The Plant Journal (2003) **34**, 753–767

Protein interaction analysis of SCF ubiquitin E3 ligase subunits from *Arabidopsis*

Eddy P. Risseeuw, Timothy E. Daskalchuk, Travis W. Banks[†], Enwu Liu, Julian Cotelesage[‡], Hanjo Hellmann[§], Mark Estelle[¶], David E. Somers[**] and William L. Crosby[*]

Gene Expression Group, NRC Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, SK, Canada S7N-0W9

Received 20 September 2002; revised 28 January 2003; accepted 5 February 2003.

[*]For correspondence (fax +1 306 966 2033; e-mail bcrosby@cs.usask.ca).

[†]Present address: Agriculture and Agri-Food Canada, Cereal Research Centre, 195 Dafoe Road, Winnipeg, MB, Canada R3T-2M9.

[‡]Present address: Department of Biochemistry, University of Saskatchewan, Health Sciences Centre, 107 Wiggins Road, Saskatoon, SK, Canada S7N-5E5.

[§]Present address: Institut für Angewandte Genetik, Molekulare Entwicklungsbiologie der Pflanzen, Albrecht-Thaer Weg 7, 14195 Berlin, Germany.

[¶]Present address: Department of Biology, Indiana University, Myers Hall 150, 915 E. 3rd Street, Bloomington, IN 47405, USA.

[**]Present address: Department of Plant Biology, Ohio State University, Columbus, OH 43210, USA.

Summary

Ubiquitin E3 ligases are a diverse family of protein complexes that mediate the ubiquitination and subsequent proteolytic turnover of proteins in a highly specific manner. Among the several classes of ubiquitin E3 ligases, the Skp1-Cullin-F-box (SCF) class is generally comprised of three 'core' subunits: Skp1 and Cullin, plus at least one F-box protein (FBP) subunit that imparts specificity for the ubiquitination of selected target proteins. Recent genetic and biochemical evidence in *Arabidopsis thaliana* suggests that post-translational turnover of proteins mediated by SCF complexes is important for the regulation of diverse developmental and environmental response pathways. In this report, we extend upon a previous annotation of the *Arabidopsis* Skp1-like (ASK) and FBP gene families to include the Cullin family of proteins. Analysis of the protein interaction profiles involving the products of all three gene families suggests a functional distinction between ASK proteins in that selected members of the protein family interact generally while others interact more specifically with members of the F-box protein family. Analysis of the interaction of Cullins with FBPs indicates that CUL1 and CUL2, but not CUL3A, persist as components of selected SCF complexes, suggesting some degree of functional specialization for these proteins. Yeast two-hybrid analyses also revealed binary protein interactions between selected members of the FBP family in *Arabidopsis*. These and related results are discussed in terms of their implications for subunit composition, stoichiometry and functional diversity of SCF complexes in *Arabidopsis*.

Keywords: E3 ligase, *Arabidopsis*, F-box gene family, yeast two-hybrid, *ASK* gene family.

Introduction

The steady-state expression and functional activity of cellular proteins are in part regulated via the complementary action of expression and synthesis versus post-translational degradation. Recently, targeted degradation of cellular proteins has emerged as an important mode of regulation for many cellular processes. In eukaryotes, targeted protein turnover is largely mediated by the conserved ubiquitin (Ub) pathway, which catalyzes the covalent attachment of a multi-Ub chain to specific proteins. The Ub chain serves as a degradation tag, leading to substrate proteolysis via the 26S-proteasome (reviewed by Pickart, 2001; Weissman, 2001). The Ub pathway involves a three-step reaction where the E1-activated Ub moiety is transferred to the substrate protein by the E2 conjugating and E3 ligating enzymes. Individual E3 complexes exhibit differential affinity for selected substrate proteins, thereby conferring specificity to the Ub pathway.

E3's have been classified on the basis of their subunit composition as the HECT, RING/U-box, SCF, and anaphase promoting complex (APC) complexes. The HECT and RING/U-box E3 complexes consist of a single polypeptide, whereas the SCF and APC classes exhibit a multisubunit structure (reviewed by Deshaies, 1999). The SCF complex is composed of Skp1, Cullin, an F-box protein (FBP) plus Rbx1 (also referred to as ROC1 and HRT1). Rbx1 is a small RING protein that may catalyze the synthesis of poly Ub chains and mediate interaction between the E2 and the C-terminal domain of the Cullin protein. The Cullin subunit acts as a large scaffold protein that ensures optimal presentation of the substrate to the E2 (Zheng *et al.*, 2002). The Skp1 protein

© 2003 Blackwell Publishing Ltd

binds tightly to the F-box domain of the FBP and mediates interaction between the FBP and the N-terminal domain of Cullin (Schulman *et al.*, 2000; Zheng *et al.*, 2002). The C-terminal domains of FBPs commonly contain protein-interaction motifs, such as LRR, WD40 and Kelch repeats, which may confer binding specificity to different substrates. SCF substrates include negative regulators of the cell cycle, as well as transcription factors, signal transduction components and developmental regulators (Blondel *et al.*, 2000; Jiang and Struhl, 1998; Skowyra *et al.*, 1997; Tsvetkov *et al.*, 1999).

The budding yeast *Saccharomyces cerevisiae* expresses a single Skp1 protein, two Cullins and 14 FBPs, thereby providing significant combinatorial diversity of SCF complexes. In multicellular organisms, the diversity of potential SCF complexes is considerably expanded by the expression of additional Cullin and Skp1 genes, accompanied by large FBP gene families that can exceed several hundred members. The *Arabidopsis* genome contains at least 10 Cullins, 21 *Skp1*-like (*ASK*) genes (Farrás *et al.*, 2001), plus not less than the 703 FBPs identified in this study combined with those in recent publications (Andrade *et al.*, 2001; Gagne *et al.*, 2002). Mutations in the FBP genes *UFO, TIR1, COI1, ZTL1, FKF1, EID1*, and *ORE9/MAX2* exhibited various phenotypes affecting flower development, auxin signaling, jasmonic acid signaling, circadian clock, light signaling, and senescence, respectively (Dieterle *et al.*, 2001; Ingram *et al.*, 1995; Nelson *et al.*, 2000; Ruegger *et al.*, 1998; Somers *et al.*, 2000; Stirnberg *et al.*, 2002; Woo *et al.*, 2001; Xie *et al.*, 1998). The loss of function *ask1-1* mutation in *Arabidopsis* exhibited a pleiotropic phenotype, suggesting involvement in multiple pathways, likely by the formation of multiple SCF complexes involving different FBPs (Yang *et al.*, 1999; Zhao *et al.*, 1999).

As a basis for this study, we assembled a comprehensive annotation of the known and predicted SCF component gene families in *Arabidopsis*. Based on previous suggestions that Skp1 proteins may interact with a subset of specific FBPs (Schulman *et al.*, 2000), we have exploited this gene family information to explore the potential for selective interaction between all three SCF subunit components, beginning with an analysis of multiple interactions between the *Arabidopsis* ASK and FBP protein families using a yeast two-hybrid approach. We have extended these analyses to investigate the interaction of tripeptide SCF complexes, where the FBP, Cullin, and ASK subunits were expressed in a yeast three-hybrid system.

Results

The ASK *gene family is relatively homogeneous in* Arabidopsis

Analysis of the completed *Arabidopsis* genome sequence has revealed 21 *Skp1*-like *ASK* genes (Farrás *et al.*, 2001; Table 1). A phylogenetic tree of the ASK protein sequences is presented in Figure 1, including the SKP1-like proteins from *Caenorhabditis elegans*, *Drosophila melanogaster*, *S. cerevisiae*, *S. pombe*, and the human Skp1. With the exception of ASK20 and ASK21, all ASK protein sequences clustered to a single clade, suggesting a common ancestor. The short branches within the ASK clade indicate that the corresponding *ASK* genes in *Arabidopsis* have diverged to a lesser extent than the *skr* genes in *C. elegans* and the *Skp1* genes in *Drosophila*.

The binding interface of the human Skp1 with the Skp2 F-box protein resembles a four-layer 'sandwich' structure formed by the H5, H6, and H7 helix domains of Skp1, in combination with the H1, H2 and H3 domains of the Skp2 F-box Skp2; the H8 domain of Skp1; and the first LRR motif within the C-terminal domain of Skp2 (Figure 2a; Schulman *et al.*, 2000). The domains composing the first two layers are strongly conserved among all Skp1 and FBP family members, in contrast to the less conserved variable interface of the second two layers. It has been suggested that the variable H8 helix of the *C. elegans* Skp1 family might confer specificity to the Skp1 F-box interaction. However, with the exception of ASK20 and ASK21, the *Arabidopsis* ASK proteins exhibited significant similarity within the H8 domain (Figure 2a), and are most closely related to the human Skp1 group of orthologs as indicated in Figure 1. Taken together, the phylogenetic relatedness would argue for a limited specificity among the ASK–FBP interactions.

Evidence for gene transcription was obtained for seven of the *ASK* genes (*1*, *2*, *3*, *4*, *11*, *18*, *20*, and *21*) from yeast two-hybrid screens, cDNA cloning, and EST database searches. We suggest that *ASK6* and *ASK15* are likely pseudogenes because the genomic sequences contain frame shifts that prematurely terminate their deduced coding regions. Several of the *ASK* genes were grouped in a tandem chromosomal arrangement, including *ASK5* with *ASK13* found on chromosome 4, *ASK7–10* on chromosome 3, and *ASK16* with *ASK19* localized to chromosome 2. The *ASK12* gene, including 149 base pairs of the predicted 5′ non-coding region, was nearly identical to *ASK11*, suggesting that both genes have arisen from a recent duplication of a common ancestral gene. A single intron was found at the identical position in *ASK1–4*. In the *ASK18* gene, an internal duplication of the first 25 codons is evident, resulting in a repeated structure of the S1, S2, and H1 domains. The ASK7 protein exhibited a 27-amino-acid deletion of the H7 and H8 domains, which are implicated for binding to the F-box motif (Figure 2a).

The *ASK20* and *ASK21* genes were distinguished by a C-terminal extension at the SKP1-like domain that exhibited little similarity with any other protein (Figure 2b). Cloning of the *ASK20* cDNA revealed an alternative splice site in the sixth intron; among 19 clones recovered, 15 exhibited a sixth intron that had been spliced 35 bp upstream from the

Table 1 Characteristics of members of the *Arabidopsis* Skp1 and Cul1-like gene families

Arabidopsis Skp1 and *CUL*-like gene	Gene ID locus	No. of amino acids (AA length)	No. of exons	EST's transcripts identified
ASK1	At1g75950	160	2	U97020
ASK2	At5g42190	171	2	U97021
ASK3	At2g25700	163	2	AY052700
ASK4	At1g20140	163	2	AY058205
ASK5[d]	At3g60020	153	1	
ASK6	At3g53060	149[FS]	1	
ASK7[b]	At3g21840	125	1	
ASK8[b]	At3g21830	152	1	
ASK9[b]	At3g21850	153	1	
ASK10[b]	At3g21860	152	1	
ASK11	At4g34210	152	1	AV556614
ASK12	At4g34470	152	1	
ASK13[d]	At3g60010	154	1	
ASK14[a]	At2g03170	149	1	
ASK15	At3g25650	177[FS]	1	
ASK16[a]	At2g03190	170	1	
ASK17	At2g20160	150	1	
ASK18	At1g10230	183	1	T42880
ASK19[a]	At2g03160	200	1	
ASK20A	At2g45950	227	10	AF370493
ASK20B	At2g45950	342	10	This study
ASK21	At3g61410/20	175/349	5/10	AI994751, this study
ElonginC	At5g59140	112	3	AV530805
CUL1	At4g02570	738	19	Gray *et al.* (1999)
CUL2	At1g02980	742	14	This study
CUL3A	At1g26830	732	2	AV562776
CUL3B	At1g69670	732	2	BE528119
CUL4	At5g46210	742	17	AV544062
	At1g43140	732[FS]	11	
	At1g59790[c]	374[N]	9	
	At1g59800[c]	255[N]	6	
	At4g12100	434[N]	5	
	At3g46910	372[NFS]	5	

[a,b,c,d]Symbols represent gene clusters.
[N]N-terminal domain.
[FS]Frame shift.

acceptor site resulting in a truncated protein of 227 amino acids (*ASK20A*). The remaining four *ASK20B* clones had been spliced in a manner consistent with EST AF370493 containing 10 exons and a 342-amino-acid open-reading frame. The homologous *ASK21* gene was localized to chromosome 3 between At3g61410 and At3g61420 with a predicted exon–intron structure identical to *ASK20.* However, upon cloning of the *ASK21* cDNA, four independent clones revealed premature polyadenylation in an adenine-rich region found at codon 175.

Arabidopsis *F-box proteins are encoded by a super family*

The F-box sequences identified in this study, supplemented by those arising from earlier genetic studies (Figure 3a) were used in a BLAST search to identify other potential FBP genes in the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000). This survey revealed a large number (703) of genes that were predicted to encode the F-box consensus sequence presented in Figure 3(a). The predicted complexity of the FBP gene family was similar to that previously described (Gagne *et al.*, 2002). Gene expression was verified for 39% (278/703) of these genes by the presence of a 'Tentative Consensus' sequence or EST in the TIGR AtGI database. Defining the F-box domain as the $+6/-60$ region with respect to the position of the first conserved proline, in 62% of the FBPs the N-terminal region to the F-box motif was less than 20 amino acids long, demonstrating that the F-box was predominantly localized to the N-terminus.

CLUSTALW analysis of the C-terminal domains identified 13 classes including protein interacting domains, such as leucine-rich repeats (LRR), Kelch repeats, or TUBBY domains (Figure 3b). The various classes are indicated in the rectangular cladogram of Figure 3(b), which was derived from the complete phylogram that can be downloaded from

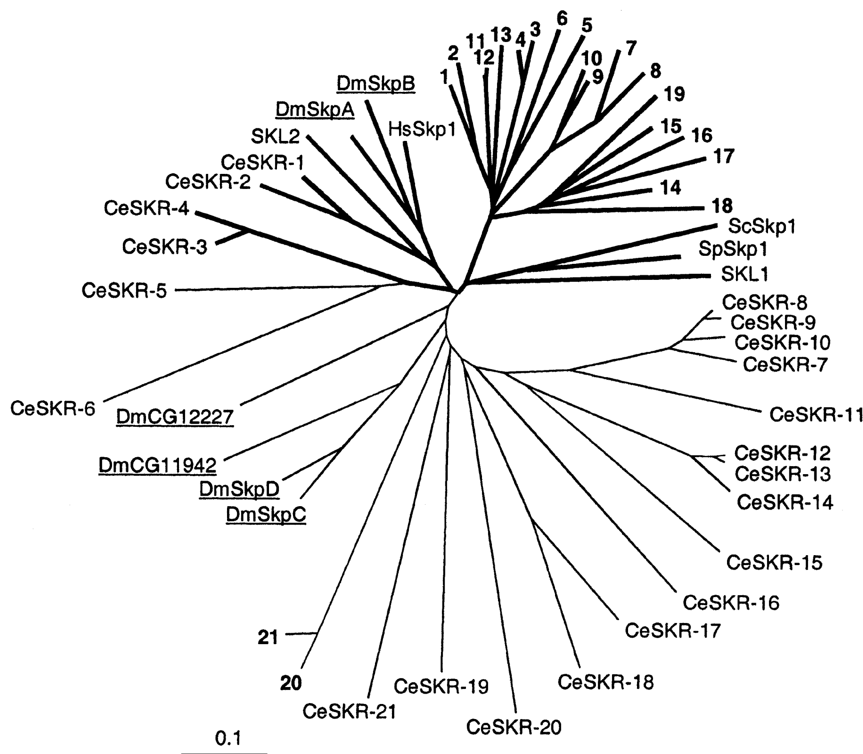

Figure 1. Phylogenetic analysis of 21 *Arabidopsis* ASK protein sequences (1–21 in bold), relative to select Skp1 proteins from other eukaryotes. Sequences that share the conserved H8 helix domains from human HsSkp1 are connected with bold lines. Hs, human; Dm, *Drosophila*, Sc, *Saccharomyces cerevisiae*, Sp, *Schizosaccharomyles pombe*; Ce, *Caenorhabditis elegans*; SKL, Skp1 proteins from unknown organisms included in Figure 5.

http://bioinfo.pbi.nrc.ca/fbox_data. An annotated list of the *Arabidopsis* FBPs can be viewed and searched using a second companion data file at the same site.

Three C-terminal classes FBL1, FBL2, and FBL3 were defined by LRR repeats represented by 169, 50, and 5 genes, respectively. Most genes in the FBL2 class, including *TIR1*, *COI1*, and *ORE9*, featured one or more predicted LRR-CC smart00367 domains. FBL1, which corresponds to the LRR-PD designated group (Gagne *et al.*, 2002), and FBL3 presented plant-specific variants of the LRR domain. Domain searches revealed Kelch repeats in all FBK8 genes, which included *ZTL*, *FKF1* and *LKP2*, and the previously described F-box-/Kelch-containing family in *Arabidopsis* (Andrade *et al.*, 2001). The other eight FBK classes contained conserved tyrosine and tryptophan residues, which are characteristic of the β3 and β4 sheets in both Kelch and WD40 repeats that comprise the blades of the tertiary propeller structure (Adams *et al.*, 2000; Smith *et al.*, 1999). FBK1, FBK2 + 3, FBK5, FBK7, and FBK9 correspond with A-I, A-II, C-II, C-I, and lectin containing FBPs, respectively, designated by Gagne *et al.* (2002). The FBK4 class comprising six members was represented by unusual floral organs (*UFO*). FBK1–3 and 8 were the largest classes represented by 117, 39, 82, and 99 genes, respectively, whereas the other classes contained fewer than 27 genes each. Finally, 10 genes were predicted to encode the conserved TUBBY domain, which grouped to a distinct class.

Diverse FBPs interact with ASK1 and ASK2

In an effort to identify additional SCF subunits, an *Arabidopsis* yeast two-hybrid library was screened using the ASK1 and ASK2 coding regions as bait. About 1×10^6 yeast transformants were generated, and 153 clones were identified on selective medium at 20°C. Of these, 100 clones were sequenced resulting in the identification of 36 unique cDNA sequences, which were named SKP1/ASK-interacting proteins (SKIP) in accordance with the previously reported SKIP proteins (Farrás *et al.*, 2001; Table 2). Interestingly, an F-box domain was identified in 28 of the SKIPs (Figure 3a).

Figure 2. Amino acid sequence comparison of the *Arabidopsis* ASK proteins with select Skp1 proteins from other eukaryotes.
(a) Alignment of the ASK-Skp1 C-terminal domains. Residues that are 80–100% similar are shaded in black; 60–80% similarity is indicated in dark gray, and 30–60% similarity is denoted in light gray. HsSkp1 residues that contact HsSkp2 are indicated at the bottom. Bars represent helices in the HsSkp1 sequence (Schulman *et al.*, 2000).
(b) Alignment of the predicted amino acid sequences of ASK1, ASK20, and ASK21. Intron boundaries are marked with (|), and exons are indicated with roman numerals. *ASK20A* and *ASK20B* were two alternative splice variants of the sixth intron. The 3′ cleavage site in the *ASK21* cDNAs (5′-UAUGCA↓AA) is indicated with #. *ASK20* is located on BAC F4I8 and corresponds to the complementary strand nucleotides 81884–82098, 82191–82305, 82457–82628, 82703–82829 A/82794B, 83211–83280, 83526–83416, 83794–83603, 84100–83876, 84681–84406, and 85129–85272. The *ASK21* cDNA is located on BAC F2A19 and corresponds to complementary strand nucleotides 17103–17125, 17303–17419, 17610–17790, 17870–17964, 18407–18476, 18769–18960, 19050–19274, 19683–19850, and 20430–26004.

(a)

```
ASK1      92  IDQA-TLFELILAANYLNIKNLLDLTCQTV-----ADMIKGKTPEEIRTTFNIKNDFTP-EEEEEVRREENQWAFE
ASK2     103  VDQG-TLFDLILAANYLNIKGLLDLTCQTV-----ADMIKGKTPEEIRKTFNIKNDFTP-EEEEEVRREENQWAFE
ASK3      95  VDHP-TLFDLIRAANYLNISGLLDLTCKAV-----ADQMRGKTIAQMREHFNIKNDYTP-EEEAEVRNENRQWAFE
ASK4      95  VDQP-TLFDLILAANYLNIGGLLDLTCKAV-----ADQMRGKTPEQMRAHFNIKNDYTP-EEEAEVRNENKQWAFE
ASK5      80  IETT-ILFDVMMAANYLNIQSLLDLTCKTVSDLLQADLLSGKTPDEIRAHFNIENDLTA-EEVAKIREENQWAFQ
ASK6      67  MEQS-ILFDVMMAANYLNIQSLLDLTFQTV-----ADLLSGKTPGEIRSYFNIENDFTA-EGPAEIRKVNQWAFE
ASK7      84  KDQY-TIFHLMNAAYDLHIKSLLALAYQTV-----ADMVN---------------------------FNKWAFE
ASK8      84  KDKS-TIFALTNAANFLNNKSLLHLAGQTV-----ADMIKGNTPKQMREFFNIENDLTP-EEEAAIRREENKWAFE
ASK9      85  KDTS-TIFDLIKAANYLNIKSLFDLACQTV-----AEIIKGNTPEQIREFFNIENDLTP-EEEAAIRREENKWAFE
ASK10     84  KYQS-TIFDLIMAANYLNIKSLLDLACQTV-----ADMIKDNTVEHTRKFFNIENDYTH-EEEEAVRREENQWGFE
ASK11     84  LEQS-TIFELILAANYLNIKSLLDLTCQTV-----ADMIKGKTPEEIRSTFNIENDFTP-EEEEAVRKENQWAFE
ASK12     84  LEQS-TIFELILAANYLNIKSLFDLTCQTV-----ADMIKGKTPEEIRSTFNIENDFTP-EEEEAVRKENQWAFE
ASK13     85  LEQSSTLFDVMLAANYLNIKDLLDLGCQTV-----ADMITGKKPDEIRALLGIENDFTP-EEEEEIRKENQWAFE
ASK14     81  FDQP-TVFQLLLAANYLNIKGLLDLSAQTV-----ADRIKDKTPEEIREIFNIENDFTP-EEEAAVRKENAWAFE
ASK15     98  IDME-TIFKLILAANYLNVEGLLGLTCQTV-----ADYIKDKTPEEVRELFNIENDFTHEEEEEAIRKENAWAFEADTKHEDPKP
ASK16     99  FDME-TVMKLILAVNYLNVQDLLGLTCQTV-----ADHMKDMSPEEVRELFNIENDYTP-EEEEAIRKENAWAFEDLK
ASK17     82  IDMD-TLFKLLDAADYLIVIGLKNLIAQAI-----ADYTADKTVNEIRELFNIENDYTP-EEEEELRKKNEWAFN
ASK18    114  LDLE-TIFKIILAANYLNFEGLLGFASQTV-----ADYIKDKTPEEVREIFNIENDFTP-EEEEEIRKENAWTFNE
ASK19    122  FDIK-TIFDIILAANYLNVQGLFDLCSKTI-----ADYIKDMTPEEVRELFNIENDFTP-EEEEAIRNENAWTFEQDGKQQVPKP
Sc Skp1  125  VDQE-MLYEIILAANYLNIKPLLDAGCKVV-----AEMIRGRSPEEIRRTFNIVNDFTP-EEEAAIRREENEWAEDR
Sp Skp1   93  VDQE-MLFEIVLASNYLDIKPLLDTGCKTV-----ANMIRGKSPEDIRKTFNIPNDFTP-EEEEQIRKENEWAEDR
SKL1     109  IEQD-ILFELILAANYMDIKSLLDLACAKV-----ASMIKGKTPEQIRETFNIVNDFTP-EEESQIREENKWCEEA
Hs Skp1   94  VDQG-TLFELILAANYLDIKGLLDVTCKTV-----ANMIKGKTPEEIRKTFNIKNDFTE-EEEAQVRKENQWCEEK
SkpA      93  VDQG-TLFELILAANYLDIKGLLEITCKTV-----ANMIKGKTPEEIRKTFNIKKDFSP-AEEEQVRKENEWCEEK
SkpB      92  VDQG-TLFELILAANYLDIQGLLDVTCKTV-----ANMIKGKSPQAIRDTFAIQNDFLP-QEEEQVRKENEWCEDK
SKL2      98  VDQG-TLFELILAATTSTLRVFLDVTCKTV-----ANMIKGKTPEEIRRTFNIKNDFTP-EEEEQIRREENAWCED
SKR-1    108  VDQG-TLFELILAANYLDIKGLLDVTCKTV-----ANMIKGKSPEEIRRTFNIKNDFTP-EEEEQIRKENAWCED
SKR-2    106  IDQG-TLFELILAANYLDIKGLLDVACQSV-----ANMIKGKSPDEIRRAFNIKDDFTA-EDREQIRKENAWCDD
SKR-3     99  VDQG-TLFEILLAANYLDIRGLLDVTTQNV-----ANMMKGKTPSQIRTLFNIENDFSE-EEREAMKKENAWCED
SKR-4     92  VDQG-TLFEIILAANYLDIRGLLEVTTQNV-----ANMMKGKTPSQVRTLFKIDN-FSE-EELEAMKKGNAWCED
SKR-5     91  VDKG-TLFELVLAATYLDIKGLFNVTCKSI-----ANSIKGKSPEEIRAVFNIGNETNME
SKR-6    137  LDQN-TLFDLVLAANYLNIENLFDVTTQFI-----ANMMKNNTPSQIRARFGVSNKHSS----AEDRSENWS
CG12227   93  MDQG-TLFELILAANYLDIPNLLNAAQMTV-----ANMIKGRTTEEIRQTFHITNDFSP-SEEDLVTMESEVPQEDEAIEYGEII
SkpC      96  VDQP-TLFEIILAANYLEIKGLVDLCCKTV-----ANMIRGKTPEEIRHTFNIPDEIP-SRTAQLGEDL
SkpD      89  VDQP-ILFEITVAANYLEIKGLEDLCCKTL-----ANMIRGKTPEEIRQTFNIEDELP-IDTAETGENL
CG11942   96  VNST-TLLEIILAAKQLQIKGLLELTYNVV-----ANMIRGKTPEEIRFIFNIPEDVSP-SVDGELRWKDLELWPMDF
ASK20     98  MDTK-RLCELTSAADSLQLKPLVDLTSRAL-----ARIIEGKNPEEIREIFHLPDDLTEEEEKLEPLKNSMDDPR
ASK21     98  MDTK-RLCELTSAADSLQLKPLVDLTSRAL-----ARIIEGKTPEEIREIFHLPDDLTEEEEKLEPLKNTMDDPR
SKR-7    111  IDND-VLFDLILVASNFLDVPGLMSYACKMV-----ANVAIGKSPDEMRVLFAIPTDEED-EAAEKAAEKKAEAEKKAITEKDAAEPSTSK
SKR-8    111  IDNE-VLFDLIVACNYLDVPGLMNYGCKMV-----ANVAIGKSPDELRIIFAIPTDEED-EAAERAAEKKAEAEKKAIADKDAAEPSTSK
SKR-9    111  IDND-VLFDLIVACNYLDVPGLMNYGCKIV-----AMVAIGKSPDELRIIFAIPTDEED-EAAERAAEKKAEAEKKAITDKDAAEPSTSK
SKR-10   109  IDNE-VLFDLIVACNYLDVPGLMNYGCKMV-----AMVAIGKSPDELRIIFAIPTDEED-EAAERAAEKKAEAEKKANAEKDAAEPSTSK
SKR-11   114  ISSD-VLFDLIVACNELDVPGLMNYGCKMV-----ADVAAGKSPEDMRTIYGIPTDKQDADAAEKAAKE-EAEKNATAQRDEAGPSAS
SKR-12   105  IEDE-ALFDLILASNFLDIKGLMYFGCKTV-----SNVAKGKTTAELREIFGINTDEQD-AAEETAQKAAAEVA
SKR-13   105  IEDE-ALFDLILASNFLDIKGLMYYGCKTV-----SNVAKGKTTAELREIFGINTDEQD-AAEETAQKAAAEVA
SKR-14   105  IEDE-ALFDLILASNFLDIKGLMYYGCKTV-----SNVAKGKTTAELREIFGINTDEQD-AAEG----GAVEVA
SKR-15   109  IEHD-ALTDLVNASNFLEVMTLLTYCCKFI-----AGIAKGMSPEEMRVFFCIPTDEED-EKAERFGKEKTEASATSADTTA
SKR-16   115  LDNQ-ELVDLINASNDLQMQQLMEYACKTV-----ANVAKGKNEAQLRELFGILTDEEE-----------------AELALNEPGPSTA
SKR-17   117  LPMS-NLFDLTAAYHLDVTGLINYGCKTV-----ANSAKGKNAEEMRELFGIPEPWEQ--------------------------PSTSTATWDD
SKR-18   120  LPMG-NLLAVIKAAYDLDITGLVNYGTQTV-----ASRINGKSAEEMREIFQLPEPCQQ-------------------------PSTSTDTWAD
SKR-19    98  VESG-VLFALLNASHALGVEDLMNVGCAAA-----AELIRGKSTEEIRKIYGIR------SDEEQVEEALANGGEGTSAMTFTTDSD
SKR-20    97  VRHN-MLFDIIRAARDFTVPGLFAVCCRVV-GQNPREIIDGLVNDE---------EEQPVYPVKKCKNHTNEVNNATLFFKFESKILFSIPYKKT
SKR-21    89  TNSA-ILLHLAEAAFRLEIVGLLDVACRAV-----SIML-GRTLNEVKLMLRVGGVESPSDEEDSLEDVLELEVDVDENEEDDVEIEIPAISAA
Hs Skp1-Skp2  Q    F  IL  N        L     CK V      A  IKGK  P  IR  F I    F    E    V   EN WC
```

```
                     H5                  H6                 H7            H8
```

(b)

```
                                                                                  ASKAEAVEGAA
ASK1      1                 MSAKKIVLKSSDGESFEVEEAVALESQTIAHMVEDDCVDNG----VPLPN-VTSKILAKVIEYCKRHVEAATSDDDLK
ASK20B    1  MSEGDLAVMKPETMKSYIWLQTADGSIQQVEQEVAMFCPMICQEVIQKGVGSSKNHAISLPQRVNPAMFSLILDYCRFHQLPGRSNKERK
ASK21     1  MSEGEMAIIKPEMMKSYIWLETADGSIQQVEQEVAMFCPMICQEVIQKGVGSSKNYAISLPQRVNPAMLSLIFDYCRFHQVPGRSNKERK
                  II    |                                   III                                    |

ASK1         AWDADFMKIDQATLFELILAANYLNIKNLLDLTCQTVADMIKGKTPEEIRTTFNIKNDFTPEEEEEVRRENQWAFE*
ASK20B   91  TYDERFIRMDTKRLCELTSAADSLQLKPLVDLTSRALARIIEGKNPEEIREIFHLPDDLTEEEKLEPLKNSMDDPRIRLLNRLYAKKRKE
ASK21    91  VYDEKFIRMDTKRLCELTSAADSLQLKPLVDLTSRALARIIEGKTPEEIREIFHLPDDLTEEEKLEPLKNTMDDPRIRLLNRLYAKKRKE
                                IV                                      |               V      #

ASK20A  181  LKERERLKNVEVEEHVDERSVDDLLSFINGRGLFGSKENSVSQITRL*
ASK20B  181  LKERERLKNVEVEEHVDERSVDDLLSFINGRDHKAVKMSKGKKKKKKKDQKIVSSN-NIHDKESHDLRSKQQCVEEIG---SSMREVPN
ASK21   181  LKEREKLKSVEVEEHVDERSVDDLLSFINGRDPKVVKTSKSKKKNKKRKEQKNGSSNGTCEALDLHNLDSKSQSAEIVDNTASCLGDVSN
                   |           VI            |         VII              |

ASK20B  271  LLSAEDDISTPNAGSEDEDIDDEIDPAMRELLDREVEDFAQRLNSNWVRSLGKERRPVHFSINGNGTTRRHTCQSP*
ASK21   271  LPSMEDDIFTPKTEFEDGYIDDEIDPALKELLDREVEDFARRLNSSWVLSIGQERQPVNFSINGNGTSRRLTCPAAGHK*
                  VIII                    |                  IX                  |  X
```

© Blackwell Publishing Ltd, *The Plant Journal*, (2003), **34**, 753–767

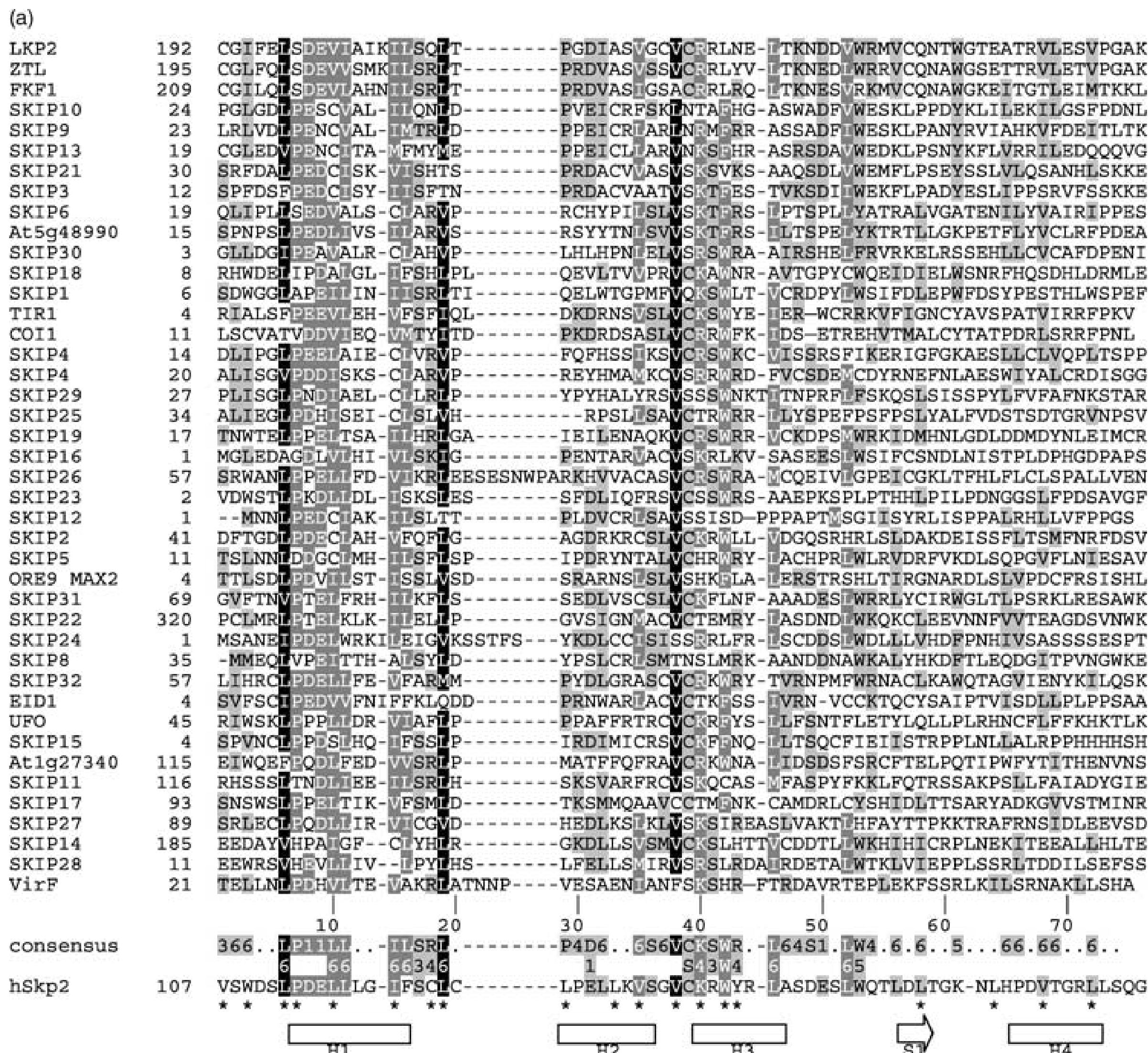

Figure 3. F-box domain comparison and classification of *Arabidopsis* FBPs interacting with ASK, and/or ASK2.
(a) Multiple alignment of F-box sequences from FBPs known to interact with ASK1 and/or ASK2. The F-box consensus sequence was compared with the F-box from the human Skp2. Shading of residues was according to Figure 2(a). Amino acid substitution groups were numbered according to the Blosum 35 matrix: (1) ED; (2) NQ; (3) ST; (4) KR; (5) FYW; and (6) LIVM. Asterisks indicate HsSkp2 residues that contact HsSkp1, and bars represent helix and beta sheet domains (Schulman *et al.*, 2000). The core F-box is composed of helixes H1, H2, and H3, whereas S1 and H4 form the first repeat of the LRR.
(b) Rectangular cladogram representing FBP classes based on clustering of the C-terminal domains (see Experimental procedures).

In these SKIPs, the F-box motif was present in every cDNA clone, suggesting that the F-box domain was required for interaction with ASK1 and ASK2. The remaining eight cDNA classes either exhibited similarity with dual-specificity phosphatases (Yuvaniyama *et al.*, 1996), were unknown genes, or were surmised as false positives.

The selected FBPs exhibited a variable C-terminal domain and represented 6 of the 13 defined classes among all three clusters. Another eight more unique genes were not classified (Table 2). SKIP15 encoded a polypeptide similar to UFO, while SKIP26 contained a TUBBY domain. Three proteins, SKIP1, SKIP6 and EID1, had been previously identified as FBPs from other studies (Dieterle *et al.*, 2001; Farrás *et al.*, 2001). Taken together, the results indicated that a structurally unrelated group of FBPs is competent to interact with ASK1 and ASK2.

Consistent with the co-evolution of the F-box and C-terminal domains (Gagne *et al.*, 2002), the F-box domain alignment from the heterogeneous FBPs interacting with ASK1 or ASK2, supplemented by those arising from earlier studies, showed poor conservation (Figure 3a). On an average, less than 9 out of 19 human Skp2 residues contacting Skp1 were similar among this group of F-box domains (Schulman *et al.*, 2000).

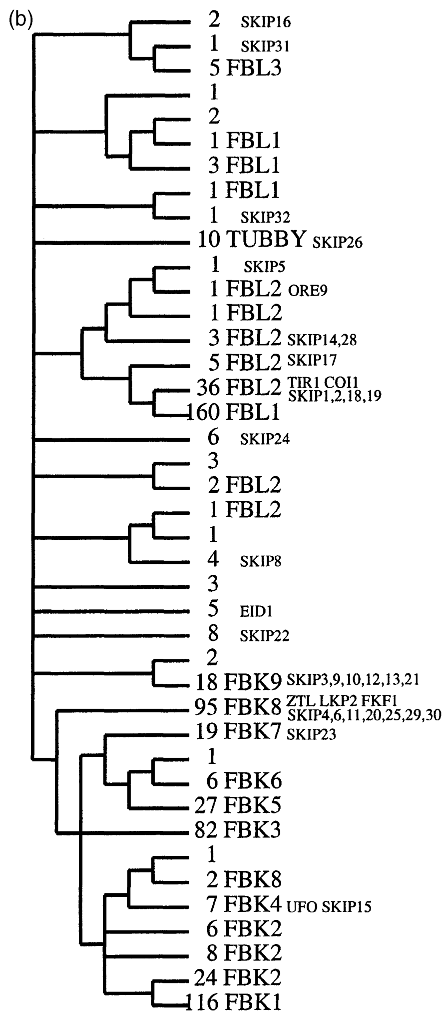

Figure 3. continued

To verify that the selected F-box domains did not belong to a selected group within the general F-box gene family, the F-box sequences were compared with each other using the Smith–Waterman algorithm. For every pairwise comparison, the comparison scores normalized for the sequence length (log length score), were arranged in a square matrix with scores from identical sequences in the median. Cumulative distributions of the log length scores in the matrices are presented in Figure 4. Scores from pairwise comparisons involving all non-identical F-box domains exhibited a 50th percentile score of 55, whereas the score for comparison between identical sequences was 420, confirming that the F-box sequences were, overall, very heterogeneous. Likewise, selected F-box domains capable of interacting with ASK1 and ASK2 exhibited a 50th percentile score of 44, indicating that the ASK-binding subset was comparably diverse as the complete gene family. Thus, based on the absence of a bias for F-box sequence similarity, the data suggest that ASK1 and ASK2 interact relatively non-specifically with most *Arabidopsis* FBPs.

FBPs can interact with other ASK proteins

The ASK protein sequences exhibited extensive similarity throughout the predicted coding region, including the H8 helix domain (Farrás *et al.*, 2001; Figure 2a); however, some degree of specificity was reported between the *Arabidopsis* ASK and FBP families (Gagne *et al.*, 2002). To further determine the complexity of the ASK–FBP interactions, a matrix of yeast two-hybrid combinations was assembled involving a different subset of FBPs in paired combination with the majority of the ASK protein family. All FBPs in the selected set, including five FBPs that did not arise from the previous library screens, showed interaction with ASK1 and ASK2, as well as with the closely related ASK11 (Figure 5). Taking into account that negative interactions can arise because of the limitations of the yeast two-hybrid system, the interaction with the other ASK proteins exhibited a more selective pattern. With some exceptions as discussed later, the overall interaction profiles of the ASK proteins, including ASK1, ASK2, and ASK11, were similar to those reported by Gagne *et al.* (2002).

Most FBPs tested also interacted with the non-plant yeast Skp1 protein and with two Skp1 orthologs, here referred to as SKL1 and SKL2 (re-named from ASK10; Schrammeijer *et al.*, 2001). The *SKL* cDNAs originated from contaminating transcripts of unknown (likely fungal or insect) origin, which were recovered from yeast two-hybrid screens as interacting clones with the FBPs SKIP17 and VirF. Interestingly, like ASK1, ASK2, and ASK11, the strongly interacting SKL1 and SKL2 proteins contained most of the human Skp1 residues contacting Skp2 (Figure 2a). Thus, even though the F-box-binding interface is conserved among most ASK proteins, a correlation is apparent between the conservation of these residues in Skp1 orthologs and their ability to bind a wide variety of F-box proteins.

Annotation of the Arabidopsis *Cullin family*

Analysis of the *Arabidopsis* genome by us and others revealed the presence of at least 10 predicted Cullin genes (Shen *et al.*, 2002; Table 1). Six of these exhibited reading frames that contained apparently intact canonical C- and N-terminal domains, whereas four Cullins consisted only of the N-terminal domain. Evidence for expression of these genes was supported by the presence of EST sequences in the AtGI database representing five of the complete Cullins defined by CUL1, CUL2, CUL3A, CUL3B, and CUL4. A sixth Cullin, encoded by the predicted gene At1g43140, exhibited an apparent coding region frame shift when compared to the most related CUL2, and may therefore not be functional.

Table 2 Yeast two-hybrid interacting clones selected with ASK1, ASK2 and CUL1 bait constructs

Yeast two-hybrid interactors	Gene ID locus	No. of times interactor identified with bait:			No. of amino acids (AA length)	No. of codons truncated at N-terminus	Proline position	C-terminal domain
		ASK1	ASK2	CUL1				
EID1	At4g02440	2	2		336	0–5	10	Leucine zipper
SKIP1	At5g57900	6	6		300	0–8	12	FBL2
SKIP6	At2g21950	1			372	14	25	FBK8
SKIP8	At4g10930	1			225	0	41	
SKIP9	At3g61060	7	3		290	0–26	29	FBK9
SKIP10	At1g63090	1			289	17	30	FBK9
SKIP11	At2g02870	1	1		467	92	122	FBK8
SKIP12	At1g80110	1	2		264	0	5	FBK9
SKIP13	At5g52120	2	1		291	4–12	25	FBK9
SKIP14	At3g26000	2	3	2	435	134–178	191	FBL2
SKIP15	At1g76920		1		374	0	10	FBK4
SKIP16	At1g06110		1		436	0	7	Human Fbx3
SKIP17	At4g02740		3		479	0–20	99	FBL2
SKIP18	At4g08980		1	2	317	3–5	16	FBL2
SKIP19	At4g05460		1	1	302	13–14	23	FBL2
SKIP20	At3g59940	2	2		418	13–14	20	FBK8
SKIP21	At2g02230	1	1		317	23	36	FBK9
SKIP22	At1g23780	1	3		475	3–310	326	Human Fbx7
SKIP23	At2g17030	1			433	0	8	FBK7
SKIP24	At1g08710	2	1		274	0	7	Leucine rich
SKIP25	At1g31350	1			395	2	40	FBK8
SKIP26	At1g25280	1			445	55	63	TUBBY
SKIP27	At4g21510	5	1		192	80	95	
SKIP28	At2g01620	1		2	290	0	17	FBL2
SKIP29	At2g24540	1			373	4	33	FBK8
SKIP30	At3g63220	1			345	3	9	FBK8
SKIP31	At5g45360	6	5		316	0–9	75	
SKIP32	At1g21760	1			328	60	63	
SKIP33	At2g04550	2			283	0–5	No	Putative phosphatase
SKIP34	AI998821		7		94	0–25	No	Similar to tomato 240K04.02
SKIP35	At3g59910	1			611	278	No	Unknown protein

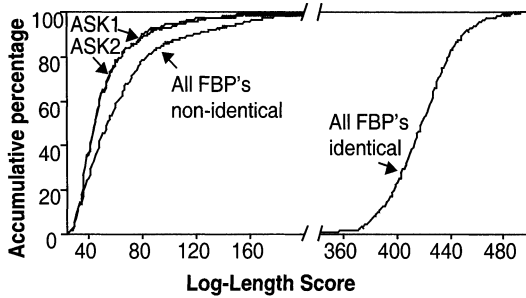

Figure 4. Pairwise comparison of F-box sequences from all FBPs versus FBPs that interact with ASK1 and ASK2.
The plots represent the cumulative distribution of the Smith–Waterman sequence similarity scores normalized for the length of the sequences, for each of the different sequence sets (see Experimental procedures).

The complete Cullins from *Arabidopsis* and from other eukaryotes were subjected to phylogenetic analysis and were found to cluster in five clades (Figure 6). Cullins from *S. pombe*, *C. elegans*, and humans that grouped in the first clade were previously shown to interact with SKP1 as well as participate in the SCF class of E3 ligases (Furukawa *et al.*, 2002; Kominami *et al.*, 1998; Nayak *et al.*, 2002; Yamanaka *et al.*, 2002). The *Arabidopsis* CUL3A and CUL3B were closely related and clustered in Clade-III, whereas CUL4 was located to a fourth clade (Figure 7). CUL1 and CUL2 were also related, but did not cluster in any of the five clades. Immuno-precipitation experiments have shown co-enrichment of CUL1 with the FBP protein TIR1 (Gray *et al.*, 1999), as well as with ASK1 (Risseeuw and Liu, unpublished results), indicating that CUL1 is associated with the SCF class of E3's in *Arabidopsis* and thus can be considered a functional homolog of the Clade-I Cullins.

Quaternary requirements for CUL–FBP interaction

The SCF structural model predicts a direct interaction between ASK and at least one member of the Cullin family (Zheng *et al.*, 2002). As no Cullins have been found by

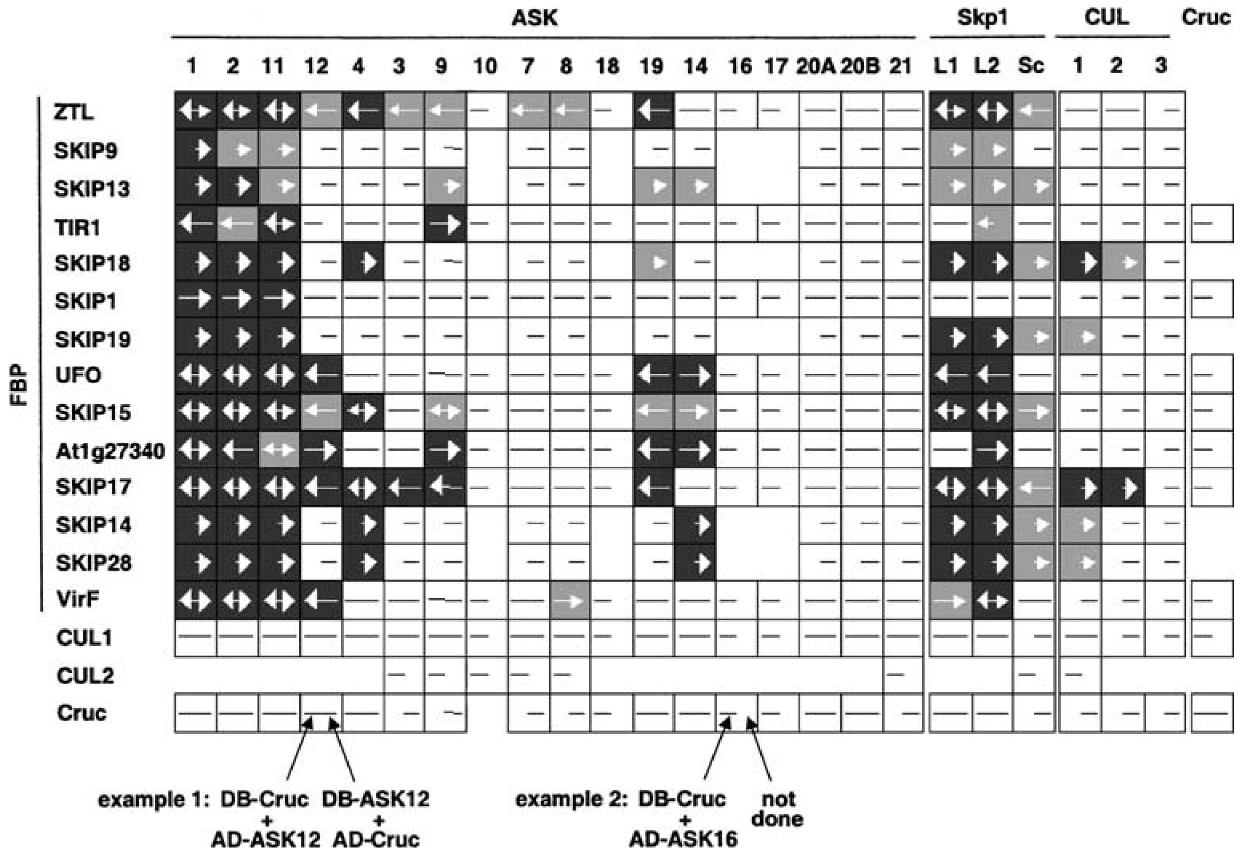

Figure 5. Direction of the arrows indicates the orientation of the interacting proteins fused to the Gal4 DNA binding (BD) and activation domains (AD): left, BD-FBP and AD-ASK/SKP1; right, BD-ASK/SKP1 and AD-FBP (see example 1).
Yeast two-hybrid interactions between the *Arabidopsis* SCF subunits FBP, ASK, and CUL. Arrows and lines indicate yeast growth on SD-LTH solid medium supplemented with 5 mM 3-AT after a 7-day incubation at 20°C: line, no growth; small arrowhead, moderate growth; big arrowhead, strong growth. The direction of the arrows indicates the orientation of the interacting proteins fused to the GAL3 DNA binding (BD) and activation domains (AD): left, BD-FBP and AD-ASK/SKP1; right, BD-ASK/SKP1 and AD-FBP (see example 1). One-sided lines and arrows indicate that the interaction was tested in one bait/prey configuration only (see example 2). Sc is *Saccharomyces cerevisiae* Skp1, whereas L1 (SKL1) and L2 (SKL2) are Skp1 homologies from unknown organisms. A full-length cDNA for the *Arabidopsis* seed storage protein cruciferin (Pang *et al.*, 1988) was used as a negative control.

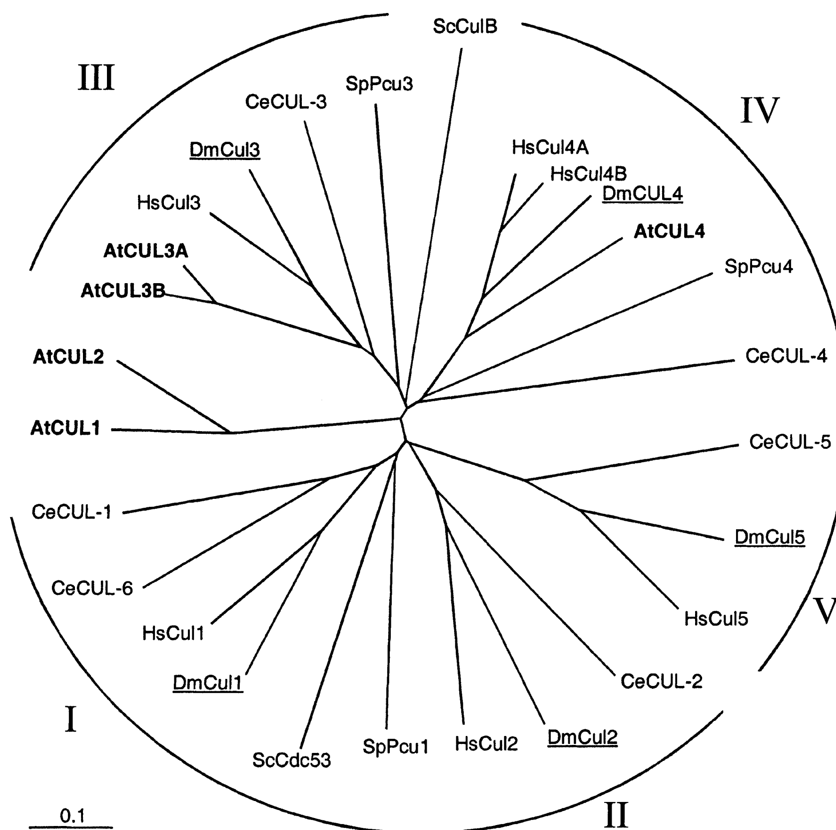

Figure 6. Phylogenetic analysis of *Arabidopsis* Cullins in relation to other eukaryotes.
At, *Arabidopsis*; Hs, human; Dm, *Drosophila*, Sc, *Saccharomyces cerevisiae*, Sp, *Schizosaccharomyles pombe*; Ce, *Caenorhabditis elegans.* Roman numerals represent the five major Cullin clades. Cullins from the anaphase promoting complex (APC) are not included.

screening the two-hybrid library with ASK1 or ASK2 'bait' constructs, reciprocal screens were performed using AtCUL1 as 'bait'. From these experiments, no ASK proteins were selected, although four interacting FBPs (SKIP14, SKIP18, SKIP19, and SKIP28) were identified that were previously identified in the ASK1 and ASK2 screens (Table 1). All seven clones recovered contained the F-box motif, suggesting that the F-box was required for the interaction. Furthermore, directed yeast two-hybrid analysis between CUL1 and other FBPs also identified SKIP1 and SKIP17 as interacting partners (Figure 5). The six FBPs all belonged to the same FBL2 class. As these FBPs showed a positive yeast two-hybrid interaction with the yeast Skp1, we speculate that Skp1 may, in some way, bridge selected CUL1–FBP interactions in the heterologous yeast system (Figure 5). The possibility of an interaction between *Arabidopsis* CUL1 and other yeast SCF components is supported by our finding, and by others, that the yeast temperature-sensitive *cdc53-2* mutant allele is complemented by expression of the *Arabidopsis CUL1* gene (Zheng *et al.*, 2002; T. Banks, unpublished results). As a consequence, the yeast Skp1 is able to compete with the ASK proteins for binding with CUL1. However, given that CUL1 and CUL2 do not detectably interact with the yeast Skp1, we concluded that the likelihood of Skp1 interference in the CUL–ASK interaction was minimal.

To test whether the CUL1–FBP interactions could be bridged by the co-expression of an ASK protein, selected ASK genes were expressed in yeast from a third expression construct, in addition to the bait-CUL1 and prey-FBP plasmids. The three-way interactions were quantified by parallel growth curve analysis under selection in liquid media as an indirect measure of two-hybrid marker gene activation (Diaz-Camino *et al.*, 2002; Figure 7). Yeast growth was enhanced in diploid strains expressing prey-SKIP14, SKIP17, SKIP18 or SKIP19 constructs, in combination with bait-CUL1, when ASK1 or ASK2 was co-expressed in the same cells (Figure 7). Interestingly, yeast growth in these same 'bait–prey' combinations was not observed when ASK7, ASK19 or yeast Skp1 was co-expressed, even though SKIP17 and SKIP18 participate in binary interactions with ASK19 in yeast two-hybrid screens (Figure 5). In contrast, ASK19 had an inhibitory effect on the interactions between CUL1 and the FBPs defined by SKIP1, SKIP19 and SKIP28, which was in accordance with the absence of binary interaction between these FBPs and ASK19, as summarized in Figure 5. Co-expression of ASK7, which lacks the H7 and H8 helixes and is therefore unlikely to interact with an F-box, also inhibited select FBP–CUL1 interactions – possibly by competing for ASK-/Skp1-binding sites on the Cullin (Figure 7).

FBPs that exhibited significant interaction with CUL1 also showed robust interaction with the closely related CUL2. This interaction was enhanced by ASK1 co-expression in the case of SKIP17, whereas no corresponding interactions

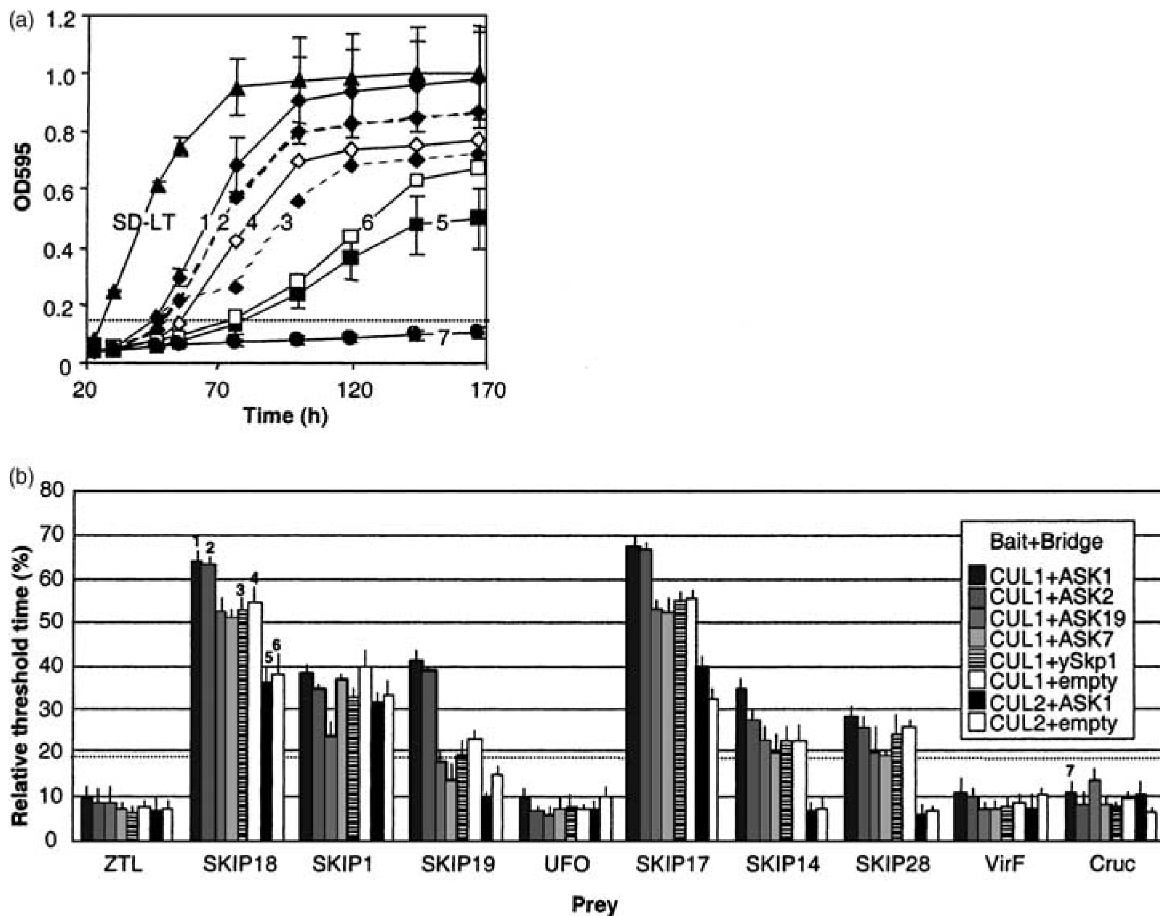

Figure 7. Yeast three-hybrid analysis of *Arabidopsis* SCF subunits.
(a) Yeast growth curve analysis of three-way interactions between prey–SKIP18 and bait–CUL1 (diamonds) or bait–CUL2 (squares), co-expressed with ASK1 (1, 5), ASK2 (2), yeast Skp1 (3), or none (4, 6) from the third Bridge vector. Yeast strains harboring different hybrid gene combinations were grown in liquid medium in six replicates, and the cell density was measured once or twice daily. Prey–cruciferin served as negative control (7). Numbers correspond to the bars in (b). SD-LT: yeast cells grown without selection for hybrid interactions. Error bars are shown only for SD-LT, combinations 1 and 5.
(b) Growth curve analysis of yeast three-hybrid interaction with bait–CUL1 or CUL2 in combination with different prey–FBPs. Select Skp1/ASK proteins were expressed from the Bridge vector. For each strain, the time was measured from the point of subculture to the time taken for the cultures to reach the threshold OD_{595} of 0.15 (dotted line in (a)). The bars represent these threshold times, which were related to threshold times in the absence of 3-AT selection, as defined by: threshold time$^{SD\text{–}LTH + 5\ mM\ 3\text{-}AT}$/threshold time$^{SD\text{–}LT}$ $\times$ 100%. The relative threshold time value of 19% indicates cultures that reached OD_{595} of 0.15 after 170 h. Values lower then 19% were calculated by extrapolation of the growth during the last measurement period.

were observed with the less structurally related CUL3A (not shown), suggesting that CUL1 and CUL2 are the dominant participants in SCF complex formation in *Arabidopsis*.

Although a three-way interaction with ASK and CUL proteins was demonstrated for selected FBPs, all other FBPs from Figure 5 that failed to interact with CUL1 in the binary setting also failed interaction in the presence of any of the ASK and Skp1 proteins listed in Figure 5. The same negative interactions were obtained in the presence of bait-CUL2 or CUL3A (selected combinations are presented in Figure 7). This result was surprising because the FBPs enjoyed robust interaction with ASK1, which, in turn, showed interaction with CUL1. As the SCF model predicts minimal involvement of the F-box domain in the interaction of Skp1 with Cullin (Zheng *et al.*, 2002), as well as the negatively interacting TIR1 was found in the form of a complex with ASK1 and CUL1 (Gray *et al.*, 1999), the data suggest that another factor is involved in the SCF assembly.

To explore the possibility that multiple FBPs are involved in SCF complexes, two-hybrid interactions were analyzed between all combinations of FBPs that are used in Figure 5. All interactions appeared negative except for two robust interactions between SKIP19 and either SKIP17 or SKIP18. The capability of FBPs to form hetero-dimers may have implications for SCF assembly and stochiometry. Taken together, the results suggest that selected FBP proteins participate directly in higher quaternary complexes, while other FBPs may require additional prerequisites such as dimerization for SCF assembly that were not provided in the heterologous yeast two-hybrid system.

Discussion

BLAST search analyses against the complete *Arabidopsis* genome sequence, using a different set of F-box queries identified in this study, resulted in a similar number of F-box-containing *Arabidopsis* genes as reported by Gagne *et al*. (2002), indicating that the annotated sets closely reflect the complete FBP family. As the analysis of the FBP family resulted in the same conclusions, we refer to Gagne *et al*. (2002) for the discussion on this part.

Are ASK1 and ASK2 'master components' of SCF complexes in Arabidopsis?

The crystal structure of the human Skp1–Skp2 dimer revealed a four-layer 'sandwich' structure within the C-terminal domain of Skp2 (Schulman *et al*., 2000), and the authors suggested that the heterogeneous H8 domain found in the large Skp1 (SKR) family of *C. elegans* could in part confer specificity of the interaction. In contrast to *C. elegans,* the *Arabidopsis* ASK protein family is markedly less divergent, including the human Skp1-homologous H8 domain that is common to 19 of the 21 predicted ASK proteins.

When exploring the specificity of ASK/F-Box interactions using the yeast two-hybrid system, all FBPs examined were found to interact with ASK1, ASK2, and ASK11, whereas a more specific interaction pattern was observed with the other ASK proteins. Several lines of evidence support the notion that ASK1, ASK2, and possibly ASK11 interact with most *Arabidopsis* FBPs. Sequence alignment and pairwise comparison of the F-box motifs that interact with ASK1 and ASK2 failed to detect any common features versus the complete FBP annotation set. Moreover, the corresponding C-terminal domains were structurally diverse based on the LRR, Kelch-like, and TUBBY content represented by six distinct classes, as well as on the more unique content of nine FBPs including VirF from *Agrobacterium tumefaciens*. The general interaction of ASK1, ASK2, and ASK11 contrasts with the more specific interaction observed by Gagne *et al*. (2002). However, we suspect that the interaction results are under-estimated based on the negative interactions of At1g76920 with ASK4 and ASK9, and At1g80440 with ASK1, which were positive in our experiments. Thus, the negative interactions reflecting specificity could also be the result of the stringent conditions (high temperature and 3-AT level) used for the two-hybrid selection, the single bait–prey orientation and the testing of specific bait–prey combinations versus library screens. For example, we noticed that many robust interactions selected at 20°C were negative at 30°C.

Notwithstanding the caution required in interpreting negative two-hybrid interactions, our results are similar to those obtained for *C. elegans* where all tested FBPs interacted with SKR1, but more specific interaction profiles were detected with the other SKRs 2–4 even though they belonged to the designated HsSkp1 H8 homology group (Yamanaka *et al*., 2002). Interestingly, in both organisms, the non-specifically interacting ASK and SKR proteins are the most homologous orthologs of the human Skp1 with respect to the 26 residues that contact the Skp2 F-box.

Moreover, additional observations support the suggestion that, like the *skr-1* gene in the nematode, the *ASK1* gene is expressed throughout the plant with higher steady-state levels observed in proliferating tissues (Porat *et al*., 1998; E. Risseeuw and E. Liu, unpublished observation). Thus, it seems that most FBPs in *Arabidopsis* and *C. elegans* are capable of interaction with a relative abundant class of 'master key' SKP1 proteins represented by ASK1, ASK2, ASK11, and SKR1, but that interaction with the other SKP1 proteins may be more specific – even in the case where the H8 helix is conserved. Indeed, the *Arabidopsis ask1-1* mutant exhibits a pleiotropic phenotype, suggesting that ASK1 protein is involved in the formation of functionally diverse SCF complexes containing different FBPs (Yang *et al*., 1999; Zhao *et al*., 1999). On the other hand, the fact that loss of ASK1 function is not lethal demonstrates that the numerous interactions between ASK1 and the wide variety of FBPs are not essential for viability. As the function of this large group of FBPs is likely essential, it can be predicted that ASK1 is partially redundant with the other general interacting ASK2 and ASK11 because of their potential differential expression profiles.

Bridging the interaction with Cullin

Arabidopsis contains at least five functional Cullin genes of which the deduced protein sequences map to Clade-I, -III, and -IV in comparison with Cullins from other organisms. Skp1 proteins from human, *C. elegans* and *S. pombe* were found to interact exclusively with Clade-I Cullins (Furukawa *et al*., 2002; Kominami *et al*., 1998; Nayak *et al*., 2002; Yamanaka *et al*., 2002). Human CUL2 (Clade-II) was shown to interact with the elonginB/C complex (Pause *et al*., 1997), while the Clade-III, -IV, and -V Cullins were predicted to recruit other unknown adapter proteins (Zheng *et al*., 2002). The conservation of Cullin clades, including their putative binding sites, suggests that these adapters along with ASK proteins have co-evolved in *Arabidopsis*. Indeed, a gene homologous to elonginC does exist in *Arabidopsis* (Table 1).

The *Arabidopsis* CUL1 was previously shown to interact with ASK1 in the SCF^{TIR1} complex (Gray *et al*., 1999). Recently, a *cul1* null mutant in *Arabidopsis* was shown to arrest early in embryogenesis, indicating that CUL1 is a component of one or more SCF complexes that function early in plant development (Shen *et al*., 2002). Consistent with the Cullin classification presented here, we found that

Clade-I representatives CUL1 and CUL2, but not CUL3A from Clade-III, interact with FBP members of the FBL2 class. As the same FBPs were able to interact with the yeast Skp1 and the SCF model predicts minimal contact between the F-box and the Cullin, we suggest that these CUL–FBL2 interactions may be 'bridged' by endogenous yeast Skp1. Indeed, using the three-hybrid system, we found that additional expression of ASK1 or ASK2 enhanced the CUL1/FBL2 interaction. Surprisingly, none of the ASKs were able to enhance an interaction with the Cullins involving other FBPs that enjoyed robust interaction with ASK1, ASK2 and others but characteristically failed to interact with yeast Skp1. In addition, all the ASKs failed to interact with CUL1 and CUL2 in reciprocal two-hybrid experiments. Based on these results, we conclude that another factor, besides ASK/Skp1, is required for the interaction between FBP and Cullin, but which is missing in the heterologous system.

Although the binding interfaces between the SCF subunits have been characterized, little is known about the stochiometry of the components in the SCF complex. In exploring the possibility that more than one FBP may participate in SCF formation, an interaction matrix involving a subset of FBPs identified two FBP pairs that could dimerize. This result indicates that at least some *Arabidopsis* FBPs have the potential to assemble in higher order SCF complexes involving more than one FBP. Indeed, dimerization has been shown to be functionally important for the WD40-containing FBPs Pop1 and Pop2 in *S. pombe*, and βTrCP1 and βTrCP2 from human cells (Suzuki *et al.*, 2000; Wolf *et al.*, 1999). Although dimerization of βTrCP1 and βTrCP2 was not dependent on Skp1 binding, we suggest the possibility that members of the expanded ASK protein family assist in the specific assembly of SCF dimers. One implication of this suggestion is that ubiquitination of a specific target protein may depend on the co-expression of multiple ASK and FBP proteins. Dimerization would also imply a higher level of potential combinatorial diversity in the formation of SCF complexes, with implications for the prominence of post-translational protein turnover as a key regulatory mechanism in plant metabolism and development.

Experimental procedures

Plasmid constructs

All cDNAs were cloned as *Sal*I/*Not*I or *Xho*I/*Not*I fragments into the corresponding *Sal*I/*Not*I sites of the yeast two-hybrid GAL4 fusion expression vectors bait pBI770 and prey pBI771 (Kohalmi *et al.*, 1998). *ASK1*, *ASK2* and *ASK4*, *SKL1*, *SKL2* and all SKIP cDNAs were recovered directly from screens of the yeast two-hybrid library (Kohalmi *et al.*, 1997), whereas other genes were PCR-amplified using primers containing terminal *Sal*I or *Not*I sites from various cDNA and/or genomic DNA sources. The *ASK* and *FBP* genes included in this study are listed in Tables 1 and 2. In the directed yeast two- and three-hybrid analyses, full-length open-reading frames were used with the following exceptions: *ASK20B (*codons 1–300), *ASK21 (*13–175), SKIP13 (4–291), SKIP14 (134–435), SKIP17 (20–479), SKIP18 (3–317), and SKIP19 (13–302). *SKL1* sequence has NCBI accession number AF527945. *SKL2* was re-named from the previous designation of *ASK10* (Schrammeijer *et al.*, 2001).

The bridge vector pBI772 used for expression of the third gene in yeast three-hybrid studies was modified from the *LYS2 ARS/CEN* containing vector pRS317 (Sikorski and Hieter, 1989) as follows. The *Not*I site in the multiple cloning sequence was removed by *Xba*I/*Sac*I digestion, followed by end-filling using Klenow polymerase and self-ligation. In the pBI770 'bait' vector, the *Hin*dIII/*Sal*I *GAL4*-BD fragment was replaced with the *Hin*dIII/*Sal*I linker AAGCTTCCGTCGAC. Finally, the expression cassette containing the *ADC1* promoter, the pBI770 multiple cloning site, and the *ADC1* terminator was cloned as an *Apa*I/*Pvu*II fragment into the corresponding *Apa*I/*Sma*I sites of the *Not*I-deleted pRS317 vector, yielding pBI772. *ASK* and *Skp1* coding sequences were subsequently cloned in the *Sal*I and *Not*I sites of pBI772.

Yeast two-hybrid analysis

Yeast two-hybrid screens were conducted as previously described (Kohalmi *et al.*, 1998), with the modification of adding one-tenth of the volume of dimethyl sulfoxide (DSMO) to the yeast cells before heat shock treatment. Briefly, the yeast two-hybrid strain YPB2 (*MATa*) carrying the bait plasmid was transformed with an *Arabidopsis* cDNA library cloned in the prey plasmid. Bait–prey interactions were selected on SD-Leu-Trp-His containing 5 mM 3-amino-1,2,4-triazole (3-AT) plates after a 7-day incubation at 20°C. Selected clones were assayed for expression of the *lacZ* reporter gene using an X-gal filter essay. The prey inserts were partially sequenced after plasmid rescue by transformation into *E. coli* or by direct sequencing of PCR amplicons derived from whole yeast cells (Wang *et al.*, 1996).

Directed yeast two- and three-hybrid interactions were conducted by the mating of YPB2 (*MATa*) carrying the bait and bridge plasmids, with LBA414α (*MAT*α) carrying the prey plasmid using standard procedures (Sherman *et al.*, 1983). Diploid cells were selected on SD-Leu-Trp and subsequently tested for protein interactions by monitoring growth on solid or in liquid SD-Leu-Trp-His media containing 5 mM 3-AT. Cells on solid medium were incubated for 7 days at 20°C, and growth was qualitatively scored as indicating strong, weak, and no interaction.

Liquid cultures were used for growth curve analysis (Diaz-Camino *et al.*, 2002). Briefly, 200 μl SD-Leu-Trp pre-cultures were grown in sterile microtiter plates at 30°C until stationary phase. Pre-cultures were diluted 840-fold in 200 μl SD-Leu-Trp-His + 5 mM 3-AT and, as a control, in 200 μl SD-Leu-Trp. Cells were incubated at 20°C with 200 r.p.m. shaking (shaking was critical for optimal growth) and OD_{595} values were measured after 23, 31, 47, 55, 77, 100, 119, 144, and 167 h of culture. Threshold times were defined as the point where cultures reached an OD_{595} value of 0.15 by linear extrapolation of the two closest data points below and above the threshold value. Interaction threshold times from cultures under 3-AT selection were normalized with the average threshold time of the same cells grown in the absence of selection (SD-Leu-Trp). Six replicate cultures were grown for each plasmid combination in both media.

Data analysis

The F-box motif was defined by the amino acid sequence spanning the −6 and +60 positions relative to the conserved proline. The

F-box gene set of 703 sequences was obtained by comparing F-box queries with the MIPS and TIGR *Arabidopsis* protein databases using standard BLAST search parameters. BLAST results were visually inspected for conserved amino acids in the F-box motif, and the corresponding predicted genes were edited using the genome viewer GENQUIRE (Lewis *et al*., 2002). A file containing the complete list of the F-box genes annotated in this study can be downloaded from http://bioinfo.pbi.nrc.ca/fbox_data.

Sequence comparison of the Skp1, Cullin and FBP protein families was conducted using CLUSTALX and CLUSTALW (Thompson *et al*., 1997). The gonnet250 protein weight matrix was selected, and the gap opening and extension parameters were 10 and 0.2, respectively. Phylogenetic trees were generated using TREEVIEW (Page, 1996), and sequence alignments were edited using GENEDOC available at http://www.cris.com/~Ketchup/genedoc.shtml.

The sequence directly downstream from the F-box was defined as the C-terminal domain. This domain was used for the classification of the FBPs. A phylogram of the deduced protein sequences of the C-terminal domains can be viewed at http://bioinfo.pbi.nrc.ca/fbox_data. A condensed version of the phylogram is presented as a rectangular cladogram in Figure 3(b). For this conversion, the phylogram branches measuring less than 0.003 amino acid substitutions per site, as indicated with the scale bar, were collapsed in the rectangular cladogram. The C-terminal domain classes of the FBPs were hand-annotated by BLAST search analysis and Smith–Waterman pairwise sequence comparison using the GeneMatcher™ service offered by the Canadian Bioinformatics Resource (http://www.cbr.nrc.ca/). Twenty-two FBPs with short or truncated C-terminal domains were not included in the phylogram, but were classified according to their full-length protein sequences.

Different sets of F-box sequences based on the interaction with ASK1 and ASK2 were analyzed using Smith–Waterman pairwise comparisons. The sequence similarity of every F-box pair within each set was reflected in the Smith–Waterman score normalized for the sequence length. For this exercise, the pam250 protein weight matrix was selected, and the gap open and extension penalties were −15 and −2, respectively. The queries were not filtered. For each set, the scores were arranged in square matrices. The distribution of the scores derived from identical and non-identical sequence pairs are presented in a cumulative distribution plot in Figure 4 (http://www.cris.com/~Ketchup/genedoc.shtml).

Acknowledgements

We thank members of the PBI Bioinformatics Group for their generous assistance with data analysis. The yeast strains Mty470 (*cdc53–2*) and LBA414α were gifts from Drs Mike Tyers (Samuel Lunenfeld Research Institute, University of Toronto) and David Thomas (McGill University, Montréal), respectively. The plasmid pRS317 was kindly provided by Dr Phil Hieter (University of British Columbia, Vancouver). This research was supported by the NRC 'Core' Program, the NRC Genomics in Health Initiative Program and a NSF '2010' Grant (MCB 0115870) to WLC.

Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ1768/TPJ1768sm.htm

Table S1 *Arabidopsis* genomic loci with a putative F-box domain

Figure S1. Phylogram of the C-terminal domains of F-box proteins.

References

Adams, J., Kelso, R. and Cooley, L. (2000) The kelch repeat superfamily of proteins: propellers of cell function. *Trends Cell Biol.* **10**, 17–24.

Andrade, M.A., Gonzalez-Guzman, M., Serrano, R. and Rodriguez, P.L. (2001) A combination of the F-box motif and kelch repeats defines a large *Arabidopsis* family of F-box proteins. *Plant Mol. Biol.* **46**, 603–614.

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796–815.

Blondel, M., Galan, J.M., Chi, Y., Lafourcade, C., Longaretti, C., Deshaies, R.J. and Peter, M. (2000) Nuclear-specific degradation of Far1 is controlled by the localization of the F-box protein Cdc4. *EMBO J.* **19**, 6085–6097.

Deshaies, R.J. (1999) SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* **15**, 435–467.

Diaz-Camino, C., Risseeuw, E., Liu, E. and Crosby, W.L. (2003) A high throughput system for two-hybrid screening based on growth curve analysis in microtiter plates. *Anal. Biochem.* (in press).

Dieterle, M., Zhou, Y.C., Schafer, E., Funk, M. and Kretsch, T. (2001) EID1, an F-box protein involved in phytochrome A-specific light signaling. *Genes Dev.* **15**, 939–944.

Farrás, R., Ferrando, A., Jasik, J., Kleinow, T., Okresz, L., Tiburcio, A., Salchert, K., del Pozo, C., Schell, J. and Koncz, C. (2001) SKP1-SnRK protein kinase interactions mediate proteasomal binding of a plant SCF ubiquitin ligase. *EMBO J.* **20**, 2742–2756.

Furukawa, M., Ohta, T. and Xiong, Y. (2002) Activation of UBC5 ubiquitin-conjugating enzyme by the RING finger of ROC1 and assembly of active ubiquitin ligases by all Cullins. *J. Biol. Chem.* **277**, 15758–15765.

Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M. and Vierstra, R.D. (2002) The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **99**, 11519–11524.

Gray, W.M., Del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H. and Estelle, M. (1999) Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes Dev.* **13**, 1678–1691.

Ingram, G.C., Goodrich, J., Wilkinson, M.D., Simon, R., Haughn, G.W. and Coen, E.S. (1995) Parallels between *UNUSUAL FLORAL ORGANS* and *FIMBRIATA*, genes controlling flower development in *Arabidopsis* and *Antirrhinum*. *Plant Cell*, **7**, 1501–1510.

Jiang, J. and Struhl, G. (1998) Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. *Nature*, **391**, 493–496.

Kohalmi, S.E., Nowak, J. and Crosby, W.L. (1997) A practical guide to using the yeast two-hybrid system. In *Differentially Expressed Genes in Plants: a Bench Manual* (Harper, G. and Hansen, E., eds). London: Taylor & Francis, pp. 63–82.

Kohalmi, S.E., Reader, L.J.V., Samach, A., Nowak, J., Haughn, G.W. and Crosby, W.L. (1998) Identification and characterization of protein interactions using the yeast two-hybrid system. *Plant Mol. Biol. Man.* **M1**, 1–30.

Kominami, K., Ochotorena, I. and Toda, T. (1998) Two F-box/WD-repeat proteins Pop1 and Pop2 form hetero- and homo-complexes together with cullin-1 in the fission yeast SCF (Skp1-Cullin-1-F-box) ubiquitin ligase. *Genes Cells*, **3**, 721–735.

Lewis, S., Gibson, M., Harris, N., Wiel, C., Wilkinson, M., Block, D., Crosby, W.L., Clamp, M. and Searle, S. (2002) *Construction*

of a Genome 'Sandbox' (Grant, R., ed). Norfolk, UK: Horizon Scientific Press.

Nayak, S., Santiago, F.E., Jin, H., Lin, D., Schedl, T. and Kipreos, E.T. (2002) The *Caenorhabditis elegans* Skp1-related gene family: diverse functions in cell proliferation, morphogenesis, and meiosis. *Curr. Biol.* **12**, 277–287.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A. and Bartel, B. (2000) *FKF1*, a clock-controlled gene that regulates the transition to flowering in *Arabidopsis*. *Cell*, **101**, 331–340.

Page, R.D.M. (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**, 357–358.

Pang, P.P., Pruitt, R.E. and Meyerowitz, E.M. (1988) Molecular cloning, genomic organization, expression and evolution of 12s seed storage protein genes of *Arabidopsis thaliana*. *Plant Mol. Biol.* **11**, 805–820.

Pause, A., Lee, S., Worrell, R.A., Chen, D.Y., Burgess, W.H., Linehan, W.M. and Klausner, R.D. (1997) The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. *Proc. Natl. Acad. Sci. USA*, **94**, 2156–2161.

Pickart, C.M. (2001) Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* **70**, 503–533.

Porat, R., Lu, P.Z. and O'Neill, S.D. (1998) *Arabidopsis* SKP1, a homologue of a cell cycle regulator gene, is predominantly expressed in meristematic cells. *Planta*, **204**, 345–351.

Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Turner, J. and Estelle, M. (1998) The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast grr1p. *Genes Dev.* **12**, 198–207.

Schrammeijer, B., Risseeuw, E., Pansegrau, W., Regensburg-Tuink, T.J., Crosby, W.L. and Hooykaas, P.J. (2001) Interaction of the virulence protein VirF of *Agrobacterium tumefaciens* with plant homologs of the yeast Skp1 protein. *Curr. Biol.* **11**, 258–262.

Schulman, B.A., Carrano, A.C., Jeffrey, P.D., Bowen, Z., Kinnucan, E.R., Finnin, M.S., Elledge, S.J., Harper, J.W., Pagano, M. and Pavletich, N.P. (2000) Insights into SCF ubiquitin ligases from the structure of the Skp1–Skp2 complex. *Nature*, **408**, 381–386.

Shen, W.H., Parmentier, Y., Hellmann, H., Lechner, E., Dong, A., Masson, J., Granier, F., Lepiniec, L., Estelle, M. and Genschik, P. (2002) Null mutation of AtCUL1 causes arrest in early embryogenesis in *Arabidopsis*. *Mol. Biol. Cell*, **13**, 1916–1928.

Sherman, F., Fink, G.R. and Hicks, J.B. (1983) *Methods in Yeast Genetics*. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory.

Sikorski, R.S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. and Harper, J.W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex (see comments). *Cell*, **91**, 209–219.

Smith, T.F., Gaitatzes, C., Saxena, K. and Neer, E.J. (1999) The WD repeat: a common architecture for diverse functions. *Trends Biochem. Sci.* **24**, 181–185.

Somers, D.E., Schultz, T.F., Milnamow, M. and Kay, S.A. (2000) *ZEITLUPE* encodes a novel clock-associated PAS protein from *Arabidopsis*. *Cell*, **101**, 319–329.

Stirnberg, P., van De, S.K. and Leyser, H.M. (2002) *MAX1* and *MAX2* control shoot lateral branching in *Arabidopsis*. *Development*, **129**, 1131–1141.

Suzuki, H., Chiba, T., Suzuki, T., Fujita, T., Ikenoue, T., Omata, M., Furuichi, K., Shikama, H. and Tanaka, K. (2000) Homodimer of two F-box proteins betaTrCP1 or betaTrCP2 binds to IkappaBalpha for signal-dependent ubiquitination. *J. Biol. Chem.* **275**, 2877–2884.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* **25**, 4876–4882.

Tsvetkov, L.M., Yeh, K.H., Lee, S.J., Sun, H. and Zhang, H. (1999) p27 (Kip1) Ubiquitination and degradation is regulated by the SCF (Skp2) complex through phosphorylated Thr187 in p27. *Curr. Biol.* **9**, 661–664.

Wang, H., Kohalmi, S.E. and Cutler, A.J. (1996) An improved method for polymerase chain reaction using whole yeast cells. *Anal. Biochem.* **237**, 145–146.

Weissman, A.M. (2001) Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell Biol.* **2**, 169–178.

Wolf, D.A., McKeon, F. and Jackson, P.K. (1999) F-box/WD-repeat proteins pop1p and Sud1p/Pop2p form complexes that bind and direct the proteolysis of cdc18p. *Curr. Biol.* **9**, 373–376.

Woo, H.R., Chung, K.M., Park, J.H., Oh, S.A., Ahn, T., Hong, S.H., Jang, S.K. and Nam, H.G. (2001) ORE9, an F-box protein that regulates leaf senescence in *Arabidopsis*. *Plant Cell*, **13**, 1779–1790.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998) *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. *Science*, **280**, 1091–1094.

Yamanaka, A., Yada, M., Imaki, H., Koga, M., Ohshima, Y. and Nakayama, K.I. (2002) Multiple Skp1-related proteins in *Caenorhabditis elegans*. Diverse patterns of interaction with Cullins and F-box proteins. *Curr. Biol.* **12**, 267–275.

Yang, M., Hu, Y., Lodhi, M., Mccombie, W.R. and Ma, H. (1999) The *Arabidopsis SKP1-LIKE1* gene is essential for male meiosis and may control homologue separation. *Proc. Natl. Acad. Sci. USA*, **96**, 11416–11421.

Yuvaniyama, J., Denu, J.M., Dixon, J.E. and Saper, M.A. (1996) Crystal structure of the dual specificity protein phosphatase VHR. *Science*, **272**, 1328–1331.

Zhao, D., Yang, M., Solava, J. and Ma, H. (1999) The *ASK1* gene regulates development and interacts with the *UFO* gene to control floral organ identity in *Arabidopsis*. *Dev. Genet.* **25**, 209–223.

Zheng, N., Schulman, B.A., Song, L. *et al.* (2002) Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature*, **416**, 703–709.