Pollen-Specific SKP1-Like Proteins are Components of Functional SCF Complexes and Essential for Lily Pollen Tube Elongation

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Introduction

Many plant development and differentiation events, such as flowering, photomorphogenesis, senescence and self-incompatibility (SI), are regulated by the degradation of specific proteins (Hochstrasser 1996, Dreher and Callis 2007). Eukaryotes are suggested to have two major protein degradation pathways: vacuolar–lysosomal and ubiquitin–proteasome pathway. The ubiquitin–proteasome pathway mediates protein degradation and is involved in diverse aspects of plant development and differentiation, including pollen tube elongation and self-incompatibility. We characterized three lily (Lilium longiflorum) SKP1-like genes, LSK1–LSK3, that are specifically expressed in late pollen developmental stages and the elongating pollen tube. The encoded peptide sequences reveal that LSK1–LSK3 share high identity with Arabidopsis ASK1 and contain a putative N-terminal CUL1- and a C-terminal F-box-interacting domain. Yeast two-hybrid and in vitro affinity binding assays revealed that the LSKs associate with lily CULLIN1. In addition, the LSK genes can functionally complement the yeast skp1 deletion mutant YDR328C. To investigate their biological functions in pollen tube elongation, an in vivo approach for green fluorescent protein (GFP)-tagged dominant-negative LSK1–LSK3 was developed. Microprojectile bombardment with N-terminally truncated LSK1–LSK3 (LSK1–LSK3Δ–GFP) significantly retarded pollen tube elongation in both in vitro germination and in vivo self- and cross-pollination after >12 h incubation. Interestingly, elongation of pollen tubes harboring overexpressed LSK2Δ–GFP and LSK3Δ–GFP was substantially inhibited within the self-pollinated styles. The elongation of most LSK2Δ–GFP-transformed pollen tubes could germinate only on the stigmatic surface of self style and showed statistically significant growth arrest as compared with control pollen tubes. Lily exhibits typical gametophytic self-incompatibility via an unknown mechanism, but LSK2 and LSK3 may be involved in this complex machinery. These results suggest critical roles for LSK1–LSK3 in regulating fundamental pollen tube elongation in vitro and in vivo and that the 26S proteasome-mediated protein pathway plays an important role in pollen tube elongation.

Keywords: Lily (Lilium longiflorum) • LLCUL1 • LSK • Pollen tube elongation • SCF complex • Self-incompatibility.

Abbreviations: AChE, acetylcholinesterase; ChAT, choline acetyltransferase; CNBr, cyanogen bromide; FAP, FIM-associated protein; 5-FOA, 5-fluoro-orotic acid; GFP, green fluorescent protein; GSI, gametophytic self-incompatibility; GST, glutathione S-transferase; LLCUL1, lily CULLIN1; LSK, lily SKP1-like protein; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; SI, self-incompatibility; SKP, S-phase kinase-associated protein; SLF, S-locus F-box; SSI, sporophytic self-incompatibility; Ub, ubiquitin.

The nucleotide sequences reported in this paper have been submitted to GenBank with the following accession numbers: FJ467291 (LSK1), FJ467292 (LSK2), FJ467293 (LSK3) and FJ467294 (LLCUL1).
(Ub)--26S proteasome-mediated proteolyses. Ub--26S proteasome-mediated proteolysis marks specific proteins for degradation by attaching K48-linked Ub chains through cycles of a three enzyme-catalyzed cascade. The Ub-activating enzyme (E1) uses ATP to activate Ub to form a thioester-linked E1--Ub intermediate; Ub is then transferred to an E2 to form a thioester-linked E2--Ub intermediate; with the help of a specific E3, Ub on E2 is then transferred to the targeted protein substrate (Patton et al. 1998); and the protein substrate marked by K48-linked Ub chains is recognized and degraded by the 26S proteasome (Hoyt 1997).

The enzymatic components of the E1--E2--E3 cascade for Ub conjugation is hierarchical—usually only one E1, a limited number of E2s and a large number of E3s—which demonstrates the delicate control of protein degradation in eukaryotes (Pickart 2001). Currently, in Arabidopsis, two, 37 and probably >1300 genes are reported to encode E1, E2 and E3 enzymes, respectively (Bachmair et al. 2001). The SCF complex is one typical multimeric E3 and consists of four major components: S-phase kinase-associated protein 1 (SKP1), CULLIN1, an F-box protein and Rbx1. SKP1 protein functions as a bridge to connect with CULLIN1 and F-box protein and forms the major scaffold of the SCF complex. Two major domains are found in most F-box proteins: the N-terminus, which contains a conserved motif of approximately 40 amino acids, i.e. the F-box, for interaction with SKP1; and the C-terminus of a variable protein--protein association domain (such as LRR, WD40 or a Kelch domain), for interaction with specific target proteins (Schulman et al. 2000). CULLIN1 is a conserved protein found in all eukaryotes, and the Nedd8/Rub modifications it operates seem to be critical for regulation of the activity of the SCF complex (del Pozo et al. 2002, Dharmasiri et al. 2003). The Rbx1 protein has been characterized as a RING-H2 domain-containing protein that physically interacts with CULLIN1 and Ub-bound E2 to execute the protein ubiquitination processes (Skowrya et al. 1999, Gray et al. 2002). No SKP1 homolog has been found in Eubacteria or Archaea, and only one functional SKP1 has been found in human and yeast. However, many SKP1-related genes have been annotated in the genomic sequence of other organisms. For instance, 21 SKP1-related genes (ASK genes) are found in the Arabidopsis genome, and seven SKP1-related genes have been found in Drosophila melanogaster (Zhao et al. 2003b, Kong et al. 2004).

Similarly to SKP1, F-box proteins comprise a large protein family. Within the Arabidopsis genome >700 F-box proteins have been identified so far (Gagne et al. 2002, Takahash et al. 2004). Several F-box proteins seem to interact with target proteins with high specificity, which suggests a role in the regulation of several developmental and physiological processes. For example, an F-box protein forms the SCF{TIR1} complex, which regulates auxin responses (Gray et al. 1999, del Pozo et al. 2002). UFO is another F-box protein that forms the SCF{UFO} complex and is critical in the regulation of B floral organ identity genes in Arabidopsis (Samach et al. 1999). The presence of a large number of F-box proteins and SKP1-related proteins in plant genomes indicates that ubiquitination specificity mediated by extremely complicated combinations of these proteins plays critical roles in regulating diverse developmental processes in plants.

Seeds and grains are staple foods around the world, and their production is the result of successful double fertilization of female gametophytes by male gametophytes. In flowering plants, the highly reduced haploid male gametophyte (pollen grain) plays a critical role in fertilization through generation of a pollen tube that delivers the two sperm cells to the egg and central cells within the embryo sac for double fertilization. Recent studies show that Ub--26S proteasome-mediated proteolyses play an important role in regulating pollen tube elongation and double fertilization, such as S-RNase-based gametophytic SI (GSI). For example, the S-locus F-box (SLF) probably forms a unique SCF{SLF} E3 ligase in the families Solanaceae and Plantaginaceae and is involved in the regulation of SI (Hua and Kao 2006, Huang et al. 2006). Recently, one pollen-specific SKP1-like gene, Antirrhinum SLF-interacting SKP1-related 1 (AhSSK1), was identified from an Antirrhinum hispanicum pollen (Huang et al. 2006). Yeast two-hybrid analyses indicate that S-locus F-box proteins AhSLF-S1 and AhSLF-S5, physically interact with AhSSK1 but not with FIM-associated proteins (FAPs), a class of widely expressed SKP1-like proteins. Glutathione S-transferase (GST) pull-down assays also revealed the formation of complexes involving AhSSK1, CULLIN1 and an S-RNase. In Antirrhinum pollen, transient expression of a CUL1-interacting domain-deleted dominant-negative construct of FAP1 but not AhSSK1 affected growth of pollen tubes.

Lily (Lilium longiflorum) has been known for a long time to exhibit typical GSI, whereby pollen tube elongation stops in one-third to half of the style by an unknown mechanism. Until recently, it has been shown that endogenous cAMP (Tsuruhara and Tezuka 2001) and choline derivatives (Tezuka et al. 2007) in the pistil may be involved in lily SI. It has been shown that their contents were significantly lower in self-pollinated styles than in cross-pollinated styles, and self-pollinated pistils pre-treated with cAMP and acetylcholine or several choline derivatives significantly enhanced elongation of self-pollinated tubes. The acetylcholine content of pistils is regulated by choline acetyltransferase (ChAT; acetylcholine-forming enzyme) and acetylcholinesterase (AChE; acetylcholine-decomposing enzyme). Both exogenous neostigmine and cAMP, a potent inhibitor of AChE and activator of ChAT, respectively, also significantly enhanced the elongation of pollen tubes in self-pollinated styles (Tezuka et al. 2007). These results imply that the threshold of endogenous acetylcholine probably regulated
by cAMP plays a critical role in self-pollinated lily gynecium; however, it is still unclear what potential male counterparts are involved in lily GSI. In this study, we identified three pollen-specific SKP1-like transcripts from lily pollen, LSK1–LSK3. LSK proteins share high amino acid sequence identity with Arabidopsis ASK1. Also, LSK proteins interacted with lily CULLIN1 in yeast two-hybrid and in vitro binding assays. They functionally complemented a yeast skp1 deletion mutant. LSK constructs with truncation on the N-terminus showed differential effects on pollen tube elongation in both in vitro germination and in vivo self- and cross-pollination. Their roles in the regulation of pollen tube elongation, pollination and probably in SI are discussed.

Results

Three lily SKP1-like transcripts are pollen specific and expressed at late pollen maturation stages

A home-made microarray of lily pollen tube cDNAs was used to screen transcripts with up-regulated expression during pollination (unpublished data). Three partial SKP1-like cDNAs were identified and designated as lily SKP1-like 1–3 (LSK1–LSK3). Full-length cDNAs were obtained by 5′ and 3′-RACE (rapid amplification of cDNA ends) approaches and encoded peptides of 161, 157 and 161 amino acids with estimated molecular mass of 18.1, 17.9 and 18.3 kDa, respectively (Fig. 1A). Comparison of the protein sequences showed that the peptides share high amino acid identity with Arabidopsis ASK1 (41–58%) and Antirrhinum FAP1 (40–61%) but less with AhSSK1 (30–35%; Fig. 1A and Supplementary Fig. S1). To confirm these data, we analyzed the phylogenetic relationship using plant SKP1-like genes previously surveyed by Huang et al. (2006). LSK3 formed a cluster with several well-characterized SKP1-like genes such as ASK1, NbSKP1 and FAP1 (Supplementary Fig. S2). In contrast, LSK2 appeared close to human and yeast SKP1 genes, and LSK1 was closely related to putative rice SKP1 genes.

Most SKP1 proteins contain two functional domains: the N-terminal CUL1- and C-terminal F-box-interacting domain (Zheng et al. 2002). Structural analysis of human SKP1 and its F-box protein SKP2 complex has suggested that the interface between SKP1 and F-box proteins is conserved among yeast, human (Schulman et al. 2000) and plants (Huang et al. 2006). All three LSKs are predicted to contain eight α-helices, as is found in most of the already characterized SKP1 proteins (Fig. 1). In addition, the highly conserved N-terminal CUL1-interacting domain (the dashed line in Fig. 1) and amino acid residues theoretically located on the SKP1 interface for CULLIN1 interaction (Zheng et al. 2002) were found in all individual SKP1-like proteins from different species, including the three LSKs (asterisks in Fig.1).

SKP1 was found to be a kinetochoore protein required for cell cycle progression. Arabidopsis ASK1 is ubiquitously expressed, with high expression in root and shoot meristems (Connelly and Hieter 1996, Portet et al. 1998). To determine whether LSK genes are highly expressed in cell cycle progression tissues, we used reverse transcription–PCR (RT–PCR) and quantitative RT–PCR to examine the spatial and temporal expression patterns of the LSK genes. As shown in Fig. 2A (left panel), LSK1 and LSK2 were specifically enriched in pollen grains (Po) and elongating pollen tubes (T) but not in roots (R), leaves (L) or pistils (Pi). Quantitative RT–PCR revealed similar expression profiles of these LSK transcripts (Fig. 2C). Nevertheless, in contrast to the expression of LSK1 and LSK2, that of LSK3 was 800–1000 times lower in elongating pollen tubes. During pollen tube elongation (right panel in Fig. 2A), the LSK genes were present in both in vivo (Vo) and in vitro grown pollen tubes, and expression levels remained high during pollen tube elongation (0–12–24 h). During microspore development, all LSK transcripts were first detected in developing pollen (stage 7 in Fig. 2B) and greatly accumulated to their maximum expression level during late pollen developmental and maturation stages (stages 8–10 in Fig. 2B). Thus, these LSKs may function as pollen- or pollen tube-specific SKP1-like proteins and are probably involved in proteasome-mediated protein degradation by formation of functional SCF complexes within the germinating lily pollen tube.

LSKs functionally complement a yeast skp1 deletion mutant and interact with lily CULLIN1 protein

Protein structure and phylogenetic analyses indicate that LSKs are a novel class of pollen-specific SKP1-like proteins. In the yeast genome, only one functional SKP1 gene is deemed essential for yeast survival (Connelly and Hieter 1996). To investigate the functional conservation of the three lily LSK genes, we performed complementation experiments with a haploid yeast skp1 deletion strain, skp1Δ [pRS316:ySKP1], harboring a shufflable plasmid-borne wild-type yeast SKP1. The skp1Δ [pRS316ySKP1] was derived from YDR328C, a heterozygous diploid mutant with one SKP1 allele replaced by a KanMX4 module (Fig. 3). YDR328C was first transformed by pRS316ySKP1, which harbored a 1.4 kb yeast genomic fragment containing full-length yeast SKP1 in the shufflable pRS316 vector (Ura3+). After sporulation, all haploid spores survived on YPD medium, and those containing the pRS316ySKP1 plasmid could survive on a selective SD-U plate (Fig. 3A). The haploid skp1Δ knockout yeast strain skp1Δ [pRS316ySKP1] containing the plasmid-borne yeast SKP1 was selected by G418 and uracil auxotrophy (–U) for the SKP1 knockout module (KanMX4 module) and pRS316ySKP1, respectively (Fig. 3A, YPD+G418 and SD-U+G418). The haploid skp1Δ [pRS316ySKP1] strain grew well under uracil auxotrophy (Fig. 3B, SD-U, lane 1) but not under uracil and leucine auxotrophy (Fig. 3B, SD-U-L, lane 1). After individual introduction of lily
LSK1–LSK3 driven by a ScRPN10 promoter in pRS425 (Leu2+), the haploid skp1Δ [pRS316ySKP1] strain grew well under uracil and leucine auxotrophy (Fig. 3B, SD-U-L, lanes 2–4 for LSK1–LSK3, respectively). The capacity for complementation by lily LSK1–LSK3 was analyzed by ejecting pRS316:ySKP1 on 5-fluoro-orotic acid (5-FOA) treatment. As shown in Fig. 3B (lanes 2–4), the haploid skp1Δ strains expressing individual LSK genes (pRS425:RPN10:LSK1–LSK3) survived without wild-type yeast SKP1, which indicated functional complementation. The haploid skp1Δ [pRS316ySKP1] was not viable after losing pRS316ySKP1 (Fig. 3B, SC+5FOA, lane 1). After 5-FOA treatment, the pRS316ySKP1 plasmid indeed was completely shuffled by pRS425:RPN10:LSK1–LSK3, as shown by the absence of growth on SD-U and by being able to grow on SD-L (Fig. 3B, SD-U and SD-L, lanes 2–4). The above results show that these LSK genes are functional SKP1-like genes and can replace yeast SKP1.

SKP1 is well known to function as the adaptor to interact with CULLIN1 and F-box protein in a typical SCF complex (Craig and Tyers 1999). To examine the potential interaction between LSKs and lily CULLIN1 (LLCUL1), the full-length LLCUL1 cDNA was derived and isolated from lily pollen by use of RT–PCR with degenerate primers deduced from Arabidopsis and rice CULLIN1 sequences, followed by 5′- and 3′-RACE experiments. LLCUL1 encodes a polypeptide of 744 amino acids with a mol. wt of about 87 kDa. The deduced protein shows high sequence identity with CULLIN1 from Arabidopsis (81.3%) and rice (84.9%) (Supplementary Fig. S3). In addition, the conserved amino acid residues on the interface of CULLIN1 for SKP1 association (Zheng et al. 2002) were conserved in LLCUL1 from Arabidopsis (81.3%) and rice (84.9%) (Supplementary Fig. S3). In addition, the conserved amino acid residues on the interface of CULLIN1 for SKP1 association (Zheng et al. 2002) were conserved in LLCUL1 from Arabidopsis (81.3%) and rice (84.9%) (Supplementary Fig. S3). In addition, the conserved amino acid residues on the interface of CULLIN1 for SKP1 association (Zheng et al. 2002) were conserved in LLCUL1 from Arabidopsis (81.3%) and rice (84.9%) (Supplementary Fig. S3). In addition, the conserved amino acid residues on the interface of CULLIN1 for SKP1 association (Zheng et al. 2002) were conserved in LLCUL1 from Arabidopsis (81.3%) and rice (84.9%) (Supplementary Fig. S3). In addition, the conserved amino acid residues on the interface of CULLIN1 for SKP1 association (Zheng et al. 2002) were conserved in LLCUL1 from Arabidopsis (81.3%) and rice (84.9%) (Supplementary Fig. S3). In addition, the conserved amino acid residues on the interface of CULLIN1 for SKP1 association (Zheng et al. 2002) were conserved in LLCUL1 from Arabidopsis (81.3%) and rice (84.9%) (Supplementary Fig. S3). In addition, the conserved amino acid residues on the interface of CULLIN1 for SKP1 association (Zheng et al. 2002) were conserved in LLCUL1 from Arabidopsis (81.3%) and rice (84.9%) (Supplementary Fig. S3).
Gal4-AD prey fusion (pGADT7:LLCUL1), and various LSKs were constructed as Gal-BD bait fusions (pGBKT7:LSK1–LSK3). As shown in Figure 4A, AH109 strains harboring pGADT7:LLCUL1 and various pGBKT7:LSK1–LSK3 grew well on SD-W-L-H-A medium, which indicates activation of the His reporter by association between prey and various bait fusions. To rule out the possible self-activation of various bait fusions, the control AH109 strains harboring the empty prey fusion (pGADT7) and various bait fusions (pGBKT7:LSK1–LSK3) were examined and shown to be incapable of growing on the SD-W-L-H-A medium. AH109 harboring pGBKT7:S3/pGADT7:T or pGBKT7:L/pGADT7 was included as a positive and negative control, respectively. In addition, the cells containing pGADT7:LLCUL1 and various LSK baits (pGBKT7:LSK1–LSK3) showed α-galactosidase activity by streaking cells onto selection media containing X-α-Gal, as did pGBKT7:S3- and pGADT7:T-transformed yeasts (data not shown).

The LSK1–LLCUL1 interaction was further confirmed by purification of LSK1-interacting proteins from affinity column assays with recombinant LSK1 protein-conjugated cyanogen bromide (CNBr) resin, then immunoblotting with an anti-AtCULLIN1 antibody. A putative lily CULLIN1-like...
Functional analysis of lily pollen-specific LSKs

A. Functional complementation of the haploid yeast skp1 deletion mutant by three LSK genes. To test the functions of three LSK genes, a skp1 heterozygous diploid yeast mutant, YDR328C, with its SKP1 gene replaced by a KanMX4 module, was used for complementation assay. (A) Isolation of the haploid skp1 knockout yeast mutant (skp1∆[ySKP1]) expressing plasmid-borne SKP1 for complementation. Four segregating spores (a–d) from a pRS316ySKP1 (yeast SKP1 gene)-transformed YDR328C tetrad grew normally on the YPD plate and the selective SD-U plate. The haploid skp1 knockout yeast mutant expressing plasmid-borne SKP1 was selected by G418 and uracil auxotrophy (–U) for the SKP1 knockout module (KanMX4 module) and pRS316ySKP1, respectively (YPD+G418 and SD-U+G418). Two spores (b and c) were obtained from this selection, and the yeast strain skp1∆[ySKP1] derived from spore b was used for the following complementary assay. (B) Functional complementation of the yeast skp1 mutant by all three LSK genes. The yeast strain skp1∆[ySKP1] grew normally on SD-U but not on SD-U-L (lane 1 in SD-U-L) plates. By transforming skp1∆[ySKP1] with individual LSK genes, pRS425:RPN10::LSK (lane 2–4 for LSK1, LSK2 and LSK3, respectively), the yeast strain skp1∆[ySKP1] could survive on the SD-U-L plate. After ejecting the pRS316ySKP1 by S-FOA treatment, the yeast skp1∆ strain containing individual transformed LSK genes survived (SC+5-FOA, lanes 2–4), which indicates functional complementation. To confirm the lack of the originally resident pRS316ySKP1 plasmid, only control strain skp1∆[ySKP1] (lane 1 in SD-U) but not S-FOA-treated LSK transformants (lanes 2–4 in SD-U) could survive on SD-U. However, the pRS425:RPN10::LSK (lanes 2–4 in SD-L)-containing cells but not the control strain skp1∆[ySKP1] (lane 1 in SD-L) could survive on SD-L plates.

B. Effects of pollen tube elongation were examined by transient assays with microprojectile bombardment. Compared with elongation of pollen tubes overexpressing GFP (Fig. 5A, B), that of pollen tubes overexpressing intact LSKs (data not shown) and LSK1∆–GFP (Fig. 5C, I) was not significantly affected within 12 h after bombardment but showed slightly retarded pollen tube elongation after 24 h incubation (Fig. 5D, I). However, overexpression of LSK2∆–GFP (Fig. 5E, F, I) or LSK3∆–GFP (Fig. 5G, H, I) significantly diminished the elongation of pollen tubes after 12 h incubation, and the prohibitive effects were even more severe after 24 h incubation. Thus, overexpression of LSK1∆, LSK2∆ and LSK3∆ impairing normal pollen tube elongation is probably due to the disturbance of the balance imposed by SCF complexes on protein degradation.

Recently, AhSSK1 was shown to interact with an S-locus F-box protein, AhSLF. The resulting SCFASAFL complex might be involved in SI reactions but had no effect on normal pollen tube elongation (Huang et al. 2006). To explore the physiological functions of lily pollen-specific LSK genes, individual intact LSKs or those with a deletion on a conserved CUL1 interaction domain (LSK∆1–3) were tagged with green fluorescent protein (GFP) and driven by a pollen-specific promoter pZM13 (pZM13::LSK1∆-GFP, pZM13::LSK2∆-GFP and pZM13::LSK3∆-GFP). The effect of various LSK∆s on pollen-tube elongation was examined by transient assays with microprojectile bombardment. Compared with elongation of pollen tubes overexpressing GFP (Fig. 5A, B), that of pollen tubes overexpressing intact LSKs (data not shown) and LSK1∆–GFP (Fig. 5C, I) was not significantly affected within 12 h after bombardment but showed slightly retarded pollen tube elongation after 24 h incubation (Fig. 5D, I). However, overexpression of LSK2∆–GFP (Fig. 5E, F, I) or LSK3∆–GFP (Fig. 5G, H, I) significantly diminished the elongation of pollen tubes after 12 h incubation, and the prohibitive effects were even more severe after 24 h incubation. Thus, overexpression of LSK1∆, LSK2∆ and LSK3∆ impairing normal pollen tube elongation is probably due to the disturbance of the balance imposed by SCF complexes on protein degradation.

After 12 h, pollen tubes overexpressing LSK1∆–GFP showed no significant impairment of pollen tube elongation, similar to overexpression of AhSSK1∆ which has been suggested to be involved in S-RNase-based SI in Antirrhinum (Huang et al. 2006). Since lily exhibits GSI, we aimed to

Fig. 3 Functional complementation of the haploid yeast skp1 deletion mutant by three LSK genes. To test the functions of three LSK genes, a skp1 heterozygous diploid yeast mutant, YDR328C, with its SKP1 gene replaced by a KanMX4 module, was used for complementation assay. (A) Isolation of the haploid skp1 knockout yeast mutant (skp1∆[ySKP1]) expressing plasmid-borne SKP1 for complementation. Four segregating spores (a–d) from a pRS316ySKP1 (yeast SKP1 gene)-transformed YDR328C tetrad grew normally on the YPD plate and the selective SD-U plate. The haploid skp1 knockout yeast mutant expressing plasmid-borne SKP1 was selected by G418 and uracil auxotrophy (–U) for the SKP1 knockout module (KanMX4 module) and pRS316ySKP1, respectively. (B) Functional complementation of the yeast skp1 mutant by all three LSK genes. The yeast strain skp1∆[ySKP1] grew normally on SD-U but not on SD-U-L (lane 1 in SD-U-L) plates. By transforming skp1∆[ySKP1] with individual LSK genes, pRS425:RPN10::LSK (lane 2–4 for LSK1, LSK2 and LSK3, respectively), the yeast strain skp1∆[ySKP1] could survive on the SD-U-L plate. After ejecting the pRS316ySKP1 by S-FOA treatment, the yeast skp1∆ strain containing individual transformed LSK genes survived (SC+5-FOA, lanes 2–4), which indicates functional complementation. To confirm the lack of the originally resident pRS316ySKP1 plasmid, only control strain skp1∆[ySKP1] (lane 1 in SD-U) but not S-FOA-treated LSK transformants (lanes 2–4 in SD-U) could survive on SD-U. However, the pRS425:RPN10::LSK (lanes 2–4 in SD-L)-containing cells but not the control strain skp1∆[ySKP1] (lane 1 in SD-L) could survive on SD-L plates.

LSKs dominant-negative mutant shows defective pollen tube growth

Recently, AhSSK1 was shown to interact with an S-locus F-box protein, AhSLF. The resulting SCFAHSLF complex might be involved in SI reactions but had no effect on normal pollen tube elongation (Huang et al. 2006). To explore the physiological functions of lily pollen-specific LSK genes, individual intact LSKs or those with a deletion on a conserved CUL1 interaction domain (LSK∆1–3) were tagged with green fluorescent protein (GFP) and driven by a pollen-specific promoter pZM13 (pZM13::LSK1∆-GFP, pZM13::LSK2∆-GFP and pZM13::LSK3∆-GFP). The effect of various LSK∆s on
explore the biological functions of LSKs in the lily SI response. We developed an in vivo approach to explore the effects of GFP-tagged dominant-negative LSKΔ constructs on pollen tube growth during pollination. Lily pollen grains bombarded with various LSKΔ constructs mentioned above were used for self- and cross-pollination. Two and a half days after pollination, the pollinated styles were cross-cut into 2 cm segments starting from the stigma and extending towards the ovary. Then the pollinated styles were dissected longitudinally to measure the length of pollen tubes at every 0.5 cm interval under a fluorescence dissection microscope. At least 10 styles were used for two biological replicates, and one

Fig. 4 The pollen-specific LSK proteins interacted with lily CULLIN1 protein. LSK1 interacts with LLCUL1 in yeast two-hybrid (A) and affinity purification (B) assays. (A) Yeast two-hybrid assay was used to examine the interaction between LSKs and LLCUL1. LLCUL1 and various LSK genes were cloned into pGBK7 (pGBK7:LSK1-3) and pGADT7 (pGADT7:LLCUL1), respectively, to produce BD-LSK and AD-LLCUL1 fusion proteins. Yeast cells harboring pGADT7:LLCUL1 and various pGBK7:LSK plasmids but not those with an empty AD fusion (pGADT7) and various pGBK7:LSK plasmids could survive on SD-W-L-H-A selection medium. AH109 harboring pGBK7:53/pGADT7:T or pGBK7:1/pGADT7:T was included as a positive and negative control, respectively. (B) Recombinant LSK1 proteins conjugated with CNBr resin beads were used for affinity purification of LLCUL1 from pollen grains. Lily pollen total native proteins were incubated with LSK1-conjugated CNBr resin beads, then underwent washing 10 times with the column volume buffer and were eluted by eluting buffer. The eluent samples were separated by SDS–PAGE and immunoblotted with anti-AtCUL1 antibody. Lane 1, total pollen native protein; lane 2, unbound flow-through proteins; lanes 3 and 4, samples from the first and second washing; lanes 5, 6 and 7, first, second and third elution solution collected from columns. The arrow indicates the expected lily CULLIN1 protein.
Fig. 5 Transient overexpression of CUL1-interacting domain-deleted LSK proteins in lily pollen caused dominant-negative impairment in pollen tube elongation. Pollen-specific promoter pZM13-driven GFP (GFP) or N-terminal CUL1 interaction domain-truncated LSK genes (pZM13::LSKsΔ-GFP) were bombarded into lily pollen grain. After 12 h (A, C, E, G) and 24 h (B, D, F, H) in vitro germination, transformed pollen tubes were observed under an epi-fluorescence microscope and the effect of overexpressed LSK genes on pollen tube elongation was measured. The quantitative data shown in (I) were from transformed pollen tubes (n >100 with three biological repeats) after germination for 12 and 24 h. Bars = 1.0 mm. Arrows indicate the transformed pollen tubes showing growth retardation.

A typical result is shown in Fig. 6. The control pollen transformed with pZM13::GFP (GFP in Fig. 5) showed normal tube elongation, about 6.8 ± 1.3 cm, in cross-pollinated styles, but in self-pollinated styles, pollen tube elongation showed typical GSI and was arrested at 1.2 ± 0.8 cm beneath the stigma. In all LSKΔ-GFP-transformed pollen tubes, tube elongation was greatly arrested, with different degrees of inhibition on both cross- and self-pollination. In cross-pollinated styles, all LSKΔ-GFP-transformed pollen tubes elongated to halfway in the styles as compared with GFP-transformed tubes, and LSK2Δ-GFP-transformed pollen tubes showed the most severe phenotype, which correlated well with the in vitro results (Fig. 5). In self-pollinated styles, only LSK1Δ-GFP-transformed pollen tubes could elongate to halfway in the styles (0.8 ± 0.35 cm) as compared with GFP-transformed tubes (1.2 ± 0.8 cm). The elongation of most LSK2Δ-GFP-transformed pollen tubes could germinate only on the stigmatic surface and showed statistically significant growth arrest as compared with control pollen tubes (inset in Fig. 6). These in vitro and in vivo pollen tube elongation results show that all LSKs are essential for normal pollen tube elongation. Interestingly, LSK2Δ-GFP- and LSK3Δ-GFP-transformed pollen tubes show more severe defects in elongation on self-pollination, which suggests that they may have other important functions to regulate pollen tube elongation with self-pollination.
Fig. 6 Overexpression of dominant-negative LSK transcripts showed different effects on pollen tube elongation during pollination. Pollen grains bombarded with pZM13::GFP (eGFP) or individual pZM13::LSKΔ-GFP plasmids (LSKΔ) were applied on self- (S, Avita) and cross- (C, Snowqueen) pollinated styles for 60 h. Pollinated styles were harvested and dissected into 2 cm segments starting from the stigma and style junction toward the ovary, and cut longitudinally to observe the longest length of fluorescent pollen tubes under a dissection fluorescence microscope. The inset shows the statistical analyses of the position of the tips of the longest pollen tubes from one typical experiment with 10 pollinated styles, which were bombarded with various constructs in the self- and cross-pollinated styles.
Discussion

Twenty-one SKP1-related genes are present in Arabidopsis, but only ASK1 has been well characterized (Porat et al. 1998, Gray et al. 1999, Yang et al. 1999, Zhao et al. 2003a, Liu et al. 2004). The expression patterns of these 21 ASK genes are diverse, but none is specifically expressed in pollen (Zhao et al. 2003b, Takahashi et al. 2004). In this study, we identified three lily pollen- or pollen tube-specific SPK1-related genes, designated LSK1–LSK3. The transcripts sharing significant similarity with SPK1 genes from other species are expressed during late pollen developmental stages and remain highly expressed in elongating pollen tubes (Fig. 2). In contrast to the expression of LSK1 and LSK2, that of LSK3 was 800–100 times lower in elongating pollen tubes. Our functional analyses showed that the products of these LSK genes function as typical SKP1-like proteins to complement the yeast skp1Δ mutant (Fig. 3), and LSK1 interacts with LLSUC1 protein, as seen in both yeast two-hybrid and affinity purification assays (Fig. 4). In addition, the results of our in vivo and in vitro functional analyses imply that all LSKs are essential for normal pollen tube elongation. Thus, LSK-containing SCF complexes are essential for normal lily pollen tube growth.

The Arabidopsis ask1-1 homozygous mutant shows male gametophytic sterility because of defective homologous chromosome separation (Yang et al. 1999). Recent studies imply that ASK1 participates in the regulation of chromosome remodeling, homolog juxtaposition, synapsis and synaptonemal complex formation during early meiotic prophase (Yang et al. 2006, Zhao et al. 2006). Temporal expression profiles of the three LSK genes suggest a different expression pattern. The development of lily anther or pollen is highly related to floral bud length, and meiosis occurs within flower buds of about 20–30 mm that finally generate four microspores. Pollen mitosis I occurs in flower buds of about 60–70 mm that eventually generate bi-nucleate microspores, and only after pollen maturation does pollen mitosis II generate two sperm cells in the lily pollen tube (Huang et al. 2000, Ko et al. 2002). However, we found that the three LSK transcripts started to accumulate in 115 mm flower buds (Fig. 2); thus, they may be not involved in chromosome remodeling and segregation during male meiosis. Overexpression of individual LSK genes with a deleted consensus CUL1 interaction domain (LSK1-Δ) showed different degrees of effects on pollen tube elongation: overexpression of LSK2-Δ and LSK3-Δ significantly impairs normal pollen tube elongation as compared with LSK1-Δ in both in vivo and in vitro pollination. LSK2 and LSK3 may have more important roles in regulating pollen tube growth than does LSK1 by disruption of SCF-mediated protein degradation. So far, the potential targets for LSKs in elongating pollen tubes are unknown. However, several lily pollen-specific proteins, such as LLA23 and LLA32, which accumulate during pollen maturation but are gradually degraded after pollen germination (Huang et al. 2000), may be the potential targets for the LSK-containing SCF complexes. A recent study suggested that LL223 is ABA-, stress- and ripening-induced (ASR) protein involved in the ABA signaling pathway (Yang et al. 2005). In addition, Arabidopsis TUBBY-like proteins (AtTLPs) contain a conserved F-box domain; one example is AtTLP9, which can interact with ASK1 and regulate ABA signaling (Lai et al. 2004). A TUBBY-like cDNA has been cloned from lily pollen (L.-C. Chang and G.-Y. Jauh, unpublished data). Examining the potential interaction between the TUBBY-like and LLAs proteins and LSKs to explore the role of ABA signaling pathways in pollen tube growth will be interesting.

SI is a unique mechanism evolved in plants presumably to avoid inbreeding and increase the genetic diversity of progeny in the face of environmental changes. Two kinds of self-incompatible mechanisms are found in the plant kingdom—sporophytic SI (SSI) and gametophytic SI (GSI)—and the Ub–proteasome pathway plays critical roles in both systems. For example, in *Brassica* SSI, ARC1, a stigma E3 ligase, is essential for the rejection of its own pollen (Stone et al. 2003). The best-characterized GSI is Solanaceae-type GSI, found in the families Solanaceae and Plantaginaceae (formerly known as Scrophulariaceae), in which the male S-determinant, an S-locus F-box protein (SLF/SFB), may form a putative SCF complex to detoxify the cytotoxic effects caused by the female S-determinant, an S-RNase (Hua et al. 2008). In *Petunia inflata*, PiSLF interacts with a RING-finger protein, *P. inflata* S-RNase-binding protein 1 (PiSBP1), and PiCUL1-G to form a novel E3 ligase, which helps recruit S-RNase which had previously been taken up for degradation (Hua and Kao 2006). A recent study of *Antirrhinum hispanicum* revealed that the pollen-specific SKP1-related protein AhSSK1, which shares only 37.4% identity with ASK1, interacts with CUL1, and AhSLF might form a functional SCFASL complex to regulate degradation of S-RNase in *Antirrhinum GSI* (Huang et al. 2006); overexpression of N-terminal truncated AhSSK1 did not disturb pollen tube elongation, which led the authors to suggest that AhSSK1 probably participates in only *Antirrhinum GSI*. However, the ubiquitously expressed FAP1, sharing 80.1% protein sequence identity with ASK1, cannot interact with AhSLFs, which implies that FAP1 may be functionally similar to ASK1 in regulating general plant growth and development instead of SI. Like FAP1, the proteins PiSK1, PiSK2 and PiSK3 from *P. inflata* share very high amino acid sequence identity with ASK1 (~80%) but during yeast two-hybrid experiments did not interact with PiSLF (Hua and Kao 2006). Although PiSLF did not interact with PiSKs, a pollen-specific SKP1-related protein able to interact with PiSLF might still exist. Recently, Hua et al. (2008) proposed an innovative biochemical model for S-RNase-based GSI in *P. inflata*: the PiSLF preferentially interacts with its cytosolic non-self S-RNase to form an Es-like complex for the degradation of non-self S-RNase through the Ub–26S proteasome machinery.
Lily exhibits typical GSI, whereby pollen tube elongation stops in one-third to half of the style by an unknown mechanism, but recent studies imply the involvement of stylar endogenous cAMP and choline derivative contents instead of γ-RNA-based GSI. Biochemical analyses found that the contents were significantly lower in self-pollinated styles than in cross-pollinated ones, and, importantly, self-pollinated pistils pre-treated with cAMP and the choline derivative acetylcholine significantly enhanced the elongation of self-pollinated pollen tubes (Tsuruhara and Tezuka 2001, Tezuka et al. 2007). These results imply that the threshold of endogenous acetylcholine probably regulated by cAMP in lily pistil plays a critical role in repressing pollen tube elongation in self-pollinated styles. Nevertheless, the potential male molecules involved in lily GSI are still unknown. Our results show that SCF complex-mediated protein degradation is essential for pollen tube elongation under both in vitro and in vivo conditions and probably is involved in enforcing the SI response in self-pollinated lily pollen tubes. Lily pollen tubes harboring overexpressed N-terminal truncated LSK genes (LSKΔ) showed significantly disturbed normal pollen tube elongation in both in vitro incubation and in vivo pollination (Figs. 5, 6). Interestingly, this disturbance was more severe in self-pollinated than in cross-pollinated pollen tubes. All LSK1Δ–GFP-transformed pollen tubes elongating in cross-pollinated styles, and those transformed by LSK1Δ–GFP grown in self-pollinated styles showed reduced elongation, by about 33–69% of that of GFP-transformed control pollen tubes. However, the length of LSK2Δ–GFP- and LSK3Δ–GFP-transformed pollen tubes showed more severe growth reduction, by 75–96%, in self-pollinated styles. In particular, almost all the LSK2Δ–GFP-transformed pollen tubes were arrested on the stigma (Fig. 6). These results not only truly reflect their functions in regulating pollen tube elongation found in Fig. 5, but also suggest that LSK2 may also have an important function in self-pollination, such as in GSI. We suggest that lily pollen-specific expressed LSK2, and probably LSK3, are essential parts of this complex GSI machinery. Overexpression of CUL1 interaction domain-deleted LSK2 or LSK3 depletes the essential components of the SCF complex, such as a putative F-box protein, to interfere with the normal function or formation of the SCF complex involved in SI. Further identification of the potential LSK-interacting F-box proteins and a target(s) in lily pollen, as well as establishing a possible connection between pistil cAMP-mediated acetylcholine production and the SCF complex in lily pollen tubes, may broaden our knowledge and elucidate the mechanism of SI in Liliaceae.

Materials and Methods

Plant materials

Easter lily (L. longiflorum Thunb. cv. Avita and Snowqueen) bulbs obtained from a local farm (Foreport Enterprises Co., Ltd., Taipei, Taiwan) were planted in a greenhouse under ambient conditions. Mature pollen grains were collected from anthers and dried on a bench for 2 d for further use. To collect in vitro germinated pollen tubes, pollen grains were placed in germination medium (1.27 mM CaCl₂, 0.162 mM H₃BO₃, 0.99 mM KNO₃, 290 mM sucrose, pH 5.2) and collected after different time intervals. To collect in vivo germinated pollen tubes, ‘Avita’ styles were pollinated 2 d after anthesis with ‘Avita’ pollen, and in vivo pollen tubes were collected as described previously (Jauh and Lord 1995, Jauh and Lord 1996). Microspore meiosis occurred in the pollen mother cells at a bud size of around 20–25 mm, which resulted in the formation of tetrads. Microspore mitosis was complete at a bud size of around 65–70 mm, and pollen subsequently entered the maturation stage of development. Flower buds ranging from 10 to 150 mm were used to isolate developing and maturing anthers (Huang et al. 2000, Ko et al. 2002). Leaves, roots, pistils and pollen tube were collected, frozen immediately in liquid nitrogen, and stored at −76°C until use. Chemicals, unless specified otherwise, were purchased from Sigma-Aldrich (St Louis, MO, USA).

LSK1, LSK2, LSK3 and LLCUL1 cloning

Partial cDNAs of LSK1, LSK2 and LSK3 obtained from lily pollen or pollen tubes (Wang et al. 2004) were used as templates for 5′- and 3′-RACE to clone their corresponding full-length cDNAs according to the SMART™ RACE cDNA Amplification Kit User Manual (Clontech, Palo Alto, CA, USA). LLCUL1 partial cDNA was isolated from RT–PCR with use of degenerated primers according to the conserved amino acids of Arabidopsis and rice CULLIN1 protein, and full-length cDNA was obtained by 5′- and 3′-RACE. Primer sets used in this study are listed in Supplementary Table S1.

RT–PCR and real-time RT–PCR analyses of the expression of genes in organs

Lily pollen development was divided into six stages: pre-meiosis, meiosis, microspore development, mitosis, pollen maturation and mature pollen on the basis of the length of floral buds (Huang et al. 2000, Ko et al. 2002, Mogami et al. 2002). To determine the expression patterns of these LSK transcripts in organs and during pollen development, real-time RT–PCR and RT–PCR were carried out to analyze the RNAs extracted from different organs and developing anthers of different sized lily floral buds. By use of the Absolutely RNA® RT–PCR Miniprep Kit (Stratagene, La Jolla, CA, USA), total RNA was isolated from 0.1 g of lily tissues: leaves, roots, pistils, pollen and anthers/stamens of various sized floral buds, including the mature anther and in vitro and in vivo grown pollen tubes. First-strand cDNAs were synthesized from 3 μg of total DNase I-treated RNA with use of an oligo(dT) primer and random primers according to the manufacturer’s protocol (M-MLV Reverse Transcriptase; Invitrogen Life Technologies, Carlsbad, CA, USA). To study the expression of genes in the
pollen grain, in vitro/in vivo germinated pollen tubes and organs, RT–PCR analyses were performed with the gene-specific primer sets listed in Supplementary Table S1. The PCR mix consisted of 1.5 mM magnesium chloride, 0.2 mM dNTPs, 0.5 µM each of sense and antisense primer, 2.5 U of Taq polymerase (MBD Bio, Taipei, Taiwan) and 1 × PCR buffer supplied with the Taq polymerase. The reaction was conducted at 94°C for 5 min; 25–35 cycles at 94°C for 1 min, annealing at 55–58°C for 1 min, 72°C for 1–3 min; final elongation at 72°C for 10 min with use of a Biometra® T3 Thermocycler (Whatman Biometra, Gottingen, Germany). The resulting PCR products were run on a 1% agarose gel containing 0.01% ethidium bromide. To monitor the efficacy of cDNA synthesis by PCR amplification, the housekeeping gene Actin was used as a positive control for PCR amplification, and a 761 bp fragment was obtained by use of the his3 (clone ID 23687) was obtained from Invitrogen. The plasmid, ∆his3::LSK1, ∆his3::LSK2 and ∆his3::LSK3 were cloned into the pGBKT7 vector and open reading frames were amplified by PCR and cloned into the BamHI site of a pGADT7 vector to fuse with the GAL4 DNA-binding domain; LSK2 and LSK3 were cloned into the EcoRI site of the pGBKT7 vector to fuse with the GAL4 DNA-binding domain; LLCLUL1 was cloned into the XhoI site of a pGADT7 vector to fuse with the GAL4 activation domain. Yeast possessing pGBK7-T7 and pGAD7-T were a positive control, and yeast possessing pGBK7-Lam and pGAD7-T were a negative control. The yeast strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, lys2Δ, GAL1::GAL1-1::LacZ) was used in these assays. Yeast two-hybrid interactions were accomplished according to CLONTECH MATCHMAKER GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech).

To obtain a yeast SKP1 haploid knockout mutant, pRS316::SKP1 was first transformed into YDR328C, then grown on an SD-U plate. The transformed clone was individually chosen from the SD-U plate and inoculated into 3 ml of YPD broth for incubation at 30°C with 200 r.p.m. rotation to an OD600 of 2.5–3.0. A 1 ml aliquot of culture was transferred into a sterile disposable 15 ml tube and centrifuged for 5 min at 1,200×g. The precipitated cells were resuspended in 5 ml of sterile water, centrifugation was repeated, and then cells were resuspended in 1 ml of liquid sporulation medium (10 g l⁻¹ potassium acetate, 1 g l⁻¹ yeast extract and 0.5 g l⁻¹ glucose). After 30°C incubation for 2 weeks, sporulation was observed by microscopy (BX51; Olympus, Tokyo, Japan). A 4 µl aliquot of sporulation culture was mixed with 6 µl of Zymolyase (10 µgml⁻¹) (Sigma-Aldrich) in a sterile microcentrifuge tube. We chose an evenly poured YPD plate and marked a line down the middle of the plate (bottom of the plate) with a black marker. A 5 µl aliquot of the mixture was put onto the YPD plate at one end and in the middle of the marker line, and the YPD plate was stood vertically for 30 min to move the mixtures downward and along the marker line. Tetrad dissection was accomplished by using micromanipulation as described (Sherman and Hicks 1991). After obtaining the yeast SKP1 haploid knockout mutant containing the pRS316::SKP1 plasmid, plasmids pRS425::RPN10NdeΔ:LSK1, pRS425::RPN10NdeΔ:LSK2 and pRS425::RPN10NdeΔ:LSK3 were transformed into the cells. For ura+ negative selection, 5 ml (100 mg ml⁻¹) of 5-FOA (Sigma-Aldrich) was added into 500 ml of synthetic complete (SC) medium. The cells containing pRS316::SKP1 and pRS425::RPN10NdeΔ:LSK plasmids were streaked onto SC+5-FOA to select yeast containing only pRS425::RPN10NdeΔ:LSK plasmids.

### Yeast functional complementation

For yeast functional complementation, the yeast SKP1 heterozygous diploid mutant YDR328C BY4743 (Mat α/α; his3Δ1/Δ1; leu2Δ0/Δ0; lys2Δ0/Δ0; MET15/Δ; ura3Δ0/Δ0; YDR328ckanMX4/YDR328c) (clone ID 23687) was obtained from Invitrogen. The plasmids pRS316 (Ura3+) and pRS425::RPN10NdeΔ (pRS425 carrying the ScRNP10 promoter) (Ura2+) were as described (Yang et al. 2004). A genomic fragment from yeast of about 1.4 kb containing the SKP1 promoter and open reading frame was amplified by PCR and cloned into the BamHI site of pRS316 to generate the pRS316::SKP1 plasmid. LSK1, LSK2 and LSK3 coding regions were amplified by PCR and cloned into the pRS425::RPN10 NdeΔ plasmid between Ndel–PstI sites and controlled by the ScRNP10 promoter to generate pRS425::RPN10NdeΔ:LSK1, pRS425::RPN10NdeΔ:LSK2 and pRS425::RPN10NdeΔ:LSK3. Yeast transformation was conducted according to the CLONTECH MATCHMAKER GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech).
1.875 mg of gold particles (1.0 μm) coated with 7 μg of plasmid pزم13:GFP or pزم13:LSK1ΔGFP (or pزم13:LSK2ΔGFP or pزم13:LSK3ΔGFP) were bombarded into pollen grains by use of the PDS-1000/He Biolistic® Particle Delivery System (Bio-Rad, Hercules, CA, USA) at the following settings: 1,100 p.s.i., 29 mmHg vacuum, 1 cm gap distance, 9 cm particle flight distance. Three consecutive bombardments were performed for each sample to increase the transformation frequency. For in vitro growth assay, bombarded pollen was washed from the filter paper with the use of germination medium and germinated in 2 ml of germination medium in a Petri dish of 3 cm in diameter, with gentle shaking at 30°C for 12 and 24 h incubation. Then the germinating pollen tubes with GFP signals were observed and photographed under an epi-fluorescence microscope (BX51; Olympus, Tokyo, Japan) through x4 and x10 (UPlanFl; Olympus, Tokyo, Japan) lenses and a GFP filter. The lengths of pollen tubes with a GFP signal were measured by use of Image J software. To evaluate the effect of overexpression of self- or cross-cultivars. After 2.5 d, the pollinated styles were cut into 2 cm segments and dissected vertically for fluorescence stereomicroscopy (CCD (DP70; Olympus, Tokyo, Japan) through an epi-fluorescence microscope (BX51; Olympus, Tokyo, Japan) lenses and a GFP filter. The open reading frame of LSK1-interacting protein GST–LSK1 recombinant protein overexpression and affinity column preparation for purification of LSK1-interacting protein

The open reading frame of LSK1 was cloned into a recombinant protein expression vector pGEX4T-1 in the EcoRI site by the PCR direct-cloning method. Escherichia coli strain BL21 was transformed by the pGEX4T-1:LSK1 plasmid and then used to induce recombinant protein GST–LSK1 overexpression. GST–LSK1 recombinant protein was purified by use of glutathione–Sepharose 4B according to the manufacturer’s instructions (Amersham Pharmacia Biotech, San Francisco, CA, USA). A 30 mg aliquot of GST–LSK1 recombinant proteins (5 μg μl–1) was dialyzed in 1,500 ml of buffer I (0.5 M NaCl in 0.1 M NaHCO3, pH 8.3) three times at 4°C for 24 h. A 400 mg aliquot of CNBr-activated resin was swollen by dipping and washing in 80 ml of 1 mM HCl three times at 4°C for 1.5 h. The swollen resin was transferred into columns and washed with 30 ml of ice-cold 1 mM HCl, then 30 ml of ice-cold buffer I. GST–LSK1 protein was dialyzed, and incubated together with the swollen resin in a 15 ml centrifuge tube with gentle shaking at 4°C overnight, then centrifuged at 2,000 × g at room temperature for 5 min to stop the binding reaction; the supernatant was removed. A 6 ml aliquot of 0.2 M glycine (pH 8.0) was added to the slurry gel pellet and shaken for 2 h. The slurry gel was packed into a column, washed with 5 ml of buffer I and then 5 ml of buffer II (0.5 M NaCl in 0.1 M sodium acetate, pH 4.0); the process was repeated four more times to remove unbound proteins. After washing with 30 ml of loading buffer, the GST–LSK1-conjugated CNBr resin was transferred into a 15 ml centrifuge tube that contained lily pollen native total proteins for incubation for 2 h at room temperature (or 4°C overnight) with agitation. After incubation, the slurry gel was slowly packed into a column and washed with excess 1×Tris-buffered saline buffer to remove the unbound proteins. The LSK1-interacting proteins were eluted by washing the column with 0.2 M glycine-HCl (pH 2.0).

## Supplementary data

Supplementary data are available at PCP online.

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