is the ubiquitin-conjugating enzyme, and E3 is the ubiquitin-protein ligase, which acts as a factor for substrate recognition (Koepp et al., 1999; Pickart, 2001). Both E1 and E2 are less specific than E3. The SKP1, cullin/CDC53, F-box protein (SCF) complexes are the largest and best studied family of E3 ubiquitin-protein ligases and are known to control cell cycle regulation, signal transduction, transcription, and other biological events (Bai et al., 1996; Hershko and Ciechanover, 1998; Schulman et al., 2000; Shen et al., 2002; Zheng et al., 2002).

Among the subunits of the SCF complex, SKP1 acts as an adapter that links cullin to one of the F-box proteins, which are highly variable (Willems et al., 1999; Schulman et al., 2000; Zheng et al., 2002). X-ray crystallography (Zheng et al., 2002) revealed that the human cullin, Cul1, is elongated and has a long stalk domain that binds to SKP1 and a globular domain that associates with Rbx1, a recently identified fourth subunit that contains a RING finger domain (Kamura et al., 1999; Seol et al., 1999). F-box proteins bind to SKP1 through the conserved F-box motif that is 40 to 50 amino acids long and is named for the human cyclin F (Skowyra et al., 1997; Winston et al., 1999; del Pozo and Estelle, 2000). Several SCF complexes have been identified that function in a myriad of vital biological processes in yeast and mammals. Some examples are SCF^{Skp2} (Zhang et al., 1995; Schulman et al., 2000) and SCF^{hCdc4} (Strohmaier et al., 2001), where the superscript indicates the specific F-box protein.

Recent studies suggest that plants make extensive use of SCF complexes to regulate multiple biological processes. Several SCF complexes have been characterized in Arabidopsis. For example, the SCF^{TIR1} complex regulates auxin response (Ruegger et al., 1998; Gray et al., 1999, 2001; Schwechheimer et al.,

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2001), and the SCF^{COI1} complex is involved in jasmonate responses (Xie et al., 1998; Xu et al., 2002). Most recently, another complex called SCF^{AtSKP2} was found to play a role in controlling cell division (del Pozo et al., 2002). The characterization of mutants affecting genes encoding F-box proteins further indicates that SCF complexes may regulate a variety of processes in plants, such as flower development (Ingram et al., 1995; Levin and Meyerowitz, 1995; Samach et al., 1999; Zhao et al., 1999, 2001), circadian clock (Mizoguchi and Coupland, 2000; Somers et al., 2000), gibberellin signaling (Sasaki et al., 2003), light signaling (Dieterle et al., 2001), defense response (Kim and Delaney, 2002), and leaf senescence (Woo et al., 2001).

In both yeast and human, there is only one known functional *SKP1* gene (Yu et al., 1998; Kipreos and Pagano, 2000). In contrast, fruitfly (*Drosophila melanogaster*) and *Caenorhabditis elegans* both have multiple *SKP1* homologs. *C. elegans* has at least 21 *SKP1-related* (*SKR*) genes that exhibit different expression patterns (Yamanaka et al., 2002). The SKR proteins also exhibit differences in their association with both cullins and F-box proteins (Nayak et al., 2002; Yamanaka et al., 2002). Loss-of-function studies using RNA interference (RNAi) show that *SKR* genes are differentially involved in regulating cell proliferation, meiosis, and morphogenesis (Nayak et al., 2002; Yamanaka et al., 2002).

The Arabidopsis genome contains 21 predicted *ASK* genes (Arabidopsis Genome Initiative, 2000; Farras et al., 2001), but only *ASK1* has been analyzed genetically. The *ask1-1* mutant is defective in both vegetative growth and reproductive development (Yang et al., 1999; Zhao et al., 1999). Compared with wild type, it has smaller rosette leaves and shorter stature. Furthermore, the mutant is defective in petal and stamen development. *ASK1* genetically interacts with *UFO* to regulate B function genes *AP3* and *PI*, probably through SCF-mediated ubiquitination and proteolysis (Samach et al., 1999; Zhao et al., 2001). In addition, the *ASK1* gene is required for homolog separation in male meiosis (Yang et al., 1999) and is also involved in auxin response (Gray et al., 1999). The pleiotropic functions of *ASK1* are consistent with its broad expression in both vegetative and reproductive tissues (Porat et al., 1998). Mutational studies of other *ASK* genes have not been reported; moreover, for most predicted *ASK* genes, it is not known whether they are active genes or pseudogenes. To learn about possible functions of the *ASK* gene family, we conducted reverse transcription-PCR (RT-PCR) and in situ hybridization experiments to characterize their expression patterns. Furthermore, we selectively suppressed the expression of a few *ASK* genes to investigate their possible functions. Our results show that the *ASK* genes have a variety of expression profiles and transgenic plants with different *ASK* RNAi constructs have distinct phenotypes, suggesting that members of the *ASK* gene family may have diverse functions in plant development and physiology.

RESULTS

Expression Analysis by RT-PCR

For the convenience of description and discussion, we divide the genes into several groups based on their sequence similarity: group 1, *ASK1* and *ASK2*; group 2, *ASK3* and *ASK4*; group 3, *ASK5* and *ASK6*; group 4, *ASK7* through *ASK10*; group 5, *ASK11* and *ASK12*; group 6, *ASK13*; group 7, *ASK14* through *ASK19*; and group 8, *ASK20* and *ASK21* (Fig. 1). Although the *ASK* genes share higher levels of amino acid sequence similarity within each group than between groups, the levels of similarity within different groups are not uniform.

To learn whether predicted *ASK* genes are expressed during normal development and to estimate the relative abundance of *ASK* messages in different organs, we performed RT-PCR using gene specific primers (Table I). To control for specificity, PCR products from genomic DNA and cDNA templates were sequenced directly, and their sequences match known sequences of the corresponding genes (except *ASK9*), other than polymorphisms between the Landsberg *erecta* (L*er*) and Columbia (Col) ecotypes. PCR using primers matching the Col *ASK9* gene yielded products that were most similar to *ASK8*. Thus, *ASK9* expression was not further analyzed. Expression of the 20 *ASK* genes was tested using RT-PCR, and the expression of 18 genes was detected in one or more part(s) of plants grown under normal conditions (Fig. 2; Table II). However, the sequence of the *ASK15* RT-PCR product matched the known *ASK15* genomic sequence, including a predicted 50-bp intron, suggesting that *ASK15* may be a pseudogene. The expression of *ASK6* and *ASK19* was not detected. As a control for RNA extraction and RT-PCR for different tissues, we detected uniformly strong RT-PCR products in all RNA samples for the *APT1* gene encoding adenine phosphoribosyltransferase (Moffatt et al., 1994).

RT-PCR produced nearly uniformly strong bands for *ASK1* and *ASK2* in all tissues tested: young seedlings, roots, leaves, floral stems, inflorescences, and siliques. *ASK3* seems highly expressed in the silique. *ASK4* expression seems to be at a higher level in inflorescence and siliques than in seedlings, roots, and stems. For group 4, the expression of *ASK7* and *ASK8* was detected only in the silique, whereas *ASK10* expression was found in all tissues tested. In group 5, *ASK11* and *ASK12* had similar expression patterns, with perhaps a slightly higher level in the inflorescence than in other tissues.

In group 3, *ASK5* is expressed in the inflorescence, as confirmed by in situ hybridization (see below). *ASK13* expression was detected as a stronger PCR

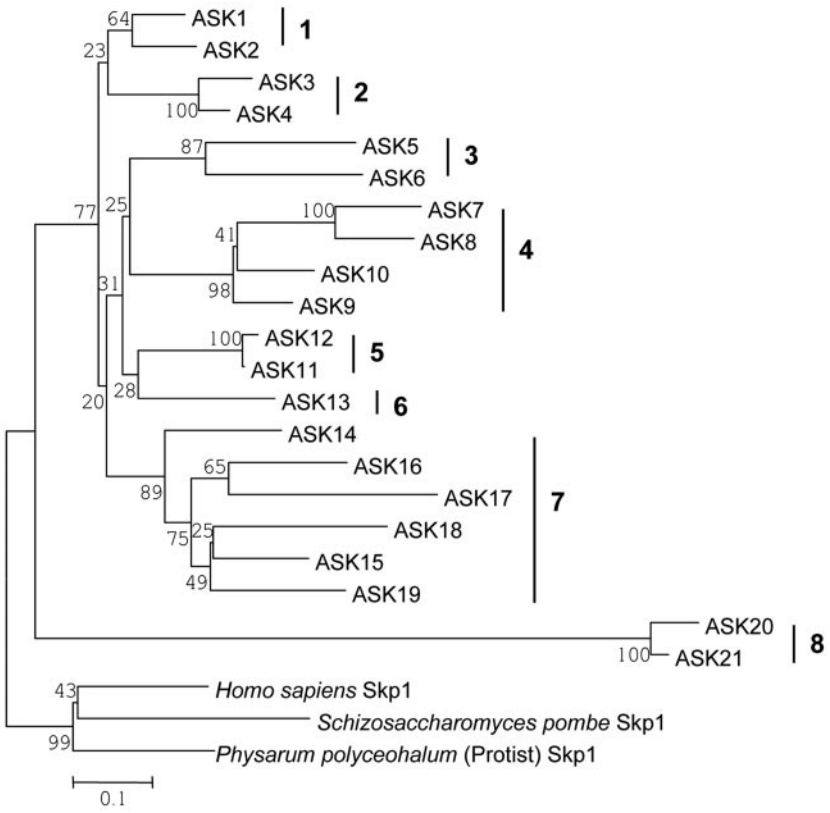


Figure 1. Phylogenetic relationships of the *ASK* genes. This neighbor joining (NJ) tree was generated in MEGA2.1 based on amino acid sequence analysis, with the "complete deletion" option selected and the poisson correction for distance estimates. The number below (or above) each clade indicates the bootstrap supports in 1,000 replicates (H. Kong and H. Ma, unpublished data). *ASK* genes have been classified into eight groups based on this tree.

band for the inflorescence than those for the seedling and silique. In group 7, *ASK14* expression was only detected in the inflorescence. *ASK15* expression was difficult to detect after one round of PCR; a second PCR using nested primers found it to be expressed in the seedling and leaf (Fig. 2). *ASK16* and *ASK17* expressions were mainly detected in the silique (Fig. 2). For *ASK18*, *ASK20*, and *ASK21*, similar levels of expression were detected in all tissues examined (Fig. 2).

Expression Analysis by in Situ Hybridization

The expression patterns of 11 *ASK* genes were further characterized using RNA in situ hybridization experiments; these 11 genes were chosen in part based on their amino acid sequence similarities. *ASK1* was included as a positive control; moreover, we wanted to compare *ASK1* and *ASK2* expression patterns because they have very similar sequences and RT-PCR results. *ASK3* and *ASK4* are highly similar to each other and, to a lesser extent, to *ASK1* and *ASK2*. *ASK14*, *ASK15*, *ASK18*, and *ASK19* are relatively similar in sequence, but differ in expression based on RT-PCR experiments. Finally, *ASK5*, *ASK9*, and *ASK11* were chosen as representatives of three other distinct groups.

To obtain gene-specific probes, we used 3′-untranslated regions (UTRs) of the *ASK* genes. Previous in situ hybridization experiments indicated that probes of 400 bps or longer do not cross-hybridize to related sequence with 83% or less nucleotide sequence identity (Yanofsky et al., 1990; Ma et al., 1991; Flanagan and Ma, 1994). The *ASK* UTRs that were used here as templates for probes are all about 250 to 350 bps long, and most of them have less than 50% identities to other *ASK* genes. Only one exception is that the *ASK9* sequence used for probe synthesis is 89% identical to *ASK10*, 89% to *ASK8*, and 86% to *ASK7*. Furthermore, in situ hybridization experiments using *ASK1* probe on *ask1* null mutant tissues, as a control, indicated that the probe was specific and did not detect expression of the other *ASK* genes (data not shown).

Expression Patterns of *ASK1* and *ASK2*

The *ASK1* and *ASK2* mRNAs were detected in all major organs examined, although *ASK2* expression was at lower levels than those of *ASK1* (Fig. 3). Both *ASK1* and *ASK2* mRNAs accumulate weakly in the cortical layer and epidermis of the root (Fig. 3, A and B) but are not detectable in the vascular tissue. In the young stem, *ASK1* and *ASK2* are expressed uniformly in the pith and vascular bundle (Fig. 3, C and D). No expression was observed for either *ASK1* or *ASK2* in the mature stem (data not shown). In the leaf, *ASK1* is uniformly expressed at a higher level than that of *ASK2* (Fig. 3, E and F). In the silique, *ASK1* is expressed throughout the valve and devel-

Table I. *Primers for RT-PCRs*

Gene Name and Identification No.[a]	OMC No.	Primer Sequence[b] 5′ to 3′	Gene Name and Identification No.[a]	OMC No.	Primer Sequence 5′ to 3′
APT1	571	TCCCAGAATCGCTAAGATTGCC	*ASK13*	589	TCAGAAAGCACACTAACGTCGAAA
	572	CCTTTCCCTTAAGCTCTG	At3g60010	590	CAAAAATCGCCACCACAAAAAGA
ASK1	221	AAGGTGATCGAGTATTGCAAGAG	*ASK14*	609	CATCACCCAAAATCTCAAAAACGA
At1g75950	383	GAAGATAGTCATGATTCATGAAG	At2g03170	610	TCATCAAAACCCTAAAACCGACAA
ASK2	420	ATCCGAAACCACGGCCGAT	*ASK15*	601	CACAGCGAAACACAAAGCTAGGG
At5g42190	593	AAATGGGTCGAGGACATGAC	At3g25650	602	TCGTTTAACACACAAAGCAAACAAA
ASK3	575	CAAACGATGGCAGAAACGAAGA		746[c]	TCTCGAACTTCCTCTGGCGTCT
At2g25700	576	AGCCGGGATTGAATGAAGAAAA		747[c]	TGTTGACTAGTTCCGATGGCGA
ASK4	622	CCATCCTCGCCAAGGTTATTGAG	*ASK16*	607	CAAAACAAACAATGTCTTCGAACAAG
At1g20140	574	AACACCCTTCAGATCCGATC	At2g03190	608	CATCGAGATCAACACTTAGAAAACGGA
ASK5	591	TCAACAACTTTGATGATCCGCTCTC	*ASK17*	605	TCGAGATCAACACTTAGACAACGGA
At3g60020	592	CGCTCCATAACAATGTCGACGAA	At2g20160	606	CAGCAAAACAAACAAAATGTCTTCG
ASK6	617	ATCGTGTTGACAAGCTCCGATGA	*ASK18*	581	GCCTTATAACAATGGCTTCTTCTTCCG
At3g53060	618	TCGCATCAAGAAAACCGAAAACA	At1g10230	582	TCGAAACGAAATTCTGCAAACCC
ASK7	616	TCCCCCACAAAGAAAACAACAATG	*ASK19*	922	GAAGGAGATGATGAAGCTGAGGA
At3g21840	666	AGCCCATTTATTGTCGTTGACC	At2g03160	923	CATTGCGAATTGCTTCTTCTTCT
ASK8	603	GAAAGAAAGCTGTTTAGGTTTTGA	*ASK20*	641	ATGTCAGAAGGTGATTTGGCCGT
At3g21830	604	TCCCCCACAAAGAAAACAACAATG	At2g45950	642	AGAGCCTCTTTGTGTCCATTCGG
ASK10	643	CATTGATTCTCCCTACGAACCGC	*ASK21*	832	TTCCACCATCGTCACTCATTGCT
At3g21860	628	AATCCCGCTTCCAGAAGTGACAG	At3g61415	833	CTGGATTGACTCGCTGTGGAAGA
ASK11	838	AATCCCTCTTGCAAACGTGGAAA			
At4g34210	839	TCGATCAAGACGTTAAAAAGAAAACA			
ASK12	838	AATCCCTCTTGCAAACGTGGAAA			
At4g34470	665	TCTACATTATTGATCAAGAAGTTGC			
	583[c]	GCTAGTTAGGTTTTTGATTCATGGG			

[a] The gene identification number is provided below the gene name. [b] The primers for each gene are listed next to the gene name and identification number, except those noted in c. [c] Primers used for nested PCR for the gene immediately above.

oping seed (Fig. 3G), again at levels higher than those of *ASK2* (Fig. 3H). *ASK1* expression is strong in the inflorescence meristem (IM) and young floral bud (Fig. 3I), at high levels in all floral organ primordia; furthermore, it is expressed at a high level in the male meiocytes in a flower at approximately stage 9, when meiosis occurs (Fig. 3K). *ASK1* expression remained high in the pollen grains (Fig. 3M). Compared with *ASK1*, *ASK2* is expressed in the IM and flower at lower levels (Fig. 3J). *ASK2* signal is also present in the male meiocytes and pollen grains (Fig. 3, L and N). These in situ hybridization results indicate that *ASK1* and *ASK2* have very similar expression patterns.

Expression Patterns of *ASK3* and *ASK4*

The *ASK3* and *ASK4* genes also have similar expression patterns (Fig. 4). In the root, young stem, and young leaf, the signals of *ASK3* (Fig. 4, A, C, and E) and *ASK4* (Fig. 4, B, D, and F) are very low if detectable at all. Both *ASK3* and *ASK4* have high levels of expression in the silique (Fig. 4, G and H); *ASK4* is very strongly expressed in the valve, septum, and developing seed (Fig. 4H). Weak signals of *ASK3* were also detected in the inflorescence (Fig. 4I). No expression of *ASK3* was detected in the IM, and *ASK3* mRNA in the flower is mostly restricted to the sepal and pedicel (Fig. 4, I and K). *ASK4* expression was detected throughout the inflorescence at a higher level in the IM than in the young flower (Fig. 4, J and L). Although *ASK3* expression was not detected in pollen grains (Fig. 4M), *ASK4* (Fig. 4N) is expressed in the pollen grains, but at a lower level than those of *ASK1* and *ASK2* (Fig. 3, M and N).

Expression Patterns of *ASK14*, *ASK15*, *ASK18*, and *ASK19*

Although the *ASK14*, *ASK15*, *ASK18*, and *ASK19* genes are similar in sequence, they have different expression patterns (Fig. 5). In the root, there is no detectable expression of *ASK14* and *ASK19* (Fig. 5, A and S), and signals of *ASK15* and *ASK18* are barely detectable (Fig. 5, G and M). *ASK15* and *ASK18* are highly expressed in the pith and vascular bundle in the stem, with the levels of *ASK15* greater than that of *ASK18* (Fig. 5, H and N). In contrast, little or no expression was seen for *ASK14* or *ASK19* in either young or mature stem (Fig. 5, B and T; data not shown). Their expression in leaf varied in levels from least to greatest: *ASK14*, *ASK19*, *ASK15*, and *ASK18* (Fig. 5, C, U, I, and O). Barely detectable expression of *ASK14* was observed in the silique (Fig. 5D). In the silique, *ASK15* and *ASK18* mRNAs are higher in inner epidermis of the valve than elsewhere (Fig. 5, J and P), and *ASK19* signals are detectable in the valve and seed coat (Fig. 5V). *ASK14*, *ASK15*, *ASK18*, and

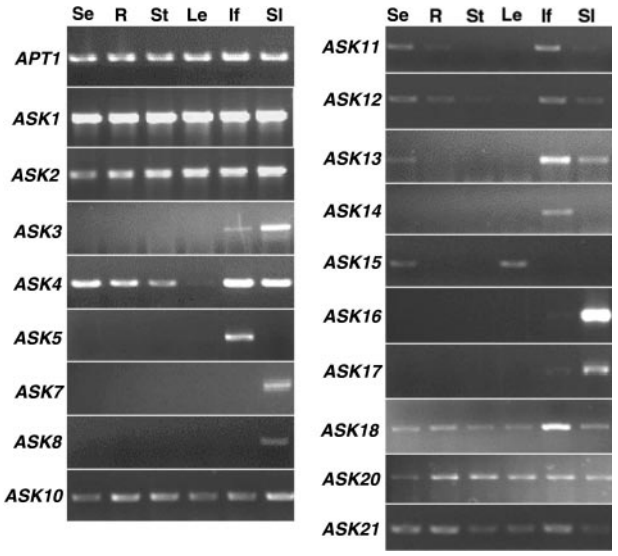


Figure 2. Analysis of *ASK* gene expression using RT-PCR. The *APT1* gene was used as an internal control, The RT-PCR results were from one round of 35 cycles, except that those for *ASK11*, *ASK12*, and *ASK15* were from two rounds of PCR (see "Materials and Methods"). Se, Seedling; R, root; St, stem; Le, leaf; If, inflorescence; Sl, silique.

ASK19 are all expressed in the inflorescence but with different spatial patterns. There is no expression of *ASK14* in the IM or pollen grains (Fig. 5E; data not shown). However, *ASK14* is expressed in the male meiocytes and even more in the tetrads (Fig. 5F; data not shown). The expression of *ASK15* and *ASK18* was found in the pedicel of young buds, with lower expression elsewhere in the flower (Fig. 5, K, Q, and R). *ASK15* is also expressed in the inner epidermis of the carpel and pedicel in the mature flower (Fig. 5L). The level of *ASK19* expression is moderate and nonspecific in the IM and flower (Fig. 5W). In the mature flower, *ASK19* expression was seen in the sepal, petal, and filament of the stamen (Fig. 5X).

Expression Patterns of *ASK5*, *ASK9*, and *ASK11*

Our results showed that the expression patterns of *ASK5* and *ASK11* were different (Fig. 6). The expression of *ASK5* was not found in the root, stem, and leaf (Fig. 6, A–C), was barely detectable in the silique (Fig. 6D), and was observed at a very low level in the inflorescence except for the IM (Fig. 6E). In the young bud, the *ASK5* mRNA is restricted to the sepal and pedicel (Fig. 6F). *ASK11* has a very weak expression in the root, stem, leaf, and silique (Fig. 6, M–P). However, *ASK11* has a slightly higher expression in the IM and young bud, particularly in the stamen (Fig. 6Q). We also detected the expression of *ASK11* in the pollen grains (Fig. 6R). As a negative control, sense probes detected very low levels of signals that matched backgrounds seen with our experimental probes (e.g. Fig. 6, S–X; data not shown). Because the *ASK9* UTR used for probe synthesis has high identity to UTRs of *ASK7*, *ASK8*, and *ASK10*, the signals detected here probably represent the combined expression of all four genes. The *ASK9*-hybridizing signal was detected in every tissue, very weakly in root (Fig. 6G), at moderate levels in the pith and vascular bundle of the young stem and in the leaf blade (Fig. 6, H and I), and at moderate levels in the valve and seed coat in silique (Fig. 6J). Signals are also present in the inflorescence except for the IM (Fig. 6K) and are especially high in the pedicel of the young flower (Fig. 6L).

Analysis of *ASK* Functions Using RNAi

To begin investigating the function of *ASK* genes, we selectively suppressed the expression of a few *ASK* genes using double-stranded RNA interference (RNAi) method. *ASK1* was chosen as a positive control because the *ask1-1* null mutant phenotypes have been described. In addition, we also wanted to know whether *ASK1* shares redundant functions with *ASK2*, or possibly other *ASK* genes, by comparing RNAi plants with the *ask1-1* mutant. *ASK11* was chosen to test the hypothesis that it plays some specific roles, because it has a more restricted expression pattern.

Table II. *Relative RT-PCR band intensities of ASK genes*

The abbreviation for the tissues is the same as in Figure 2. The number of + stands for the relative amount of PCR products; more + symbols stand for a higher expression level.

Gene Name	Se	R	St	Le	If	Sl	Gene Name	Se	R	St	Le	If	Sl
ASK1	+++	+++	+++	+++	+++	+++	*ASK12*	+	+	+	+	+	+
ASK2	+++	+++	+++	+++	+++	+++	*ASK13*	+				++	+
ASK3					+	++	*ASK14*					+	
ASK4	++	++	+	+/−	+++	+++	*ASK15*	+			+		
ASK5					+		*ASK16*						++
ASK6							*ASK17*					+	++
ASK7						+	*ASK18*	+	+	+	+	++	+
ASK8						+	*ASK19*						
ASK10	+	+	+	+	+	+	*ASK20*	+	+	+	+	+	+
ASK11	+	+			+	+	*ASK21*	+	+	+	+	+	+

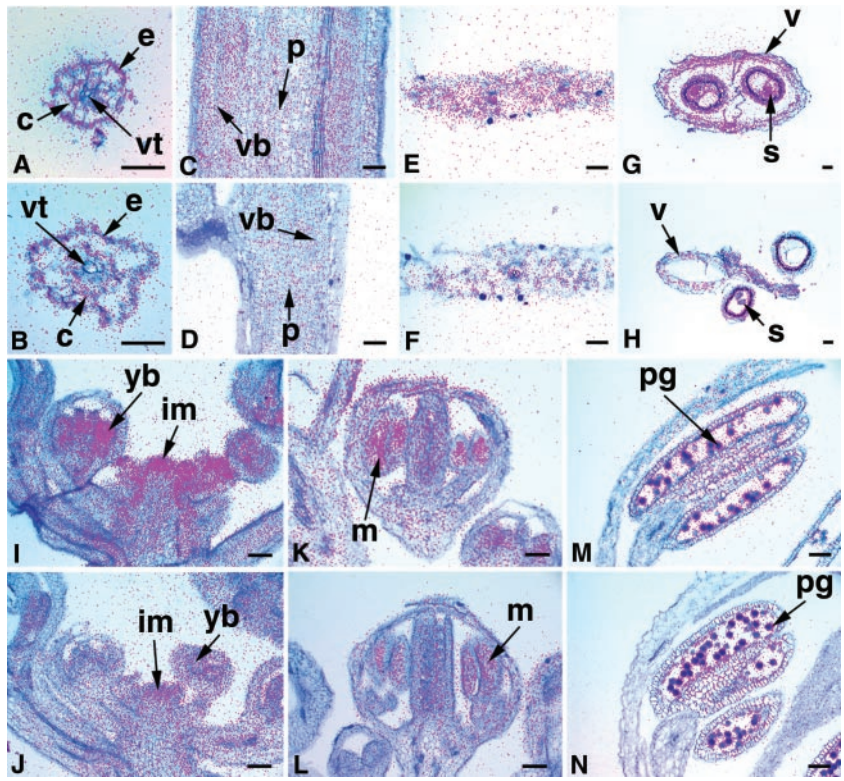


Figure 3. In situ hybridizations with *ASK1* and *ASK2*. A, C, E, G, I, K, and M, *ASK1*; B, D, F, H, J, L, and N, *ASK2*. A and B, Root cross sections with expression signal in the cortical layer (c) and epidermal cells (e), but not in the vascular tissue (vt). C and D, Longitudinal sections of a young stem with moderately expression in the pith (p) and vascular bundle (vb). E and F, Leaf cross sections showing that the *ASK1* mRNA (E) is present at a higher level than the *ASK2* mRNA (F). G and H, Silique cross sections showing expression in the valve (v) and developing seeds (s). I and J, Longitudinal sections of an inflorescence with signals in the IM (im) and young buds (yd). K and L, Two sections of a stage-9 bud with expression detected in male meiocytes (m). M and N, Anther sections of mature flowers showing expression in pollen grains (pg). Scale bar = 50 μm.

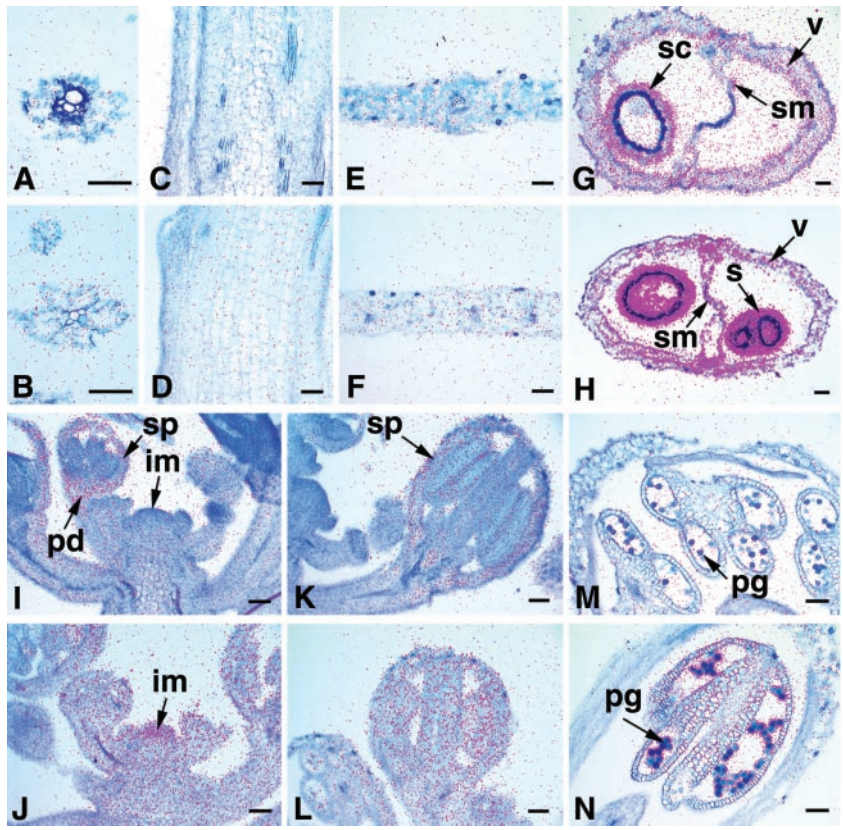


Figure 4. In situ hybridizations with *ASK3* and *ASK4*. A, C, E, G, I, K, and M, *ASK3*; B, D, F, H, J, L, and N, *ASK4*. A and B, Root cross sections with no *ASK3* expression (A) and just detectable *ASK4* expression (B). C and D, Young stem longitudinal sections with no *ASK3* (C) and weak *ASK4* expression (D). E and F, Cross sections of a leaf showing *ASK3* (E) and *ASK4* (F) mRNAs. G, Cross sections of a silique indicating that the *ASK3* signal is present in the valve (v) and seed coat (sc). H, The *ASK4* gene is very highly expressed in the valve, septum (sm), and all developing seeds (s). I, Longitudinal sections of an inflorescence showing low level *ASK3* expression in the sepal (sp) and pedicel (pd) of a young bud but not in the IM (im). J, *ASK4* expression is present in the IM but weak in young buds. K and L, Sections of an about stage-10 flower having *ASK3* expression (K) in the sepal and weak uniform *ASK4* expression (L). M and N, Sections of anther having *ASK4* (N) but no *ASK3* (M) expression in the pollen grains (pg). Scale bar = 50 μm.

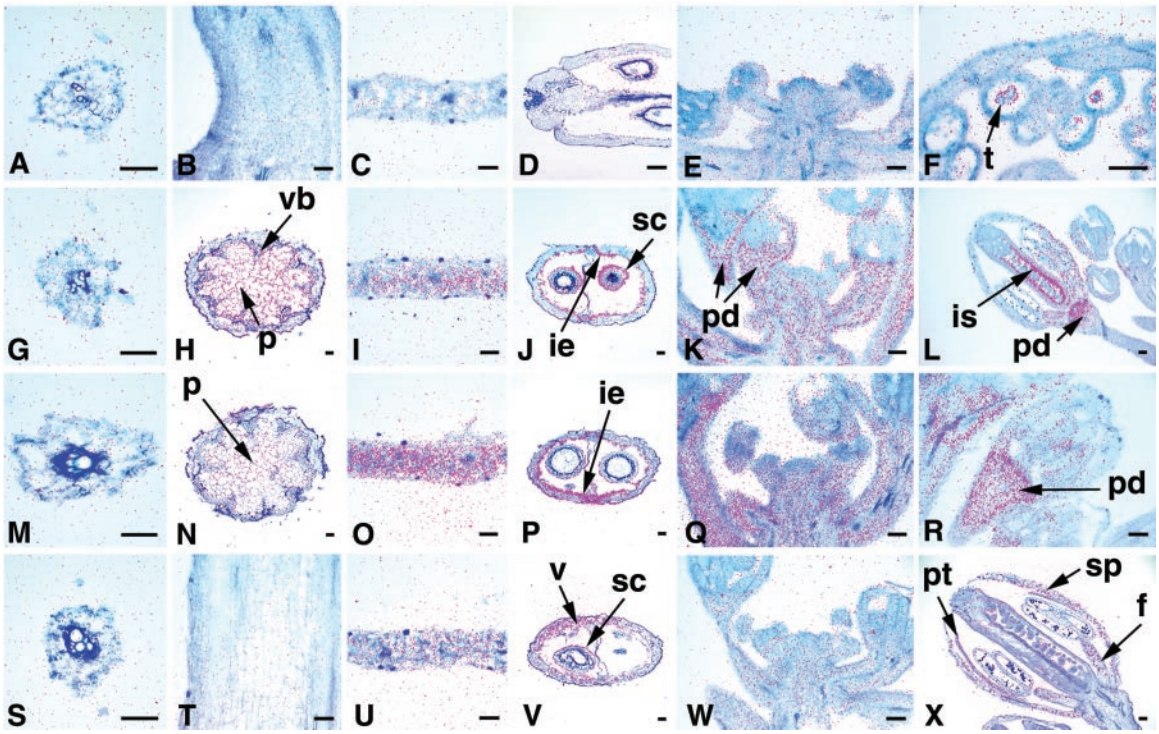


Figure 5. In situ hybridizations with *ASK14*, *ASK15*, *ASK18*, and *ASK19*. A to D, Sections showing no *ASK14* expression in the root (A) and young stem (B), but very weak expression in the leaf (C) and silique (D). E, Low level uniform *ASK14* expression in the inflorescence. F, *ASK14* expression in tetrads (t). G, No *ASK15* expression in the root. H, *ASK15* gene is highly expressed in the pith (p) and vascular bundle (vb) of a stem. I, *ASK15* expression in the leaf. J, *ASK15* is present in the inner epidermis (ie) of the valve and seed coat (sc). K, In the inflorescence, *ASK15* is more restricted to the pedicel (pd) but much less expressed in other parts of the flower. L, *ASK15* expression in the inner surface (is) of the carpel and pedicel. M, Barely detectable *ASK18* expression in the root. N, Low *ASK18* expression in the stem. O, *ASK18* is very strongly expressed in the leaf. P, *ASK18* is present in the valve; more expression in the inner epidermis of the silique. Q and R, In the inflorescence (Q) and a young bud (R), *ASK18* is restricted to the pedicel and is minimally expressed in other parts of the flower. S to W, *ASK19* had no expression in the root (S) and young stem (T), low level expression in the leaf (U), moderate expression in the valve and seed coat (V), and low and uniform expression in the inflorescence (W). X, *ASK19* expression in the sepal (sp), petal (pt), and filament (f) of mature flower. Scale bar = 50 μm.

Phenotypes from *ASK1* RNAi Plants

We generated approximately 200 independent *ASK1* RNAi lines; more than 80% of the transgenic lines showed some degrees of floral defects similar to those of the *ask1-1* mutant, including reduced fertility, indicating that the suppression is very efficient. *ASK1* RNAi lines with a weak phenotype usually had normal vegetative growth, normal stature, mild defects in flower development, and normal or partially reduced fertility. In contrast, lines with a strong phenotype had abnormal vegetative growth, short stature, and severe defects in flower development, and were usually male sterile. A strong (*ASK1*-Line 3) line and relative weak (*ASK1*-Line 11 and 14) lines were further examined.

During vegetative development, we observed a slight reduction of leaf size and plant stature in the *ask1-1* mutant, similar to previous findings (Fig. 7, B and F; Zhao et al., 1999). The strong *ASK1*-Line 3 showed dark-green, wrinkled leaves with a very short leaf petiole, whereas the width of the leaf was not affected much (Fig. 7C). In addition, the plant was much shorter in stature than the *ask1-1* mutant (Fig. 7G). RT-PCR analysis showed that *ASK1* expression was not detectable in the rosette leaf of *ASK1*-Line 3, and *ASK2* expression was also reduced (Fig. 8A). In the weak lines *ASK1*-Line 11 and *ASK1*-Line 14, vegetative development was generally normal (Fig. 7H). RT-PCR analysis further confirmed only a slight reduction of *ASK1* expression and nearly normal level of *ASK2* expression in rosette leaf in *ASK1*-Line 14 (Fig. 8A). In addition to these lines, we also observed several dwarf, dark-green plants that died before flowering, one of which is shown in Figure 7D. RT-PCR results indicate that *ASK1* expression was not detected in this plant, and *ASK2* expression was reduced (Fig. 8B).

The *ask1-1* mutant flowers had a slightly reduced number of petals, staminoid petals, and short filaments (Fig. 7J; Zhao et al., 1999). In *ASK1*-Line 3, stamens were similar to those in *ask1-1* flowers, whereas petal number was further reduced. The most

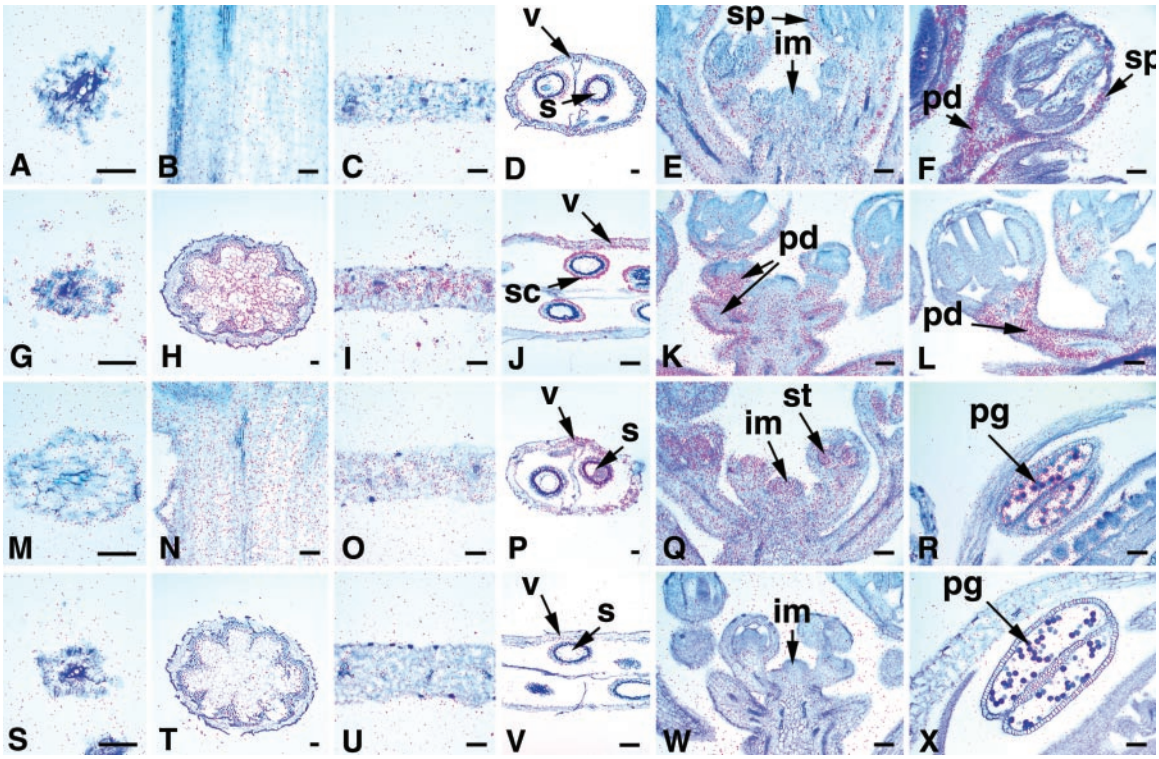
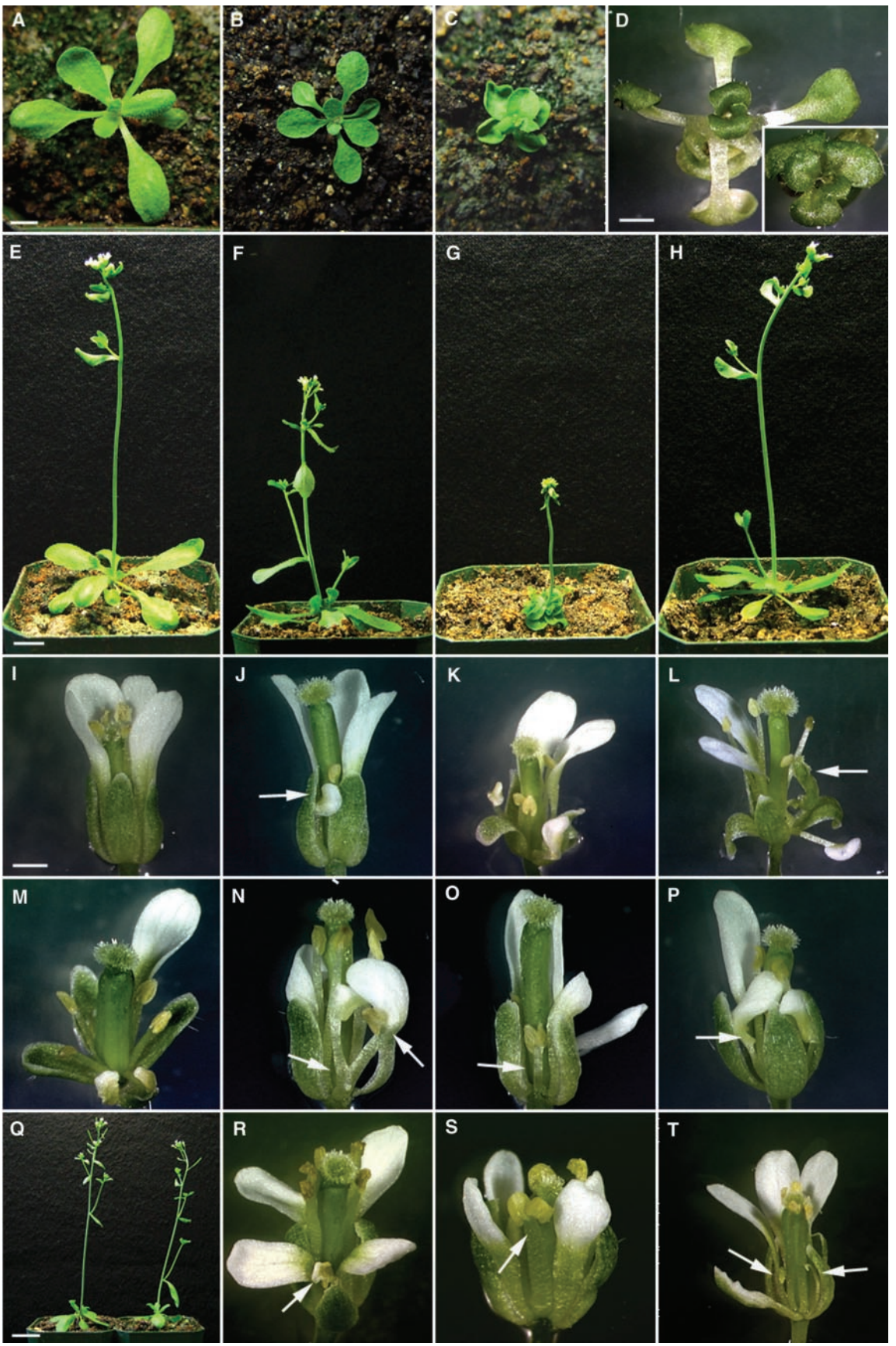


Figure 6. In situ hybridizations with *ASK5*, *ASK9*, and *ASK11*. A to D, No *ASK5* expression in the root (A), young stem (B), and leaf (C), but just detectable expression in the silique (D). E, Very low *ASK5* expression in flowers, but no expression in the IM (im). F, *ASK5* expression in both the sepal (sp) and pedicel (pd). G to J, *ASK9*-hybridizing signal is present at a very low level in the root (G), at a moderate level in the stem (H) and leaf (I), and at low levels in the valve (v) and a seed coat (sc; J). K and L, In the inflorescence (K) and young bud (L), *ASK9*-hybridizing signal is present mostly in the pedicel but much less in other parts of the flower. M to P, *ASK11* was just detected in the root (M), and expressed in the stem (N), leaf (O), and valve and seed (P) at low levels. Q, *ASK11* was more highly expressed in the IM and stamen (st) in young buds. R, *ASK11* is present in pollen grains (pg). S to X, Sections hybridized with *ASK9* sense probe only showing signals at the background level in the root (S), stem (T), leaf (U), silique (V), inflorescence (W), and anther (X). Scale bar = 50 μm.

distinctive phenotype in this line is that the outer whorl has sepals that usually curved downward (Fig. 7, K and L), with occasional carpel-like structures (Fig. 7L). In some plants, the number of carpels was increased (Fig. 7M). In weak *ASK1* RNAi lines, sepal and carpels were normal, and the reduction of filament length was not as much as that of the *ask1-1* mutant. Staminoid petals were also frequently observed. In addition, fused petals, fused stamens, or petal-stamen chimeras were frequently found (Fig. 7, N–P). Similar to the *ask1-1* mutant, the strong *ASK1*-Line 3 was male sterile, whereas the weak lines (*ASK1*-Line 11 and *ASK1*-Line 14) were partially sterile. Consistent with the phenotypes of the transgenic plants, *ASK1* expression was suppressed to a greater extent in the strong line than in weak lines (Fig. 8C). In addition, *ASK2*, and possibly *ASK4*, expression levels also seemed to be suppressed (Fig. 8C). The expression of *ASK11*, *ASK12*, and *ASK18* was detected only after increased numbers of PCR cycles; therefore, it is not certain whether they are also affected by the *ASK1* RNAi transgene.

Figure 7. Phenotypes of *ASK1* and *ASK11* RNAi transgenic plants. A to C, Seedlings of the wild type, *ask1-1* mutant, and strong *ASK1*-Line 3 at the age of 3 weeks. D, Two-week-old seedlings of the wild type and a strong *ASK1* RNAi plant (inset), which was dwarf and dark-green and died before flowering. E to H, Wild-type, *ask1-1* mutant, *ASK1*-Line 3, and the weak *ASK1*-Line 14 plants at the age of 4 weeks. The plant from Line 3 had a much shorter stature than the *ask1* mutant (G), whereas the plant from Line 14 had a normal stature and normal vegetative growth compared with that of wild type (H). I, Wild-type flower. J, An *ask1* flower showing a staminoid petal (arrow) and short stamens. K to M, Flowers from the strong *ASK1*-Line 3 exhibiting down-curved sepals, reduced petal identity, short stamens (K), carpel-like structures in outer whorl (L, arrow), and four carpels (M). N to P, Flowers from weak *ASK1* RNAi plants having fused organ between petals, petal and stamen (N, Line 11; P, Line14), stamens (O, line 11). Q, Five-week-old wild-type (left) and *ASK11* RNAi plants (right) with a normal vegetative growth and seed setting. R to T, Flowers from the *ASK11* RNAi plants showing a petaloid stamen (R), fused stamen (S), and filament-like structure or abnormal anther (T). One petal in T was removed. Scale bars = 0.5 cm (A–C, I–P, and R–T), 1 mm (D); 0.75 cm (E–H); and 2 cm (Q).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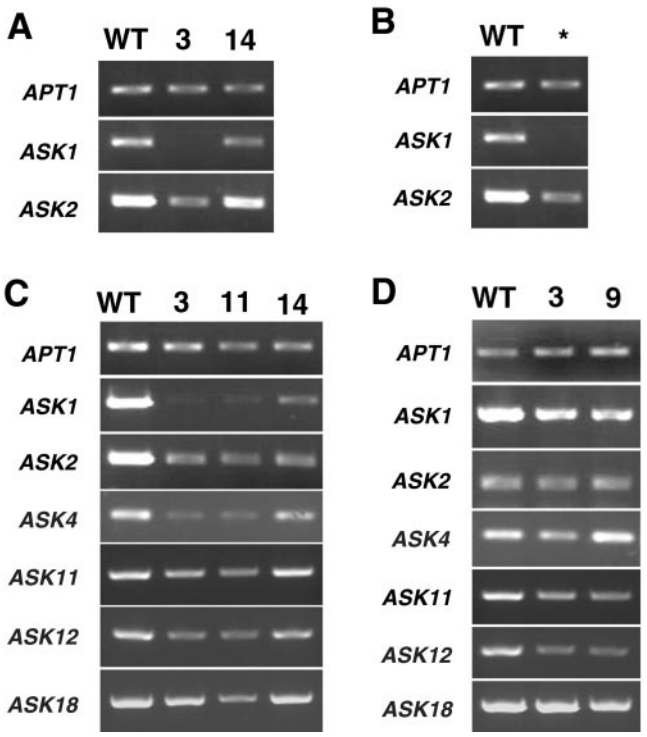

Figure 8. RT-PCR analysis of the RNAi transgenic plants. A, Leaf tissue from the wild type, *ASK1*-Line 3, and Line 14. B, Seedling from the wild type and a strong *ASK1* RNAi plant (*). C, Inflorescences from the wild type, *ASK1*-Line 3, and weak Line 11 and Line 14. D, Inflorescences from the wild-type and *ASK11* RNAi transgenic lines.

Phenotypes from *ASK11* RNAi Plants

ASK11 is almost identical to *ASK12* in the coding region; in addition, our RT-PCR results for *ASK11* and *ASK12* were very similar. Therefore, *ASK11* and *ASK12* may play very similar roles and might be functionally redundant. Consequently, the phenotype observed in *ASK11* RNAi plants, if any, might be the result of a reduction of both *ASK11* and *ASK12* functions. Approximately 100 independent transgenic *ASK11* RNAi lines were generated, and no obvious phenotype was observed during vegetative development. All transgenic lines had normal fertility (Fig. 7Q). In other words, none of the *ASK11* RNAi plants had vegetative or fertility defects that were observed in typical *ASK1* RNAi plants.

However, we observed slight defects in early flowers during reproductive development, although more severe phenotypes were observed in the flowers and inflorescences at late stages. Flowers of *ASK11* RNAi plants had normal sepals, petals, and carpels. The only defects were petaloid or fused stamens in the third whorl of about 15% of the first 15 flowers (Fig. 7, R and S). Occasionally filament or abnormal anthers were also found in the transgenic plants, whereas other stamens in the same flower still had normal length and produced viable pollen (Fig. 7T). The phenotypes of the *ASK11* RNAi plants differ from the *ASK1* RNAi plants in that even weak *ASK1* RNAi plants exhibit defects in both second and third whorls, as do the *ask1-1* mutant flowers. Comparison of the RT-PCR results between the control *APT1* gene and various *ASK* genes suggested that the expression of *ASK11* and *ASK12* was slightly reduced in the transgenic plants. In addition, *ASK1* expression was reduced slightly, but *ASK2*, *ASK4*, and *ASK18* expression was close to normal (Fig. 8D).

DISCUSSION

ASK Genes Exhibit Several Different Expression Patterns

Our RT-PCR and RNA in situ hybridization experiments indicate that nearly all of the *ASK* genes predicted from genomic sequences are expressed at different levels and exhibit a variety of spatial patterns. In addition, highly similar genes within each of several groups tend to have similar expression levels and patterns, although members of some groups show different expression patterns. In particular, *ASK1* and *ASK2* are both highly expressed in all of the tissues that were tested. In situ hybridization experiments further indicate that these two genes share very similar spatial patterns of expression throughout the plant, suggesting that they may be functionally redundant. This is further supported by the results that both ASK1 and ASK2 proteins interact with one of several F-box proteins in vitro and/or in yeast two-hybrid assays (Gray et al., 1999; Samach et al., 1999; Gagne et al., 2002; Xu et al., 2002). Also, similar expression patterns for *ASK3* and *ASK4* in the silique and the inflorescence suggest possible redundancy. Furthermore, whereas the group 4 genes (*ASK7*, *8* and *10*) are preferentially expressed in the silique, the group 5 genes (*ASK11* and *12*) are more abundant in inflorescence. The highly conserved sequences and very similar expression patterns suggest that the members of each of groups 1, 2, 4, and 5 are the result of relatively recent gene duplication events. These ideas are supported by the fact that group 4 genes form a tandem array of direct repeats on chromosome 3 and *ASK11* and *ASK12* are closely linked to each other on chromosome 4 (see below for more discussion of *ASK11* and *ASK12* functions).

In contrast with the above *ASK* genes, those in groups 3, 6, 7, and 8 exhibit greater divergence in sequence and/or in expression pattern, suggesting that they may have evolved to have different functions and/or lost their original functions. As mentioned above, no *ASK6* expression was detected. This and the fact that its sequence predicts a truncated protein lacking parts of both the highly conserved N- and C-terminal domains strongly suggest that it is a pseudogene. In addition, *ASK15* mRNA was found to contain a predicted 50-bp intron, making it a possible pseudogene.

A moderate expression level of *ASK15* and *ASK19* was detected from our in situ experiments, whereas RT-PCR results suggest that they are expressed at very low levels. One explanation for this is that the tissues used for those two experiments were not identical; alternatively, the primers for *ASK19* RT-PCR may be inefficient.

Our results clearly indicate that some *ASK* genes are widely expressed, suggesting that they may have pleiotropic functions, whereas others have more restricted expression and perhaps specific functions. Sequence divergence and expression differences between some groups of *ASK* genes also suggest that genes of these different groups may have evolved to have distinct functions. Yeast two-hybrid results showed that ASK1, ASK2, ASK11, and ASK19 can interact with about half or more of the 23 F-box proteins tested, and ASK4 interacts with several F-box proteins, whereas ASK5, ASK16, and ASK18 can interact only with a few of the F-box proteins (Gagne et al., 2002). If these interactions reflect in vivo activities, then they also support the ideas that ASK1, ASK2, ASK11, ASK13, and perhaps ASK4, have more basic or broad functions, whereas the others may have more restricted functions. In addition, ASK9 and ASK17 cannot interact with any of F-box proteins tested (Gagne et al., 2002), suggesting that they may interact with F-box proteins other than those tested and have divergent functions from other ASK proteins. Tissue-specific expression was also found in some of the F-box protein genes (Ingram et al., 1995; Kuroda et al., 2002). It is expected that only the ASK proteins and F-box proteins that are expressed in the same plant cells can interact in vivo to confer a biological function. Thus it would be very informative to compare the expression of the *ASK* genes and genes encoding F-box proteins.

Many *ASK* Genes May Function during Reproductive Development

Our results show that most of the *ASK* genes are either expressed in all tissues tested or are preferentially expressed in the inflorescence and/or fruit. This is consistent with the fact that flowers and fruits are more complex structures than roots or leaves. It is possible that reproductive development and physiology require many regulatory proteolytic pathways, not only those found in vegetative tissues, but also those specific to reproductive structures. For example, the expression of *ASK1*, *ASK2*, *ASK4*, and *ASK11* in the shoot apical meristem suggests that they are involved in regulating cellular events in the meristem. Their expression in the pollen at relatively high levels suggests that they may also play a role in pollen development. In addition, the fact that *ASK15* and *ASK18* were expressed in the pedicel of young flowers suggests roles during pedicel development. *ASK1* is known to regulate male meiosis; the detection of *ASK2* expression in meiotic cells suggests that it may also be involved in male meiosis. Furthermore, the *ASK14* mRNA is present at a high level in the microspores but is absent in the pollen grains, suggesting that the *ASK14* gene may function in the newly formed microspores.

The expression of most *ASK* genes was detected in the silique, suggesting that they play important roles during embryo, seed, or fruit development. However, the details of the expression patterns were different. For example, the *ASK4* gene is expressed at high levels in several tissues, including the valve, septum, and developing seed, but *ASK1* expression is higher in the embryo than other parts. The signals of *ASK3* and *ASK19* were more restricted to the seed coat, whereas, most interestingly, the *ASK15* and *ASK18* mRNAs are mostly present in the inner epidermis of the valve. Therefore, different *ASK* genes may have distinct functions in the development of seed and/or fruit. Recently, a mutation in the *AtCUL1* gene was found to cause an arrest of embryo and endosperm development before either the first or second mitosis of the zygote/embryo (Shen et al., 2002). Therefore, ubiquitin-dependent proteolysis is likely to be critical for embryogenesis, possibly involving one or more of the *ASK* genes.

ASK1 May Participate in Multiple Pathways Regulated by SCF Complex and Share Redundant Function with Other *ASK* Genes

ASK1 is strongly expressed in all tissues tested, consistent with previous reports on its expression (Porat et al., 1998). Previous studies of the *ask1-1* mutant, which is a null mutant with a *Ds* insertion, indicate that it is required for male meiosis and is important for flower development and auxin response (Gray et al., 1999; Yang et al., 1999; Zhao et al., 1999). Other studies demonstrated that ASK1 was able to interact with one of several F-box proteins in yeast two-hybrid and in vitro-binding assays (Gray et al., 1999; Samach et al., 1999; Dieterle et al., 2001; Woo et al., 2001; del Pozo et al., 2002; Gagne et al., 2002; Xu et al., 2002). Therefore, *ASK1* is a likely component of multiple SCF complexes that regulate many important pathways. Further support for *ASK1* having a more general function comes from a structure and evolution analysis of the *SKP1* gene family, which showed that *ASK1* is more conserved and may evolve more slowly than other *ASK* genes (H. Kong and H. Ma, unpublished data).

On the other hand, the relatively weak vegetative and floral phenotypes observed in the *ask1-1* null mutant indicate that other *ASK* genes also play a role and may share redundant functions with *ASK1*; one likely candidate is the *ASK2* gene, as suggested by its expression pattern (see above). This is supported by the enhanced phenotypes in the strong *ASK1* RNAi line together with a reduction in expression of both *ASK1* and *ASK2* and perhaps that of other *ASK* genes.

The dwarf phenotype and the dark-green leaves of the strong *ASK1* RNAi transgenic plant are similar to those found in GA-deficient or -insensitive mutants (Koornneef and van der Veen, 1980; Koornneef et al., 1985). It has been shown that putative transcription repressors (DELLA proteins), such as RGA and GAI, regulate GA signal transduction through transcriptional regulation (Olszewski et al., 2002). Recent studies have shown that the rice (*Oryza sativa*) *GID2* gene encodes an F-box protein that controls the degradation of the rice DELLA protein SLR1 through GA-dependent phosphorylation (Sasaki et al., 2003). In Arabidopsis, a mutation in *SLY1*, a *GID2* homolog, causes a deficiency in GA response and an accumulation of RGA protein (Steber et al., 1998; McGinnis et al., 2003). Our results suggest that part of the GA signaling in Arabidopsis might be mediated through the degradation of DELLA proteins by SCF complexes that involve ASK1, ASK2, and perhaps other ASK proteins.

Previous studies in our lab have demonstrated that ASK1 is required for the regulation of B function of the ABC model in flower development through interacting with an F-box protein UFO (Zhao et al., 1999, 2001). In addition to the severe reduction of B function in the strong *ASK1* RNAi line, we also observed various defects in both sepals and carpels. The presence of carpel-like structures in outer whorls suggests the expansion of C function. In addition, fused organs between petals and stamens indicate that *ASK1* and other *ASK* genes are also required for normal activity of the flower meristem, probably through the regulation of cell division.

ASK11 and *ASK12* Might Be Involved in Flower Development

ASK11 and *ASK12* are extremely similar in both sequence and expression patterns, suggesting that they have similar functions. ASK11 was able to interact with a similar set of F-box proteins as ASK1 and ASK2 in yeast two-hybrid assays (Gagne et al., 2002). Because *ASK11* and *ASK12* expression seems to be at low levels during normal development, their functions might not be very critical for development. We found that the growth of *ASK11* RNAi plants was generally normal, but we did observe some defects in the stamen of the transgenic plants, which is consistent with the expression of *ASK11/12* in stamen primordia, as observed in RNA in situ hybridization experiments.

Although the expression of *ASK11* and *ASK12* was not eliminated in the RNAi lines, weaker RT-PCR bands were reproducibly obtained from the RNAi lines than from the wild-type plants. Because additional RT-PCR cycles were needed to detect expression, the RT-PCR results suggest a reduction of *ASK11/12* expression from already-low wild-type levels. It is possible that the RNAi construct with the 35S promoter was not able to completely eliminate weak expression. However, because the reduction was observed after an increased number of PCR cycles, the interpretation of a role of *ASK11/12* in stamen development must be regarded as tentative. More definitive understanding of the function of these genes will require the analysis of mutations in these genes.

We have also noticed that *ASK1* expression seems to be reduced slightly in the *ASK11* RNAi plants, suggesting that the reduction of *ASK1* might have contributed to the observed phenotypes. Nevertheless, the *ASK11* RNAi flowers were different from flowers of *ASK1* RNAi transgenic lines, supporting a possible distinct role for *ASK11* (*ASK12*) in flower development.

CONCLUSIONS

Clearly, the *ASK* gene family contains a spectrum of actively expressed members that exhibit a variety of patterns. The encoded proteins can potentially form a multitude of SCF complexes that regulate plant development and physiology at different stages. It has been shown that SKP1 may form a complex lacking cullin, and results from *C. elegans* indicate that some SKP1 homolog do not interact with cullins (Yamanaka et al., 2002). Therefore, some ASK proteins could function by forming complexes different from SCF. In summary, *ASK* genes may play a wide range of important regulatory roles, and future studies using reverse genetics, biochemistry, and cell biology promise to uncover additional functions for this important regulatory gene family.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis plants were of the L*er* ecotype. Except otherwise noted, they were grown on Metro-Mix 360 (Scotts-Sierra Horticultural Products, Maryville, OH) at 22°C (16 h of light and 8 h of dark).

RT-PCR

For RT-PCR experiments, RNA was isolated from 3- to 4-d-old seedlings, roots of 2-week-old plants, leaves of 3- to 4-week-old plants, inflorescence stems and inflorescences of 5-week-old plants, and 2- to 10-d-after-pollination siliques. Total RNA was isolated using the RNeasy mini kit (Qiagen USA, Valencia, CA) and was treated with DNase I (Invitrogen, Carlsbad, CA). One microgram of RNA from different tissues was reverse transcribed into cDNA with oligo(dT) (16-mer) using Super Script II reverse transcriptase (Invitrogen). A fraction (about 1/20) of the first strand cDNAs was used as a template for PCR with gene-specific primers (Table I). PCR was carried out under standard conditions using 10 pmol of each primer and 27 (for *APT1* gene) or 35 cycles (for most *ASK* genes) of 94°C for 30 s, 56°C to 66°C for 40 s, and 72°C for 60 s. A second PCR of 12 cycles (for *ASK11* and *ASK15*) or 20 cycles (for *ASK12*) was performed with the same or nested primers (Table I). Control PCRs without reverse transcriptase did not produce any PCR bands. Fifty microliters of PCR products was separated on 1.5% (w/v) agarose gels containing ethidium bromide and visualized by UV light.

In Situ Hybridization

Samples of root, leaf, inflorescence stem, inflorescence, and silique were isolated from 3- or 4 week-old plants and were immediately fixed in an formaldehyde-acetic acid fixative. RNA in situ hybridizations with radioactive probes were performed as previously described (Drews et al., 1991; Flanagan and Ma, 1994). To avoid cross-hybridization, the sequences downstream of the stop codon were amplified by PCR with gene-specific primers (Table III) and cloned into pGEM-T vector (Promega, Madison, WI) for synthesizing probes.

Plasmid Constructs and Arabidopsis Transgenic Lines

The RNAi vector pRR2222 was kindly provided by Dr. Ramesh Raina (Pennsylvania State University). It was derived from another RNAi vector pFGC1008 (kindly provided by Dr. Carolyn Napoli [University of Arizona, Tucson]) by replacing the β-glucuronidase fragment flanked by AscI and *Bam*HI restriction sites in pFGC1008 with the first intron of the Arabidopsis *GPA1* gene. To generate the *ASK1* RNAi construct, the *ASK1* coding region was amplified from the *ASK1* cDNA with primers oMC529 (5′-TCACTAGTGAGCTCATAACCATGTCTGCGAAGAA-3′) and oMC 530 (5′-TCGGATCCGGCGCGCCGATAGTCATGATTCATGAAG-3′; an AscI site is underlined). The PCR product was cloned into the pCRII-TOPO vector (Invitrogen), resulting in the pMC2550 plasmid. The cloned *ASK1* cDNA was then digested with *Xho*I (in *ASK1* coding region) and AscI (introduced by oMC530) and was cloned into the *Xho*I and AscI sites of pRR2222 in the sense orientation. Finally, pMC2550 was digested with *Bam*HI (introduced by oMC530) and *Xba*I (from pCRII-TOPO) and cloned in the antisense orientation into the *Bam*HI and *Spe*I site of the pRR2222-derived plasmid with the sense *ASK1* fragment to produce pMC2570 (Fig. 9A).

The *ASK11* RNAi vector was constructed in a similar way. The *ASK11* cDNA was amplified from inflorescence of L*er* ecotype using primers oMC 611 (5′-CCTCCTCCACAAGGAACACACAATG-3′) and oMC612 (5′-GCTAGTTAGGGTTTTGATTCATGGG-3′) and cloned into the pCRII-TOPO vector, resulting the plasmid pMC2569. The cloned *ASK11* cDNA was then digested with *Xho*I and *Spe*I and cloned in the sense orientation into the *Xho*I and *Avr*II sites of pMC2560, a vector modified from pRR2222, yielding pMC2594. The pMC2560 plasmid was derived from pRR2222 by replacing the fragment flanked by *Xho*I and AscI restriction enzyme sites with the 3′-UTR of *ASK1* gene amplified by oMC438 (Table III) and oMC577, the latter of which contains a same sequence with oMC439 (Table III) plus *Avr*II and AscI sites. The antisense *ASK11* fragment was cloned into pMC2594 in the same way as that for *ASK1*, yielding pMC2600 (Fig. 9B). All constructs described above were confirmed by sequencing and were introduced into Arabidopsis plants by *Agrobacterium tumefaciens*-mediated in planta transformation (Clough and Bent, 1998). All plants were grown in the same condition, and the first 15 flowers were used for analysis.

To detect the expression of *ASK* genes in the transgenic plant, RT-PCR was carried out, with 25 cycles for *ASK1* and *ASK2*, 28 cycles for *ASK4*, and 25 cycles followed by another 20 cycles for *ASK11* and *ASK12*, and 35 cycles for *ASK18*. For each reaction, three replications were performed. Primers for *ASK1* were oMC529 (describe above) and oMC799 (5′-GAGTAAGAAA-CATTGGTTCTTG-3′); primers for other genes are shown in Table I.

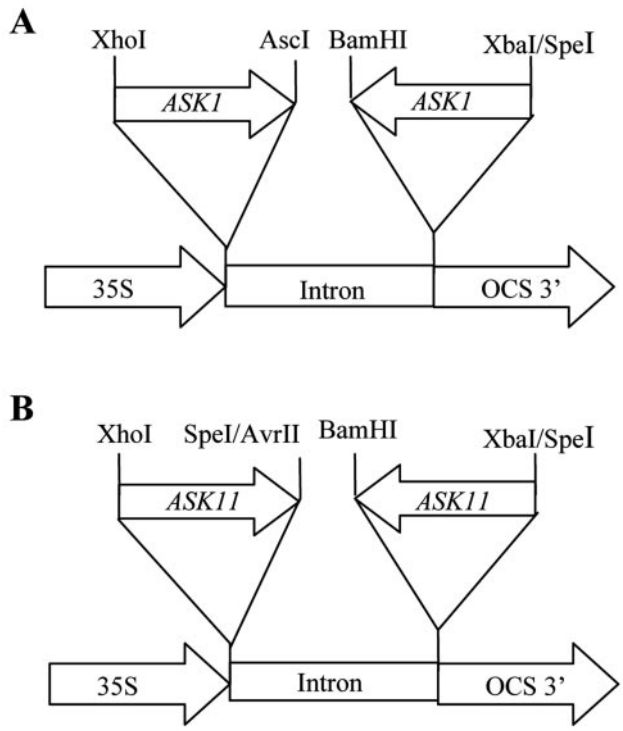

Figure 9. *ASK1* and *ASK11* RNAi constructs.

Table III. *Primers for cloning in situ hybridization probes*

Gene Name	OMC No.	Primer Sequence 5′ to 3′	Clone pMC No.
ASK1	438	AAAAGCAGCAAGCAACCAGT	2391
	439	TTAGCATTCTGTGGCGATTG	
ASK2	440	GTCATGTCCTCGACCCATTT	2392
	441	CAATCTCTCGTGTGTGCTTGT	
ASK3	659	TTGGAAAAGAATTTAAGAACATTTGA	2581
	660	CTCGGGCAAACATGTTATTGTA	
ASK4	442	GATCGGATCTGAAGGGTGTT	2406
	443	TCCATTCGTTGTAACCAGCA	
ASK5	456	TCATCAAAGTTGTTGATGCAAA	2398
	457	TCTGTTTCGGATTAGAAAACTCAA	
ASK9	452	GCGGATTATCAAAACCTAAACA	2580
	453	AACAAGCCATAACCTGTCAGAAA	
ASK11	444	GCCCATGAATCAAAACCCTA	2393
	445	CTCCCAAGAGGTGATATGCAG	
ASK14	448	TTGTCGGTTTTAGGGTTTTGA	2395
	449	GGCCAATCATTTAGTTCCCA	
ASK15	458	TGTGTTTCGCTGTGTTGTTTC	2399
	459	CGAAAATTTTGTTTGCTCTTCA	
ASK18	450	ATTTCGTTTCGAATTGCACA	2396
	451	CATACACGCGTCGATCATTC	
ASK19	446	TCCTTTTACATGTATGATTTGTTTCTG	2394
	447	TTTTTAACGGCGGCGACT	

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