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Interaction analyses of *Arabidopsis* tubby-like proteins with ASK proteins

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ABSTRACT. In this study, we have investigated protein-protein interactions between 10 *Arabidopsis* TUBBY-like proteins (AtTLPs) and various *Arabidopsis* Skp1-like proteins (ASKs) using the yeast two-hybrid system. The results indicate that six AtTLPs (AtTLP1, 2, 3, 9, 10, 11) are able to specifically interact with ASK1. AtTLP6 is able to interact with ASK1, ASK2, and ASK11, while AtTLP5, AtTLP7, and AtTLP8 did not show any significant interaction with any ASK protein. Serial deletion analyses of AtTLP2 have demonstrated that both F-box domain and tubby domain are required for AtTLP2-ASK1 interaction. Domain swapping suggests that variations at the N-terminus of AtTLP7 and C-terminus of AtTLP5 result in the inability to interact with ASK1. Using site-directed mutagenesis, we further demonstrated that naturally occurring proline-to-leucine and cysteine-to-serine variations in F-box domain might result in the dysfunction of AtTLP7's interaction with ASK1. Taking these observations together, we propose that AtTLP1, 2, 3, 9, 10, and 11 can specifically interact with ASK1 and may have an overlapping function. Since AtTLP5, 7, and 8 cannot interact with any ASK, it is suggested that they may have lost their original function(s) and/or acquired new function(s) during evolution.

Keywords: *Arabidopsis*; ASK; F-box domain; F-box protein; Protein-protein; Interaction; Ttubby domain; TULP; Yeast two-hybrid.

INTRODUCTION

The family of TUBBY-like proteins (TULPs), which includes TUBBY, TULP1, 2, 3 (TULP1-TULP3), and TUBBY superfamily protein (TUSP), has been identified in humans and mice (Kleyn et al., 1996; Noben-Trauth et al., 1996; North et al., 1997; Nishina et al., 1998; Li et al., 2001). Their products contain a conserved domain in the C-terminal region, which is called the tubby domain. The results for comparison of TULPs from different organisms revealed that sequences corresponding to the tubby domain are conserved in unicellular and multi-cellular organisms, but the N-termial of TULP is varied, suggesting functional divergence and differentiation of TULPs (North et al., 1997; Lai et al., 2011). In mammals, the TULP gene family exhibits a different tissue expression pattern and plays important role in development and physiology (Kleyn et al., 1996; Noben-Trauth et al., 1996; North et al., 1997; Nishina et al., 1998; Ikeda et al., 2000; Ikeda et al., 2001). The tubby domain has a remarkable dual binding function as it is capable of interacting with both DNA and phosphotidylinositol (Boggon et al., 1999; Santagata et al., 2001). Santagata et al. (2001) demonstrated that a tubby domain is attached to the plasma membrane via binds PI (4, 5) P_2. During activation of $G\alpha_{q/11}$ proteins, the induction of hydrolysis of PI (4, 5) P_2, and release of TULP from plasma membrane occur. The released TULP itself can regulate gene expression by serving as a transcription factor. but the gene regulated by TULP remains to be elucidated (Santagata et al., 2001). Our knowledge about the proteins which can interact with TULP are still very limited. Previous study showed that in mammals TULP1 interacts with Dynamin-1, that indicates that TULP1 may be involved in the vesicular trafficking of photoreceptor proteins (Xi et al., 2007). Amino terminus of the TULP3 that binds the IFT-A complex can promote trafficking of a subset of G protein-coupled receptors (GPCRs) into primary cilia (Mukhopadhyay et al., 2010). In plants, Lai et al. (2004) demonstrated that AtTLP9 can interact with ASK1, and may participate in the ABA signalling pathway during seed germination and early development (Lai et al., 2004).

We have identified a TUBBY-like protein gene family with 11 members in *Arabidopsis*, named *AtTLP1-11* (Lai et al., 2004). Interestingly, unlike the highly diverse N-terminal region of animal TULPs, most plant TULP members contain a conserved F-box domain (51-57 residues) (Yang et al., 2008). Instead of the transactivation domain that is

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present in animals, the conserved F-box domain in plant TULP suggests that the functional mechanism of TUBBY-like proteins in plant may be different from those in mammals. The F-box protein, named after the cyclin F, contains a 50-60 amino acid residue F-box domain that is composed of a highly degenerated hydrophobic sequence (Bai et al., 1996) regulating the cell cycle, immune response, signaling cascades, and developmental programs in SCF protein complexes [named after their main components, S-phase kinase association protein 1 (SKP1), Cullin (CDC53), and an F-box protein] and non-SCF protein complexes (Russell et al., 1999; Kitagawa et al., 2003; Vierstra, 2003; Smalle and Vierstra, 2004). The best-studied F-box protein is a component of SCF complexes, which bind substrates for ubiquitin-mediated proteolysis (Deshaies, 1999; Kipreos and Pagano, 2000). The functions of several *Arabidopsis* SCF complexes such as SCF(TIR1, transport inhibitor response 1), SCF(COI1, coronatine-insensitive 1), and SCF(AtSKP2, Arabidopsis homolog of homolog of human SKP2) in auxin signaling, jasmonic acid signaling, and cell division have been verified (Gray et al., 1999; Xu et al., 2002; del Pozo et al., 2002).

The F-box protein interacts with the SKP1-related protein via an N-terminal F-box domain and with the specific substrate via a protein-protein interaction domain in the C-terminal region (Patton et al., 1998; Xiao and Jang, 2000). F-box proteins are classified according to their potential protein-protein interaction domain at the C-terminus, including leucine-rich repeats (LRR), kelch repeats, WD40 repeat, leucine zipper, actin-like, lectin-like, and tubby domains (Gagne et al., 2002; Risseeuw et al., 2003). Some of these domains are common to F-box proteins from yeast and animals (for example, LRR and WD40 repeat), while others are unique to plant F-box proteins such as the actin-like, lectin-like, and tubby domains (Gagne et al., 2002). The diversity of C-terminal protein interaction domains confers substrate recognition specificity on the F-box proteins.

Yeast two-hybrid studies and/or co-immunoprecipitation assays have shown that F-box proteins containing LRRs (TIR1, ORE9 and COI1) or leucine zipper domain (EID1) are able to interact with ASK1 or ASK2 through their F-box domains (Dieterle et al., 2001; Gray et al., 2001; Woo et al., 2001; Devoto et al., 2002). The *ASK1* gene, first isolated by yeast two-hybrid screening using the TIR1 and unusual floral organs (UFO) F-box proteins as bait, is a *SKP1* homolog in *Arabidopsis* (Gray et al., 1999; Samach et al., 1999). Only one known functional SKP1 protein interacts with different F-box proteins to degrade different substrates through ubiquitin-mediated proteolysis in humans and yeasts (Deshaies, 1999; Schulman et al., 2000; Ilyin et al., 2002). In contrast, at least 21 *ASK* genes exhibit different expression patterns in *Arabidopsis* (Schulman et al., 2000; Ilyin et al., 2002), and the ASK proteins show differences in their association with both cullins and F-box proteins (Gagne et al., 2002; Risseeuw et al., 2003). The 21 *ASK* genes can be separated into eight groups according to their amino sequence similarity: group 1, *ASK1* and *ASK2*; group 2, *ASK3* and *ASK4*; group 3, *ASK5* and *ASK6*; group 4, *ASK7*, *ASK8*, *ASK9*, and *ASK10*; group 5, *ASK11* and *ASK12*; group 6, *ASK13*; group 7, *ASK14* through *ASK19*; and group 8, *ASK20* and *ASK21*. Except for *ASK6* and *ASK19*, expression of these genes was detected in one or more organs of plants grown under normal conditions through RT-PCR (Zhao et al., 2003).

Our preliminary study has demonstrated that AtTLP9 can interact with ASK1 (Lai et al., 2004). However, the biochemical functions of the plant tubby proteins are still unclear. In the present work, 10 representative ASKs were selected from 7 groups of ASK gene family, which can form SCF ubiquitin E3 ligase subunits from *Arabidopsis* as it have been demonstrated previously (Zhao et al., 2003; Risseeuw et al., 2003). We have performed comprehensive interaction studies between 10 AtTLPs and 10 representative ASKs members using a yeast two-hybrid system. Our data have shown that only six AtTLPs (AtTLP1, 2, 3, 9, 10, 11) can interact specifically with ASK1, that AtTLP6 can interact with three ASKs (ASK1, 2, 11), and that three AtTLPs (AtTLP5, 7, 8) did not show any significant interaction with any ASKs tested. Surprisingly, unlike AtTLP8, both AtTLP5 and 7 contain F-box domains. Domain swapping studies suggest that variations at the F-box of AtTLP7 and the tubby domain of AtTLP5 result in the inability to interact with ASK1. These results suggest that plant TULP family members might have developed different functional mechanisms during evolution through different types of mutations at either the F-box domain or tubby domain.

MATERIALS AND METHODS

Construction of the AtTLPs and ASKs genes for the yeast two-hybrid system

For general cloning, insert DNA fragments were amplified by PCR using Ex Taq DNA polymerase (Takara Bio Inc, Shiga, Japan). The primers used to amplify individual DNA fragments for yeast two-hybrid experiments are shown in Table 1 in Supplementary Material online. The amplified DNA fragments were purified by gel extraction. For the point mutation and domain swap experiments, the DNA fragments were amplified by two rounds of PCR. Two (large and small) DNA fragments were amplified by *pfu* DNA polymerase (Promega, Madison, WI, USA) separately with the first round primers, described in Table 1 in Supplementary Material online, and then purified by gel extraction. These purified large and small DNA fragments were mixed in a 1:1 molar ratio and used as the template for the second round of PCR. The second round PCR was performed by Ex Taq DNA polymerase with the second round primer pairs (Table 1 in Supplementary Material online). The amplified DNA fragments from second round PCR were purified by gel extraction. These purified DNA fragments were ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) using T_4 DNA ligase (Promega, Madison, WI, USA) and transformed into *E.coli* DH5α

Table 1. Constructions used in yeast two-hybrid experiments.

Construction	Preparation of fragments for cloning	
	Vector[a]	Primers of Insert (5' to 3')
BD-AtTLP2	pBD-GAL4	F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTACCCTTCACATGCCGG (*Pst*I)
BD-AtTLP2$^{1\text{-}110}$	pBD-GAL4	F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTATTTCAATGAGATTGG (*Pst*I)
BD-AtTLP2$^{1\text{-}178}$	pBD-GAL4	F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTAAGTACTGCTGCTCCGTGAGAA (*Pst*I)
BD-AtTLP2$^{1\text{-}320}$	pBD-GAL4	F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTAGTTGAGGCACCAGCACTGCAA (*Pst*I)
BD-AtTLP2$^{1\text{-}373}$	pBD-GAL4	F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTATAGAGGGTAGCGATAA (*Pst*I)
BD-AtTLP2$^{44\text{-}394}$	pBD-GAL4	F: GAATTCCCACAGAGCCCATGGGCTTCT (*Eco*RI) R: CTGCAGTTACCCTTCACATGCCGG (*Pst*I)
BD-AtTLP2$^{111\text{-}394}$	pBD-GAL4	F: GAATTCCAGCCGGGGCCTCGAGAC (*Eco*RI) R: CTGCAGTTACCCTTCACATGCCGG (*Pst*I)
AD-ASK1	pAD-GAL4-2.1	F: CGGAATTCATGTCTGCGAAGAAGAT (*Eco*RI) R: CTGCAGTCATTCAAAAGCCCATTG (*Pst*I)
AD-ASK2	pAD-GAL4-2.1	F: GGATCCATGTCGACGGTGAGAAAA (*Bam*HI) R: CTGCAGTTCAAACGCCCACTGATT (*Pst*I)
AD-ASK3	PAD-GAL4-2.1	F: GGATCCATGGCAGAAACGAAGAAG (*Bam*HI) R: CTGCAGCTCGAACGCCCACTTGTT (*Pst*I)
AD-ASK5	pAD-GAL4-2.1	F: GAGAATTCATGTCGACGAAGATCAT (*Eco*RI) R: CTCTGCAGTTGAAAAGCCCATTGA (*Pst*I)
AD-ASK7	pAD-GAL4-2.1	F: GAGAATTCATGTCGACAAAAAAGAT(*Eco*RI) R: CTCTGCAGTTCAAAAGCCCATTTA (*Pst*I)
AD-ASK11	pAD-GAL4-2.1	F: GGATCCATGTCTTCGAAGATGATC (*Bam*HI) R: CTGCAGTTCAAAAGCCCATTGA (*Pst*I)
AD-ASK12	pAD-GAL4-2.1	F: CGGAATTCATGTCTGCGAAGAAGAT (*Eco*RI) R: CTGCAGTCATTCAAAAGCCCATTG (*Pst*I)
AD-ASK13	pAD-GAL4-2.1	F: GGATCCATGTCGAAGATGGTTATG (*Bam*HI) R: CTGCAGTTCAAAAGCCCATTGATT (*Pst*I)
AD-ASK17	pAD-GAL4-2.1	F: CGGAATTCATGTCTGCGAAGAAGAT (*Eco*RI) R: CTGCAGTCATTCAAAAGCCCATTG (*Pst*I)
AD-ASK18	pAD-GAL4-2.1	F: GGATCCATGGCTTCTTCTTCCGAA (*Eco*RI) R: CTGCAGCTCATTAAAAGTCCAAGC (*Pst*I)
BD-AtTLP2^{P52L}	pBD-GAL4	First round PCR: 1-171 bp fragment from *AtTLP2* F: GAATTCATGTCTTTGAAAAGCATC R: ATGAAGCAACTCAGGGAGCAAAGA 148-1185 bp fragment from *AtTLP2* F: TCTTTGCTCCCTGAGTTGCTTCAT R: CTGCAGTTATTTCAATGAGATTGG Second round PCR: F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTATTTCAATGAGATTGG (*Pst*I)

Table 1. (Continued)

Construction	Preparation of fragments for cloning	
	Vector[a]	Primers of Insert (5' to 3')
BD-AtTLP2^{C83S}	pBD-GAL4	First round PCR: 1-266 bp fragment from *AtTLP2* F: GAATTCATGTCTTTGAAAAGCATC R: ATTCCTCTCCATGATTTAGATACTGAA 240-1185 bp fragment from *AtTLP2* F: TTCAGTATCTAAATCATGGAGAGGAAT R: CTGCAGTTATTTCAATGAGATTGG Second round PCR: F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTATTTCAATGAGATTGG (*Pst*I)
BD-AtTLP2^{C382S}	pBD-GAL4	PCR: 1-1185 bp fragment from *AtTLP2* F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTACCCTTCACATGCCGGTTTGGTGTCAAAGCTGCTAATGGAT (*Pst*I)
BD-AtTLP2$^{P52L-C83S}$	pBD-GAL4	First round PCR: 1-266 bp fragment from *AtTLP2^{P52L}* F: GAATTCATGTCTTTGAAAAGCATC R: ATTCCTCTCCATGATTTAGATACTGAA 240-1185 bp fragment from *AtTLP2^{P52L}* F: TTCAGTATCTAAATCATGGAGAGGAAT R: CTGCAGTTATTTCAATGAGATTGG Second round PCR: F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: CTGCAGTTATTTCAATGAGATTGG (*Pst*I)
BD-AtTLP2:7^{tubby}	pBD-GAL4	First round PCR: 1-330 bp fragment from *AtTLP2* F: GAATTCATGTCTTTGAAAAGCATC R: TCTAGGACCTGGCAATTTCAATGAGATTGGAAA 319-1137 bp fragment from *AtTLP7* F: TTTCCAATCTCATTGAAACAGCCCGGGCCTAGGGAT R: TACTGCAGAACTCGCAGGCAAGTTT Second round PCR: F: GAATTCATGTCTTTGAAAAGCATC (*Eco*RI) R: TACTGCAGAACTCGCAGGCAAGTTT (*Pst*I)
BD-AtTLP5:2^{tubby}	pBD-GAL4	First round PCR: 1-351 bp fragment from *AtTLP5* F: CGGAATTCATGTCGTTTCTGAGTAT R: AGTCTCGAGGCCCCGGCTGTTTCAACGAAACTGGAAAA 331-1185 bp fragment from *AtTLP2* F: TTTCCAGTTTCGTTGAAACAGCCGGGGCCTCGAGACT R: CTGCAGTTATTTCAATGAGATTGG Second round PCR: F: GGAATTCATGTCGTTTCTGAGTAT (*Eco*RI) R: CTGCAGTTATTTCAATGAGATTGG (*Pst*I)
BD-AtTLP7^{L48P}	pBD-GAL4	First round PCR: 1-156 bp fragment from *AtTLP7* F: CTTGAATTCATGCCTTTGTCACGGTCC R: TAATAATTCAGGCGGCATCGCCGACCA 130-1137 bp fragment from *AtTLP7* F: TGGTCGGCGATGCCGCCTGAATTATTA R: TGCTGCAGTCTCGCAGGCAAGTTTAGT

Table 1. (Continued)

Construction	Preparation of fragments for cloning	
	Vector[a]	Primers of Insert (5' to 3')
BD-AtTLP7^{L48P}	pBD-GAL4	Second round PCR: F: CTTGAATTCATGCCTTTGTCACGGTCC (*Eco*RI) R: TGCTGCAGTCTCGCAGGCAAGTTTAGT (*Pst*I)
BD-AtTLP7^{S79C}	pBD-GAL4	First round PCR: 1-248 bp fragment from *AtTLP7* F: CTTGAATTCATGCCTTTGTCACGGTCC R: TCTCCATTTCTTACAAACGCAAGCGCA 223-1137 bp fragment from *AtTLP7* F: TGCGCTTGCGTTTGTAAGAAATGGAGA R: TGCTGCAGTCTCGCAGGCAAGTTTAGT Second round PCR: F: CTTGAATTCATGCCTTTGTCACGGTCC (*Eco*RI) R: TGCTGCAGTCTCGCAGGCAAGTTTAGT (*Pst*I)
BD-AtTLP7$^{L48P-S79C}$	pBD-GAL4	First round PCR: 1-248 bp fragment from *AtTLP7*^{L48P} F: CTTGAATTCATGCCTTTGTCACGGTCC R: TCTCCATTTCTTACAAACGCAAGCGCA 223-1137 bp fragment from *AtTLP7*^{L48P} F: TGCGCTTGCGTTTGTAAGAAATGGAGA R: TGCTGCAGTCTCGCAGGCAAGTTTAGT Second round PCR: F: CTTGAATTCATGCCTTTGTCACGGTCC (*Eco*RI) R: TGCTGCAGTCTCGCAGGCAAGTTTAGT (*Pst*I)
BD-AtTLP7:2^{tubby}	pBD-GAL4	First round PCR: 1-318 bp fragment from *AtTLP7* F: CGGAATTCATGCCTTTGTCACGGTC R: AGTCTCGAGGCCCCGGCTGTTTGAGGCAAGAAGGGAA 331-1185 bp fragment from *AtTLP2* F: TTCCCTTCTTGCCTCAAACAGCCGGGGCCTCGAGACT R: CTGCAGTTATTTCAATGAGATTGG Second round PCR: F: GGAATTCATGCCTTTGTCACGGTC (*Eco*RI) R: CTGCAGTTATTTCAATGAGATTGG (*Pst*I)

[a]The restriction enzymes used to create the vector constructs are showed.

competent cells (Genemark, Taichung, Taiwan). The cloned DNA fragments were to be verified without nucleotide substitutions during PCR by DNA sequencing. The sequenced insert DNA fragments in the pGEM-T easy vectors were subsequently cloned into the target vectors. The target vectors and DNA fragments that had been inserted in pGEM-T Easy vectors were digested by appropriate restriction enzymes (Promega, Madison, WI, USA). The digested insert DNA fragments from pGEM-T easy and the linear vectors were purified by gel extraction, mixed, and then ligated by T_4 DNA ligase (Promega, Madison, WI, USA). The ligated plasmids were transformed into *E. coli* DH5α competent cells (Genemark, Taichung, Taiwan) and verified to be without frame shift by DNA sequencing.

Yeast two-hybrid experiment

The HybriZAP 2.1 two-hybrid System (Stratagene, La Jolla, CA, USA) was used to detect the protein interaction between the bait and the target proteins. The yeast two-hybrid vectors, pAD-*GAL4*-2.1 and pBD-*GAL4* Cam, were used for C-terminal GAL4 AD and BD fusion constructions. The β-galactosidase **(***LacZ***)** and *His3* reporter genes were used to measure the interaction between the bait and the target proteins in yeast. The activation of *LacZ* and *His3* was evaluated to test the viability of yeast cells on medium lacking histidine and by assays of β-galactosidase, respectively. The pBD-GAL4 Cam vector containing DNA encoding the bait protein (bait plasmid) must be transformed into yeast and assayed for

expression of *LacZ* and *His3* reporter genes. If the bait plasmid is capable of inducing expression of the *LacZ* and *His3* reporter genes in the absence of the pAD-GAL4-2.1 vector containing an insert, the bait plasmid is deemed unsuitable in detecting protein-protein interaction in this system. Subsequently, yeast containing the bait plasmid was used to prepare the competent cells and was transformed with the target plasmid(s).

Interaction between the bait and the target protein

The yeast strain YRG-2 [*MAT*a ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1UAS-GAL1TATA-HIS3 URA3::(GAL43 × 17mer)-CYC1TATA-lacZ] was used in this study. For coupled bait and target interaction experiments, yeast containing the bait plasmid was transformed with the target plasmid using the lithium acetate method. The transformed cells were plated on SD medium lacking tryptophan and leucine (SD-Trp-Leu) agar plates and were incubated at 30°C for 2 to 4 days until the colonies appeared. Colonies that grew on the same plate were re-streaked on the SD-Trp-Leu-His plates, and their *LacZ* reporter gene expression was tested using a quantitative β-galactosidase assay. For the analysis of β-galactosidase activity, mid-to-late exponential-phase yeast cells were allowed to grow further and resuspended in a Z buffer containing *o*-nitrophenyl β-D-galactopyranoside (ONPG) (4 mg/ml in Z buffer) as a substrate. The activity unit was calculated using the formula: $[OD_{420} \times 1.7]/[0.0045 \times \text{protein (mg/ml)} \times \text{extract volume (ml)} \times \text{time (h)}]$. Specific activity was expressed as nmoles *o*-nitrophenol/h/ mg yeast protein. All tests were performed at least three times.

Expression of ASK1 and AtTLP2 recombinant protein

To express ASK1-His(6x) or MBP-AtTLP2 fusion protein, the *E. coli* strain BL21 (DE3) (Promega, Madison, WI, USA) harboring the plasmid pET20-ASK1 or pMalp2x-AtTLP2 was used. Recombinant fusion proteins ASK1-His(6x) were expressed in BL21 (DE3) strain after induction by 0.4 mM isopropyl-D-thio-galactopyranoside (IPTG) (Sigma-Aldrich, St.Louis, MO, USA) for 16 h at 22°C and purified on Ni-NTA (Qiagen, Valencia, CA), following the manufacturer's recommendation (P6611; Sigma-Aldrich, St.Louis, MO, USA). Recombinant fusion proteins MBP-AtTLP2 were expressed in BL21 (DE3) strain after induction by 0.4 mM IPTG for 40 h at 15°C, and these were purified from the supernatant of induced whole cell lysate using the amylose resin. They were then eluted by column buffer at a final concentration of 10 mM maltose. To separate the AtTLP2 from MBP, Factor Xa was used to cleave the MBP-AtTLP2. A total of 25 µg of MBP-AtTLP2 was incubated with 0.5 µg of Factor Xa at 23°C for 4 h. The digested mixture was incubated with 50 µl of the amylose resin (50/50 slurry) for 30 min at 4°C on a 360 degree rolling machine. Beads were pelleted down at 3000 rpm for 1 min. The purification procedure was confirmed by Western blotting with anti-AtTLP2 antiserum.

In vitro binding assay

In vitro binding assay was performed using purified AtTLP2 and ASK1 proteins. A total of 50 µl of the Ni^{2+} charged chelating sepharose (50/50 slurry) were washed with 500 µl binding buffer thrice and resuspended in 100 µl of binding buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40). Then 10 µg of the ASK1-His (6x) protein was added to the beads and incubated at 4°C for 1 h on a 360 degrees rolling machine. Beads were pelleted down at 3000 rpm for 1 min and washed with 500 µl of binding buffer twice. The beads were resuspended in 100 µl binding buffer. A total of 10 µg of AtTLP2 protein was added to the beads and incubated at 4°C for 2 h at on a 360 degrees rolling machine. Beads were pelleted down at 3000 rpm for 1 min and washed with 500 µl binding buffer 4 times. The washed beads were pelleted down at 3000 rpm for 2 min and boiled for 10 min in 25 µl of 2x SDS sample dye. To detect the bound AtTLP2, 15 µl of the sample was separated by 12% SDS-PAGE and detected by Western blotting using anti-AtTLP2 antiserum.

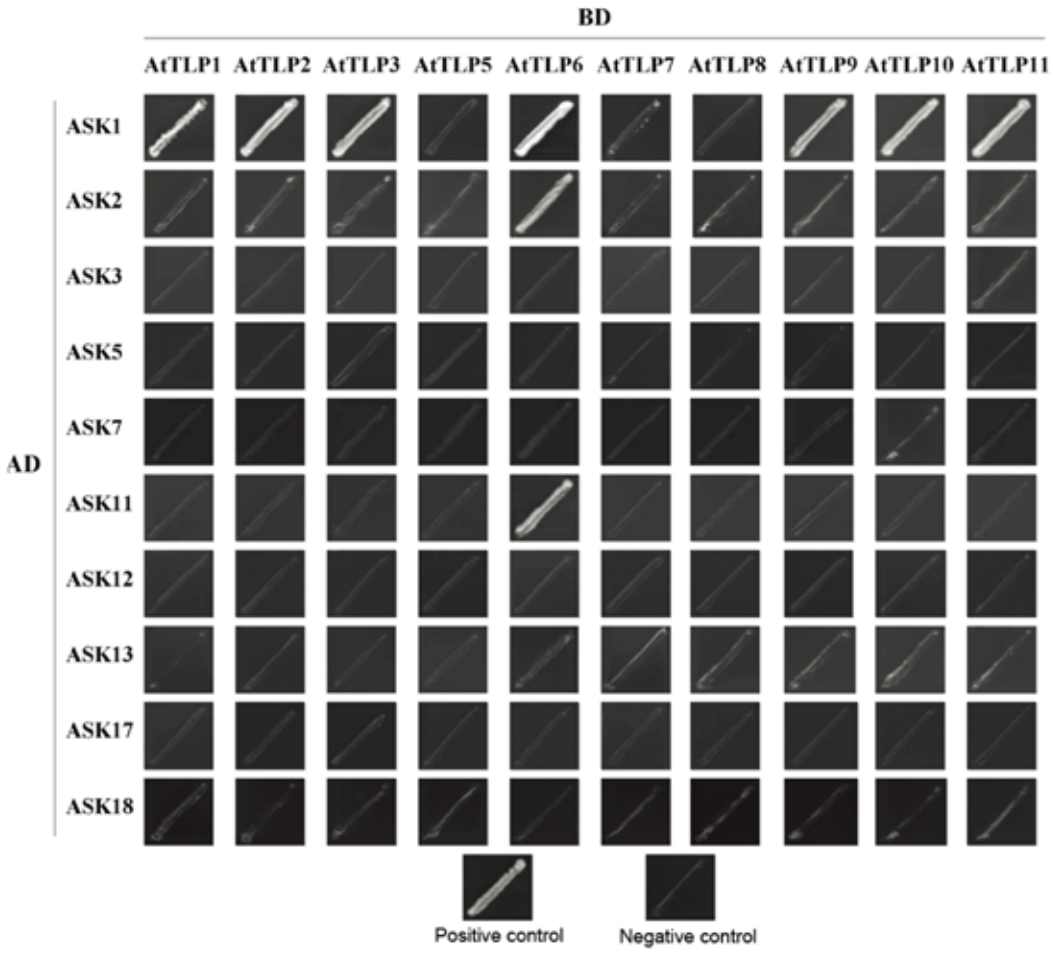
RESULTS

Interaction of AtTLPs with various ASKs

To understand the specificity of the interaction between AtTLPs and various ASKs, 10 expressed AtTLPs (we were not able to clone AtTLP4, which is too low to be detected or is a pseudo gene (Lai et al., 2004) were tested in a pairwise manner against representative ASK proteins using the yeast two-hybrid system for survival on SD-Trp-Leu-His medium and for β-galactosidase activity. Ten representative ASK proteins (ASK1, 2, 3, 5, 7, 11, 12, 13, 17, and 18) were selected according to their amino sequence similarity and gene expression patterns. In all cases, full-length AtTLPs and ASKs were expressed as C-terminal fusion with the GAL4-BD domain and GAL4-AD, respectively. As shown in Figure 1, the activation of *HIS3* and *LacZ* reporter gene indicates that the six AtTLPs (AtTLP1, 2, 3, 9, 10, and 11) were able to specifically interact with ASK1, and that AtTLP6 was able to interact with ASK1, ASK2, and ASK11. No significant interactions with ASKs were observed using AtTLP5, 7 and AtTLP8 as bait in the yeast two-hybrid system (Figure 1). The function of these AtTLPs through interaction with ASK1 is supported because the expression patterns of AtTLP1, 2, 3, 6, 9, 10, 11, and ASK1 transcript overlap (Zhao et al., 2003; Lai et al., 2004). Those AtTLPs can specifically interact with ASK1 and show similar expression patterns in different organs, so it is suggested that they may have an overlapping function. The loss of binding ability by AtTLP8 with all ASKs can be predicted since the F-box domain is absent and the C-terminal sequence of the tubby domain is highly diverse compared with other AtTLPs (Lai et al., 2004).

To further confirm this data, a physical interaction anal-

A



B

AD[a]	BD[b]											
	AtTLP1	AtTLP2	AtTLP3	AtTLP5	AtTLP6	AtTLP7	AtTLP8	AtTLP9	AtTLP10	AtTLP11	P53	Lamin C
ASK1	139.8 ±23.0	682.3 ±71.1	252.1 ±38.3	13.7 ± 1.2*	412.9 ±20.5	45.3 ± 9.8*	8.4 ± 1.8*	131.2 ±51.8	190.0 ±14.3	397.0 ±58.2	–	–
ASK2	10.2 ± 0.5*	15.6 ± 5.2*	30.8 ± 9.0*	9.1 ± 1.9*	133.8 ±30.2	10.8 ± 3.9*	10.2 ± 1.9*	20.1 ± 1.7*	41.9 ± 6.4*	14.2 ± 2.3*	–	–
ASK3	14.2 ± 0.2*	12.1 ± 0.7*	11.7 ± 1.0*	12.0 ± 1.0*	11.4 ± 1.0*	10.3 ± 0.5*	10.7 ± 1.6*	12.5 ± 1.8*	12.1 ± 0.8*	10.0 ± 1.9*	–	–
ASK5	4.2 ± 1.8*	4.1 ± 1.6*	2.4 ± 3.1*	1.1 ± 3.9*	7.7 ± 15.3*	10.3 ± 13.8*	11.7 ± 12.1*	5.0 ± 2.3*	4.4 ± 1.7*	5.4 ± 0.2*	–	–
ASK7	7.8 ± 0.8*	15.1 ± 4.0*	19.3 ± 0.9*	11.8 ± 0.9*	26.3 ± 4.5*	9.0 ± 0.7*	13.8 ± 0.8*	15.7 ± 4.4*	14.7 ± 1.7*	16.8 ± 6.4*	–	–
ASK11	20.1 ± 1.8*	19.4 ± 3.9*	47.4 ± 3.8*	6.5 ± 0.8*	104.3 ±10.3	16.0 ± 2.1*	6.3 ± 1.4*	28.1 ± 4.9*	8.3 ± 0.5*	28.1 ± 6.7*	–	–
ASK12	15.2 ± 1.7*	14.2 ± 1.8*	13.9 ± 0.9*	11.2 ± 1.7*	12.3 ± 2.3*	12.7 ± 0.9*	8.7 ± 1.2*	11.1 ± 3.5*	9.3 ± 0.6*	9.3 ± 1.1*	–	–
ASK13	6.6 ± 0.8*	13.4 ± 4.6*	22.7 ± 3.4*	13.1 ± 1.6*	33.5 ± 5.6*	11.2 ± 0.4*	15.1 ± 0.6*	15.1 ± 0.9*	18.0 ± 3.2*	19.1 ± 2.8*	–	–
ASK17	12.5 ± 1.9*	12.0 ± 0.7*	16.8 ± 2.9*	13.0 ± 2.0*	11.9 ± 0.8*	13.5 ± 1.1*	10.6 ± 0.5*	13.0 ± 1.0*	12.4 ± 1.8*	10.6 ± 1.4*	–	–
ASK18	9.5 ± 0.7*	7.7 ± 1.1*	5.6 ± 0.4*	4.1 ± 0.9*	14.0 ± 1.3*	4.5 ± 1.6*	4.0 ± 1.0*	5.5 ± 0.7*	4.7 ± 0.9*	5.7 ± 0.3*	–	–
SV40	–	–	–	–	–	–	–	–	–	–	230.3±27.3	18.6± 1.1

[a]Full length *Arabidopsis* ASKs cDNA were constructed with activating domain respectively.
[b]Full length *Arabidopsis* TLPs cDNA were constructed with binding domain respectively.
*indicates significant difference in AtTLP-ASK relative to p53-SV40 (Student's t-test; *P* value < 0.05).

Figure 1. Interaction of AtTLPs with various ASKs using yeast two-hybrid. p53-SV40 (SV40 T-antigen) and LaminC-SV40 represent known positive and negative control protein partners, respectively. A, Interaction of AtTLPs and ASKs with yeast two-hybrid histidine auxotrophic growth; B, Interaction of AtTLPs and ASKs detected by the yeast-two hybrid method using LacZ reporter. The strength of the interaction pairs were estimated by β-galactosidase activity. β-galactoside activity (nmol *o*-nitrophenol/h/mg yeast protein) was quantified with ONPG. Values represent the means ± SD of assays from at least three independent transformants. The *P* value for the Student's t-test was calculated. Transformants judged to non-interaction exhibited a significant decrease (*P* value <0.05) in β-galactosidase activity in comparison to p53-SV40 protein partners.

ysis between AtTLP2 and ASK1 was performed *in vitro* using affinity tag purified AtTLP2 and ASK1 recombinant proteins from *E. coli*. ASK1-His (6x) protein bound to the Ni^{2+}-resin was incubated with MBP-AtTLP2. Ni^{2+}-resin alone was used as a control. After washing, the samples were separated using SDS-PAGE, and the presence of bound AtTLP2 was detected by Western blotting with anti-AtTLP2 antiserum. As shown in Figure 2, the AtTLP2 protein showed binding to the ASK1-His (6x); no binding took place with Ni^{2+}-resin. This result indicates that AtTLP2 is able to physically interact with ASK1 *in vitro*, which confirms the result of yeast two-hybrid analysis.

Identification of the ASK1 interacting region in AtTLP2

The strong interaction between AtTLP2 and ASK1 was used to determine the region responsible for the interaction. A series of amino- and carboxyl- terminal deletions of AtTLP2 protein was generated to test their interactions with ASK1 using the yeast two-hybrid system for survival on SD-Trp-Leu-His medium and for β-galactosidase activity. As shown in Figure 3, the AtTLP2$^{44\text{-}394}$ transformant still retained 56% of its interacting activity with ASK1, suggesting that the sequences preceding the F-box domain (residues 1-43) are not essential for AtTLP2-ASK1 association. The failure of an F-box-deleted AtTLP2 protein (residues 111-394) and ASK1 to bind confirmed that the F-box domain is required in the interaction with ASK1. All interactions between the C-terminal deleted version of the AtTLP2 protein and ASK1 were completely abolished in spite of the F-box domain in these proteins being intact. Based on our study's results, the F-box domain of AtTLP is necessary but not sufficient for a high-affinity interaction with ASK1, and that the intact AtTLP C-terminal tubby domain is also essential for ASK1 binding (Figure 3). Previous reports showed that most F-box proteins using the F-box domain interact with the ASK protein. However,

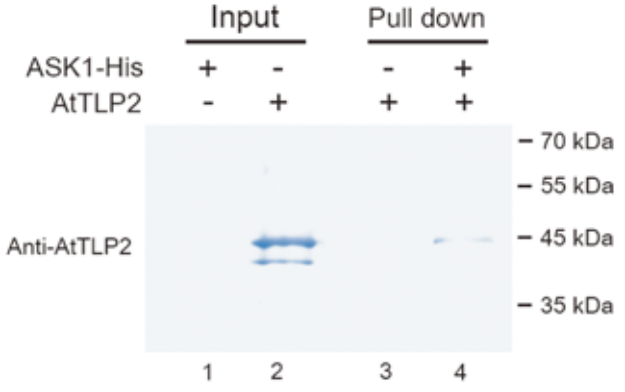


Figure 2. Interaction between AtTLP2 and ASK1 *in vitro.* AtTLP2 incubated with Ni^{2+}-resin (lane 3) or with Ni^{2+}-resin bound ASK1-His(6x) (lane 4) and 5% aliquots of the input proteins (lane1 and 2) were separated by 12% SDS-PAGE and detected by Western blotting with anti-AtTLP2 antiserum. The theoretical molecular weight of AtTLP2 is 43.88 kDa.

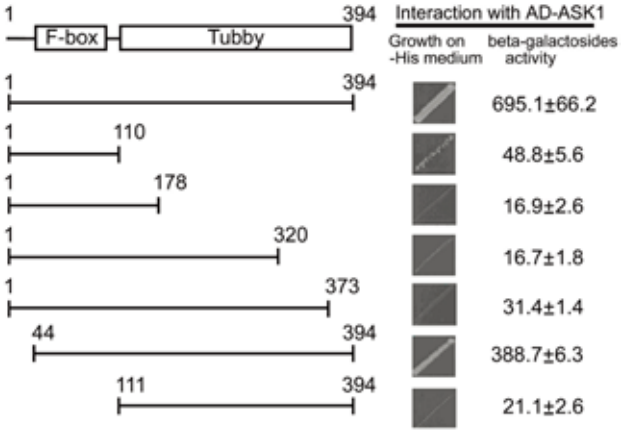


Figure 3. Identification of ASK1 binding region in AtTLP2 using the yeast two-hybrid system. Interaction of various truncations of AtTLP2 and ASK1 were evaluated by testing the viability of yeast cells on SD-Trp-Leu-His medium and by assays of β-galactosidase activity. The various segments of AtTLP2 used in the assay are shown schematically with their amino acid positions. β-galactoside activity (nmol *o*-nitrophenol/h/mg yeast protein) was quantified with ONPG. Values represent the means ± SD from three independent transformants. Numbers on the horizontal bar represent positions of amino acid residues of AtTLP2. The expressions of all constructs in yeast were verified by western blots with anti-BD monoclonal antibody (Santa Cruz, CA, USA) (data not shown).

it has been reported that the C-terminal region of TIR1 containing LRR repeats is known to be involved in ASK1-TIR1 interaction (Gray et al., 2001). It is demonstrated here for the first time that the tubby domain in AtTLP is necessary for ASK1-AtTLP interaction.

Domain swap analysis of AtTLP5 and AtTLP7

The inability of AtTLP5 and AtTLP7 to interact with ASK1 is an interesting observation because both the F-box and tubby domain in AtTLP5 and AtTLP7 were conserved when compared to other AtTLPs. The overall structures of AtTLP5 and AtTLP7 were conserved, so it is likely that the disruption of the interaction with ASK1 might have resulted from variations of some residues among these two proteins. Serial deletion analyses of AtTLP2 indicated that both F-box and tubby domain are important for the interaction with ASK1 (Figure 4), so domain swap analysis was further investigated to pinpoint the domain variations, which might lead to the loss of function of AtTLP5 and AtTLP7.

The N-terminal and C-terminal regions of AtTLP5 and AtTLP7 were exchanged with the corresponding region from AtTLP2, creating domain swap constructs. As shown in Figure 4, four chimeric proteins, AtTLP2:5^{tubby}, AtTLP2:7^{tubby}, AtTLP5:2^{tubby}, and AtTLP7:2^{tubby}, were created by exchanging their amino- and carboxyl- regions via a conserved nine-residue linker between the F-box and the tubby domain. In the AtTLP2:5^{tubby} and AtTLP2:7^{tubby} chi-

Domain swap construsts			Interaction with ASK1	
Names	**N-terminus**	**C-terminus**	**Growth on -His medium**	**β-galactosidase activity**
AtTLP2:5^{tubby}	AtTLP2 (1-110)	AtTLP5 (118-429)	−	18.2 ± 2.1
AtTLP2:7^{tubby}	AtTLP2 (1-110)	AtTLP7 (107-379)	+	279.6 ± 9.4
AtTLP5:2^{tubby}	AtTLP5 (1-117)	AtTLP2 (111-394)	+	520.3 ± 42.7
AtTLP7:2^{tubby}	AtTLP7 (1-106)	AtTLP2 (111-394)	−	20.9 ± 1.4

Figure 4. Interaction of different AtTLP domains swap constructs with ASK1 using the yeast two-hybrid system. Interaction of various domain swap constructs of AtTLPs and ASK1 were evaluated by testing the viability of yeast cells on SD-Trp-Leu-His medium and by assays of β-galactosidase activity. Numbers in parentheses represent positions of amino acid residues of AtTLPs used to create the chimeric proteins. β-galactosides activity (nmol *o*-nitrophenol/h/mg yeast protein) was quantified with ONPG. Values represent the means ± SD from three independent transformants. Plus and minus signs indicate positive interactions and negative interactions, respectively.

mera, the tubby domain in AtTLP2 is exchanged with that of AtTLP5 and AtTLP7, respectively. In the AtTLP5:2^{tubby} and AtTLP7:2^{tubby} chimera, the tubby domains in AtTLP5 and ATLP7 were exchanged with that of AtTLP2. These proteins were expressed as C-terminal fusion with GAL4-BD. The interactions between these chimeric proteins and ASK1 were tested using the yeast two-hybrid system. As shown in Figure 4, the activation of *HIS3* and *LacZ* reporter gene indicates that both AtTLP5:2^{tubby} and AtTLP2:7^{tubby} are capable of interacting with ASK1, while AtTLP2:5^{tubby} and AtTLP7:2^{tubby} are not. These data reveal that the inability of AtTLP5 and AtTLP7 to interact with ASK1 results from variations at the F-box domain of AtTLP7 and the tubby domain of AtTLP5, respectively.

Crystallographic study has indicated that the residues in the F-box domain are critical for human SKP2–SKP1 association (Schulman et al., 2000). In comparison with the amino acid sequences of the F-box domain in AtTLPs and human SKP2, some of these residues are conserved in AtTLPs (Lai et al., 2004). Among these conserved amino acids are the P113 and C136 of SKP2, which are crucial for the binding to SKP1. The amino acids of AtTLP1, 2, 3, 5, 6, 7, 9, 10, and 11 corresponding to P113 in SKP2 were P61, P52, P56, P59, P48, L48, P37, P63, and P34, respectively. In AtTLP1, 2, 3, 5, 6, 7, 9, 10, and 11, the amino acids corresponding to C136 in SKP2 were C92, C83, C87, C90, C79, S79, C69, C94, and C66, respectively. The unique change of P48L and/or C79S in AtTLP7 may account for its inability to interact with ASK1.

To elucidate whether the variation is critical to the interaction with ASK1, point mutations changing the corresponding Pro52 of AtTLP2 to Leu and/or Cys83 of AtTLP2 to Ser were performed. As shown in Figure 5A, despite the fact that the pBD-AtTLP2^{P52L}/pAD-ASK1 transformant is able to grow on the SD-Trp-Leu-His medium, the β-galactosidase activity of this transformant was reduced to one-fifth compared to pBD-AtTLP2/pAD-ASK1. The pBD-AtTLP2^{C83S}/pAD-ASK1 transformant lost the ability to interact with ASK1. Double mutants changing Pro52 of AtTLP2 to Leu and Cys83 of AtTLP2

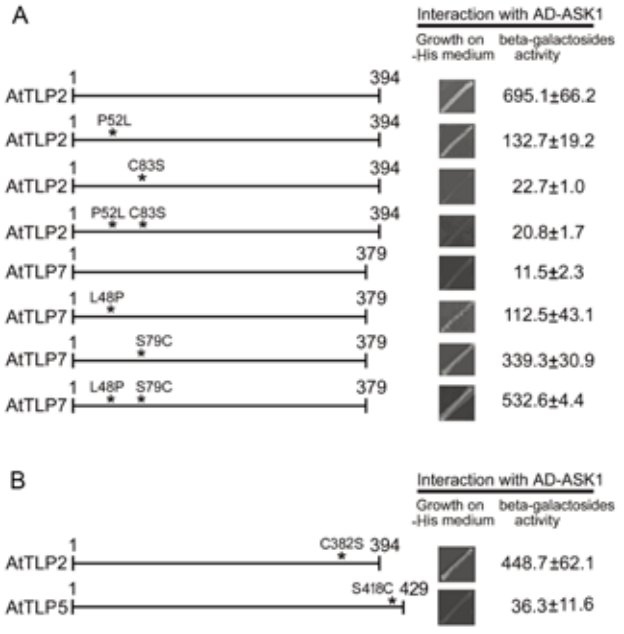


Figure 5. The identification of putative interacting residues of tASK1 in AtTLP. A, The point mutations in F-box domain of AtTLP. Interaction of AtTLP2^{P52L}, AtTLP2^{C83S}, AtTLP2$^{P52L-C83S}$, AtTLP7^{L48P}, AtTLP7^{S79C}, and AtTLP7$^{L48P-S79C}$ with ASK1 were tested by yeast two-hybrid; B, The point mutations in tubby domain of AtTLP. Interaction of AtTLP2^{C382S} and AtTLP5^{S418C} with ASK1 were evaluated by testing the viability of yeast cells on SD-Trp-Leu-His medium and by assays of β-galactosidase activity. β-galactoside activity (nmol *o*-nitrophenol/h/mg yeast protein) was quantified with ONPG. Values represent the means ± SD from three independent transformants.

to Ser also completely lost the ability to interact with ASK1. This result clearly indicated that proline (Pro, P) within the F-box domain strengthens AtTLP2-ASK1 interaction while cysteine (Cys, C) is essential for the AtTLP2-ASK1 interaction. Furthermore, amino acid substitution for these two amino acid residues through site-specific mutagenesis was employed in AtTLP7. When the Leucine

(Leu, L) residue at position 48 was replaced by a Proline, and/or a Serine (Ser, S) residue at position 79 was replaced by a Cycteine in AtTLP7$^{L48P-S79C}$, the ability to interact with ASK1 was restored. Therefore, we suggest that the Pro to Leu and Cys to Ser variations in AtTLP7 are responsible for the disruption of its interaction with ASK1.

In mouse TUBBY, the C-terminal tubby domain forms a 12-stranded β-barrel conformation traversed by a hydrophobic α-helix (α-helix 12). This α-helix 12 is important for the structural integrity of the tubby domain (Boggon et al., 1999). When analyzing this domain in detail, the amino acids corresponding to the α-helix 12 are highly conserved among AtTLP1, 2, 3, 5, 6, 7, 9, 10 and 11, but one amino acid change occurs in AtTLP5. The unique change of C418S in AtTLP5 might account for its inability to interact with ASK1. As shown in Figure 5B, despite the fact that the pBD-AtTLP2^{C382S}/pAD-ASK1 transformant is able to grow on the SD-Trp-Leu-His medium, the β-galactosidase activity of this transformant was reduced to about 65% compared to pBD-AtTLP2/pAD-ASK1. This suggested that this Cys382 to Ser variation within the tubby domain of AtTLP5 accounted for the weakening of its interaction with ASK1. However, when the Serine residue at position 418 was replaced by a Cysteine in AtTLP5^{S418C}, the ability to interact with ASK1 did not recover. We suggest that, not only is α-helix 12 behind the disruption of its interaction with ASK1, but that other regions in the tubby domain of AtTLP5 are also responsible.

DISCUSSION

In *Arabidopsis*, the TUBBY-like protein gene family has been identified as a new type of F-box protein (Xiao and Jang, 2000; Xi et al., 2007). This combination of an F-box and a tubby domain in one protein is specific to this plant species. Previous analyses revealed the importance of this gene family in plant phytohormone ABA signaling (Lai et al., 2004), but the function and the molecular mechanism of AtTLP are still unclear. In this study, molecular analyses of various AtTLP interactions with ten representative ASKs by a yeast two-hybrid system revealed AtTLP1, 2, 3, 9, 10 and 11 were able to specifically interact with ASK1 while AtTLP6 could interact with ASK1, 2 and 11 among the ASK proteins tested (Figure 1). The function of these AtTLPs through interacting with ASK1 is supported since the expression patterns of the AtTLP1, 2, 3, 6, 9, 10, 11 and the ASK1 transcript overlap (Zhao et al., 2003; Lai et al., 2004). Since those AtTLPs can specifically interact with ASK1 and show similar expression patterns in different organs, they may have an overlapping function.

To date, near 700 F-box proteins have been identified in the *Arabidopsis* genome (Gagne et al., 2002; Risseeuw et al., 2003), and some of them have been characterized as components of the SCF complexes involved in plant growth and development (Vierstra, 2003; Smalle and Vierstra, 2004). It has been shown that F-box proteins and SKP1 may function in protein complexes other than the SCF. The F-box protein centromere transmission fidelity 13 (Ctf13) is involved in forming an SKP1-based non-SCF protein complex called centromere binding factor 3 (CBF3), which is essential for the spindle check point pathway in *Saccharomyces cerevisiae* (Russell et al., 1999; Kitagawa et al., 2003). Studies in *C. elegans* have also indicated that some SKP1 homologs do not interact with Cullins (Clifford et al., 2000). Whether AtTLPs which can interact with ASK are able to form the SCF complex *in vivo* needs to be elucidated.

No significant interactions with ASKs were observed using AtTLP5, 7 and AtTLP8 as bait in the yeast two-hybrid system (Figure 1). The loss of binding ability by AtTLP8 with all ASKs can be predicted since its F-box and tubby domains are truncated. According our results, the F-box domain of AtTLP is necessary but not sufficient for a high-affinity interaction with ASK1, and the intact AtTLP C-terminal tubby domain is also essential for ASK1 binding (Figure 3). Previous reports showed that most F-box proteins using the F-box domain interact with ASK protein. However, it has been reported, the C-terminal region of TIR1 that contains LRR repeats is known to be involved in ASK1-TIR1 interaction (Gray et al., 2001). It is demonstrated here for the first time that the tubby domain in AtTLP is necessary for ASK1-AtTLP interaction.

Although AtTLP5 and 7 contained both F-box domain and tubby domain, it seems that some of the AtTLP residues important for interaction with the ASK1 protein have changed from the variations at the N-terminus of AtTLP7 and the C-terminus of AtTLP5 (Figure 4). We further demonstrated that naturally occurring proline-to-leucine and cysteine-to-serine changes in the F-box domain might result in the dysfunction of AtTLP7's interaction with ASK1 (Figure 5). However, it is difficult to predict how the amino acids in the tubby domain of AtTLP5 critically effect the interaction with ASK1. Amino acid substitution for five amino acid residues through site-specific mutagenesis was employed in the tubby domain of AtTLP5 (AtTLP5$^{T323N-L329H-S355A-H392Q-S418C}$), but it still did not recover the ability to interact with ASK1 (data not shown). The residues involved in the interaction with ASK1 at C-terminus of AtTLP5 have not been proven yet. Crystallographic studies are needed for further idenfication of the residues critical for AtTLP-ASK protein interaction.

Judging from these observations, AtTLP5, 7 and 8 can be assumed to have unique functions other than protein ubiquitination through the SCF complex. The gene expression of *AtTLP5*, *7* and *8* are usually more limited in expression, suggesting that they may have lost their original function(s) and/or acquired new function(s) during evolution.

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阿拉伯芥 TULP 和 ASK 蛋白質之交互作用分析

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本研究利用酵母菌雙雜合系統 (yeast two-hybrid system) 研究 10 個阿拉伯芥類肥胖蛋白質 (AtTLPs) 和不同的阿拉伯芥 ASKs 間的交互作用情形，結果顯示 6 個 ATTLPs (AtTLP1、2,、3、9、10 及 11) 能夠專一性的和 ASK1 作用；AtTLP6 能和 ASK1、ASK2 及 ASK11 交互作用，而 AtTLP5、AtTLP7 及 AtTLP8 則和任何的 ASK 蛋白質無交互作用之情形；利用 AtTLP2 進行系列剃除 (Serial deletion) 分析，結果顯示 F-box domain 及 tubby domain 均為 AtTLP2 和 ASK1 交互作用所需，應用功能性區塊互換 (domain swapping) 的技術進行分析後，我們推論 AtTLP7 的 N 端 F-box domain 上及 AtTLP5 的 C 端 tubby domain 上的胺基酸自然變異，可能導致 AtTLP7 及 AtTLP5 無法和 ASK1 交互作用 ; 進一步應用定點突變 (site-directed mutagenesis) 之技術，我們進一步驗證 AtTLP7 之 F-box domain 上脯胺酸 (proline) 突變成白胺酸 (leucine) 及半胱胺酸 (cysteine) 突變成絲胺酸 (serine) 的自然變異，可能為 AtTLP7 無法和 ASK1 交互作用的原因。綜合這些發現，我們推論可以專一性和 ASK1 作用之 AtTLP1、2、3、9、10 和 11 可能具有重疊之功能，而 AtTLP5、7 及 AtTLP8 不能和任何的 ASK 作用，推論可能在演化過程喪失原始功能和 / 或獲得新的功能。

關鍵詞：類肥胖基因；F-box 蛋白質；阿拉伯芥；酵母菌雙雜合系統；蛋白質 - 蛋白質交互作用；ASK。