

Proteomic identification of potential target proteins regulated by an ASK1-mediated proteolysis pathway

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The ASK1 (ARABIDOPSIS SKP1-LIKE) protein is a critical component of the SCF (Skp1-Cullin-F box protein) ubiquitin ligase complexes that recruit target proteins for degradation by the 26S proteasome. To investigate proteins that are affected by the ASK1-mediated proteolysis pathway in *Arabidopsis* flowers, we compared the proteomes of the *Arabidopsis* wild type and *ask1* mutant flower buds using two-dimensional electrophoresis (2-DE). Ten protein spots with higher or lower abundance in the *ask1* mutant flowers compared to wild type flowers were excised and subjected to further mass spectrometry (MS) analysis. The results showed that they were proteins involved in photomorphogenesis, circadian oscillation, post-translation process, stress-responses and cell expansion or elongation, suggesting that those processes were affected in the *ask1* mutant. The transcript levels of these genes were also compared based on the Affymetrix gene chip microarray data. No significant difference was observed for most of the genes, suggesting that the proteins with elevated levels of accumulation in the *ask1* mutant could be candidate targets regulated by an ASK1-mediated proteolysis pathway. These results help to elucidate the pleiotropic functions of ASK1 in *Arabidopsis* developmental processes and also demonstrate the importance and necessity of studying protein levels with respect to gene functions.

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Introduction

Floral development is one of the most complicated developmental processes in plants that involve a series of genes interacting with each other to regulate the entire process. One of the more recently identified regulatory genes of flower development is *ASK1* (*Arabidopsis SKP1-like 1*), which is required for normal floral organ development and homeotic gene expression [1-3]. SKP1 (S-phase kinase-

associated protein) was originally identified in human and yeast as an essential component of the ubiquitin-ligase complex called SCF (SKP1, Cullin/Cdc53p and F-box containing protein) [4-6]. The SCF complex targets several specific proteins in mitotic cell cycle for proteolysis [6, 7]. In the *Arabidopsis* genome, 21 *ASK* genes are found while the functions of most of them remain unknown [8, 9].

The first mutant in an *ASK* gene, *ask1-1*, was isolated following transposon insertional mutagenesis, allowing genetic analysis of *ASK1* function [1]. This mutant is defective in both vegetative and reproductive development, including smaller stature than normal, reduced number and size of petals and stamens, and reduced stamen filament length [1, 2]. Further microscopic examination revealed that it is defective in male meiosis, exhibiting an uneven distribution of chromosomes and a failure in separation of some homologous chromosomes during male anaphase I, thus

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resulting in male-sterility. ASK1 may regulate homologous chromosome separation by promoting the degradation or removal of a protein that is required for the association of two homologous chromosomes [1].

In addition to a role in meiosis, ASK1 was also shown to interact with UFO (UNUSAL FLORAL ORGANS) protein to regulate B function gene expression during the flower morphogenesis process [3, 10]. Moreover, ASK1 was reported to interact with several other F-box proteins to regulate plant growth and development, including TIR1 in auxin response [11, 12], COI1 in jasmonic acid-regulated defense response [13, 14], EID1 and COP9 in light signaling [15, 16], and ZTL and FKF1 in the control of the circadian clock [17, 18]. *ASK1* is strongly and widely expressed in plants [8], implying an important function in various plant tissues. In particular, *ASK1* is important for embryo and seedling development [19].

Many functionally important proteins in eukaryotes are short-lived and should be rapidly removed when not needed. One way to attain this goal is degradation through the ubiquitin-dependent proteolysis pathway [9]. Ubiquitin is a 76-residue protein that can be covalently attached to target proteins through an enzymatic conjugation cascade involving three enzymes: the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) [7, 10, 14]. E1 and E2 are non-specific, whereas E3 confers substrate specificity and thus attracts more attention [20]. In *Arabidopsis*, the ASK1 protein is a critical component of SCF E3 complexes that also contains Cullin (CDC53 in yeast), Rbx1/Hrt1/Roc1 and one of F-box containing proteins [5, 6, 7]. Through the catalysis by E1, E2 and E3, target proteins are polyubiquitinated and rapidly presented to the 26S proteasome for degradation. In both auxin and GA responses, degradation of transcriptional repressors of hormone-inducible genes is mediated by SCF complexes containing specific F-box proteins [12, 21].

In recent years, ubiquitin-dependent proteolysis has been shown to be important in the regulation of various cellular processes, including cell cycle progression, signal transduction, transcription, DNA repairing, and protein quality control [6-11]. Although multiple F-box proteins interacting with ASK1 have been reported, little is known about its down-stream substrates that are recruited by the SCF E3 complexes. To investigate the potential protein targets degraded by the ASK1-mediated proteolysis pathway and also the roles of ASK1 in the *Arabidopsis* floral development, we compared the proteomes between *Arabidopsis* wild type and the *ask1* mutant flower buds using the tool of two-dimensional electrophoresis. More than 3000 protein spots were resolved in the 2-DE gels. 10 protein spots showing abundance difference between the wild type and the *ask1* mutant were isolated for mass spectrometry analysis.

Seven of them had much higher levels in the *ask1* mutant than in wild type. Their transcript levels in wild type and in the *ask1* mutant were also compared based on the gene chip microarray data. For most of the proteins identified in our experiments, there is no significant transcript difference between the wild type and the *ask1* mutant, which is consistent with the idea that the levels of these proteins are regulated in the post-transcription manner and they might be target proteins degraded by an ASK1 mediated ubiquitin-dependent proteolysis pathway. These results contributed to the understanding of the pleiotropic functions of the *ASK1* gene in reproductive development, and also illustrate the importance of determining protein levels when studying gene functions.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis* wild type and the *ask1* mutant plants used in this study are of the Landsberg *erecta* (*Ler*) ecotype. All plants were grown at 23 °C under a long-day condition (16 h light and 8 h dark). The closed flower buds up to stage 11 to 12 were harvested and stored at -70 °C until use. Proteomes from at least three independently grown populations of wild type and the *ask1* mutant were compared.

Protein preparation and quantification

20 g flower buds were ground in a mortar with liquid nitrogen, and then suspended in a buffer (10% w/v trichloroacetic acid in acetone and 0.07% v/v 2-mercaptoethanol). After incubation at -20 °C for 45min, the samples were centrifuged at the 35 000 g for 15 min. The supernatant was discarded and the pellet was washed in cold (-20 °C) acetone containing 0.07% v/v 2-mercaptoethanol and 1mM Phenylmethylsulfonyl Fluoride (PMSF, Sigma, St.Louis, USA), and freeze-dried. 20 mg protein powders were resuspended in 400 µl sample solution containing 6 M urea, 2 M thiourea (Sigma, St. Louis, USA), 2%w/v CHAPS (Amersham Pharmacia Biotech, Buckinghamshire, England), 2%w/v sulfobetaine 10 (SB3-10, Sigma, St. Louis, USA), 65 mM dithiothreitol (DTT, Amersham Pharmacia Biotech, Buckinghamshire, England), 0.001% bromophenol blue (Sigma, St.Louis, USA), and 0.2% pH 3-10 carrier Biolyte ampholytes (Bio-Rad, Hercules, USA). Occasional stirring is necessary to accelerate the resuspension. After centrifugation to remove the insoluble debris, protein concentration was estimated by the method of Bradford [22].

Two-dimensional gel electrophoresis

All experimental procedures were carried out as recommended by the manufacturer (Bio-Rad, Hercules, USA) with a few modifications. Equal amounts of wild type and *ask1* flower proteins were applied to immobilized pH gradient (IPG) strips (Bio-Rad, 17cm, linear pH 5-8, and linear pH 3-10). The IPG strips were passively rehydrated with the protein samples overnight, then focused at 10 000 V for a total of 60 000 Vh at 18 °C. Following a two-step equilibration (15 min incubation in DTT buffer, and another 15 min in iodoacetamide buffer), each IPG strip was positioned on a 12.5% polyacrylamide gel. A sample of protein markers (Invitrogen Benchmark™, New

York, USA) was loaded besides the IPG strip. Low-melting agarose gel with bromophenol blue was overlaid on the IPG strip to embed the strip. The electrophoresis was run at 70 mA until the blue dye reached the bottom of the gel. Gels were fixed and stained in Coomassie Brilliant R-250 solution (50% v/v ethanol, 5% v/v acetic acid, and 0.2% w/v R-250) overnight, then destained in a solution containing 7% v/v ethanol and 7% v/v acetic acid until the spots on the gels can be clearly visualized. The gels were scanned with a laser scanner (Molecular Image FX, Bio-Rad, Hercules, USA).

Image analysis

Digitalized gel images were analyzed with PDQuest software (Bio-Rad, Hercules, USA). Spot features of the images were automatically detected with the same setting parameters. The image of the gel with the wild type sample was selected as the template for the master gel. Each image was divided into six sections for the convenience of analysis. By comparison with the molecular weights of the protein markers on the side of the gels and known Swiss-prot 2-DE protein spots on our gels, the *pI* and *Mw* of each excised proteins were estimated. By repeatedly comparing several batches of parallel gels, those protein spots with at least 2-fold difference in abundance calculated by the computer software and also confirmed by naked eyes were selected for further mass spectrometry analysis.

Protein identification by ESI-Q-TOF

Protein spots were excised and then subjected to alkylation and tryptic digestion. The gel pieces containing proteins were destained three times with a solution containing 25 mM NH_4HCO_3 in 50% acetonitrile, then dried and re-hydrated with a reducing solution (10 mM DTT in 100mM NH_4HCO_3) for 1 h at 56 °C. After cooling to room temperature, the reduced proteins were alkylated in 100 mM NH_4HCO_3 with 0.27 mM iodoacetamide for 45 min. The gel

pieces were washed with 100 mM NH_4HCO_3 and dehydrated with acetonitrile, then re-hydrated in a digestion solution containing 50 mM NH_4HCO_3 and 10 ng/ μl of trypsin (Promega, Madison, USA) at 37 °C overnight. After the trypsin digestion, the gel pieces were further extracted once with 20 mM NH_4HCO_3 , twice with 5% formic acid in 50% acetonitrile. The combined solution were dried and dissolved in 10 μl of 2% acetonitrile, 0.1% formic acid. Tryptic-digested peptide masses were measured using an ESI-Q-TOF tandem mass spectrometer. The peptide masses were used to search against the Mascot database (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF) and a protein score of more than 50 was considered as a positive identification.

Microarray experiments

The microarray experiments were performed as described before [23]. Briefly, RNA from young inflorescence (stage 1-9) was isolated from three independent *ask1* inflorescence samples and two wild type inflorescence samples. 15-20 μg of fragmented cRNA was used to hybridize the *Arabidopsis* 25 K GeneChip array (Affymetrix, Santa Clara, USA). The scan data were converted to expression levels using the R-Affy package in Bioconductor (version 1.3.25, Irizarry, Gautier, and Bolstad.) The probe-level data were normalized using RNA normalization across arrays together with arrays from the wild type inflorescence and stage 12 flower produced in the lab before [23].

Results and discussion

To identify proteins that are potentially targeted for degradation by ASK1-containing SCF complexes, protein samples from the wild type and the *ask1* mutant flowers were prepared and separated by 2-DE electrophoresis in

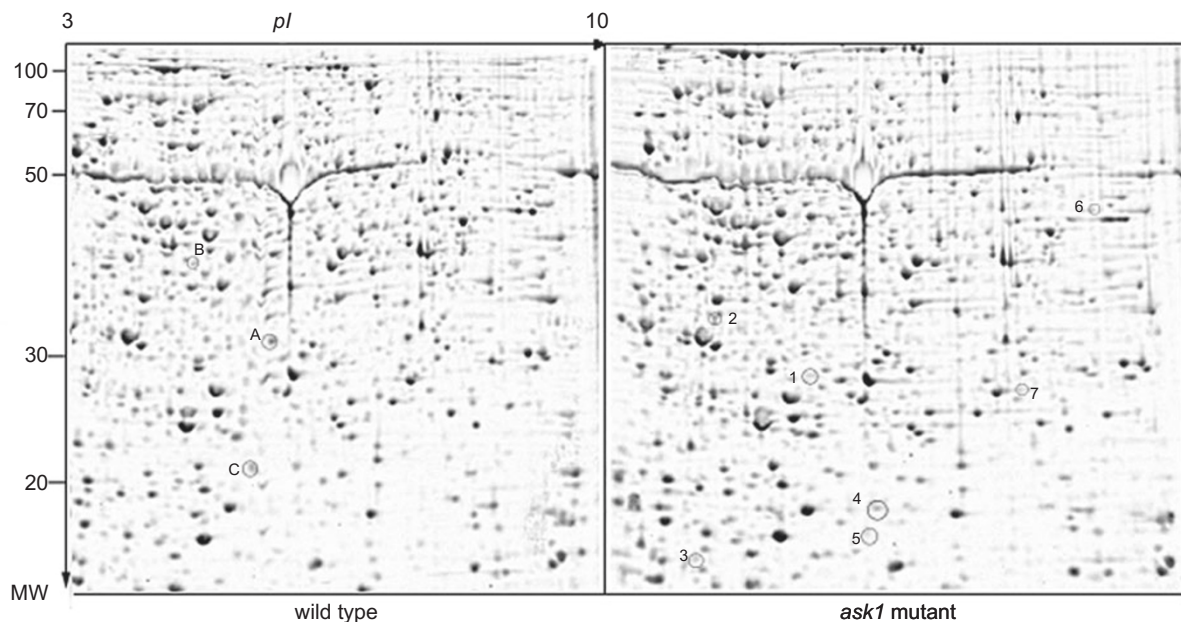


Figure 1 The overview pictures of the 2-D gels obtained for *Arabidopsis* wild type and *ask1* flowers.

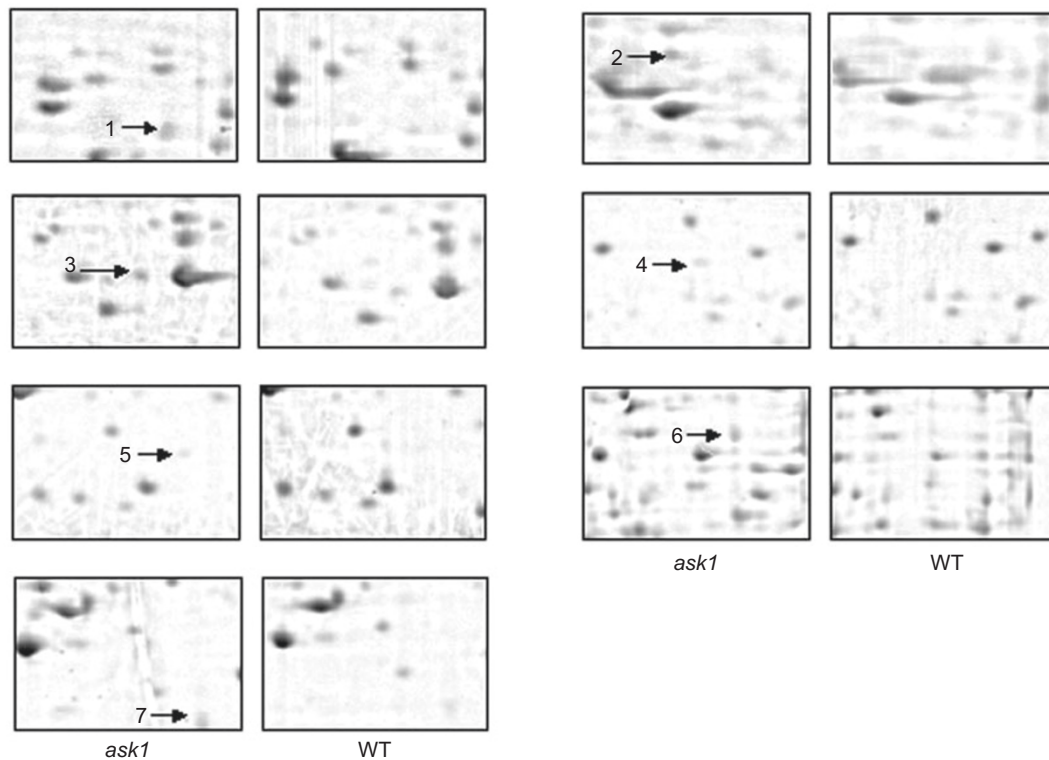


Figure 2 Proteins with higher abundance in the *ask1* mutant flowers compared to the wild type flowers. The enlarged images are spot images after digitalization and spot matching. The proteins with different abundance are marked with arrows and numbered. 1, heme oxygenase 1; 2, aldose 1-epimerase family protein; 3, oxygen-evolving complex-related; 4, 17.8 kDa class I heat shock protein; 5, glycine-rich RNA-binding protein (GRP7), BetVI allergen family protein, nascent polypeptide-associated protein; 6, aspartyl protease family protein and 7, expressed protein.

parallel. We used the broad range IPG strips of pH3-10 first to obtain the overview pictures of the wild type and *ask1* mutant proteomes, and then use pH5-8 IPG strips to ascertain the differences of some protein spots (data not shown). Digital image analysis was performed using the same setting parameters for each gel. The enlarged overview pictures of wild type and the *ask1* mutant floral proteomes are shown side by side in Figure 1. The two gels were first auto-matched with the same parameters and then divided into six sections for editing convenience. By repeatedly matching among batches of wild type and the *ask1* mutant gels, those differently expressed protein spots with high reproducibility were sorted and numbered.

Proteins that accumulated at a higher level in the ask1 mutant flowers compared to the wild type

The goal of the present study is to investigate the potential protein targets degraded by the ASK1-mediated proteolysis pathway. The working hypothesis is that, when *ASK1* is defective, the protein targets may not be degraded by the 26S proteasome and thus remain in the protein extract. By

using 2-D gel electrophoresis, we may be able to identify some of those proteins. Based on this hypothesis, the target proteins should exhibit higher abundance in the *ask1* mutant flowers than in the wild type flowers. Comparison of the proteomes of wild type and the *ask1* flowers revealed that seven protein spots (Figure 2) were always more abundant in the *ask1* mutant than in the wild type for different batches of protein samples. They are numbered from 1 to 7. Following further analysis using peptide digestions and MS by ESI-Q-TOF, the protein spots were identified as follows: 1, heme oxygenase 1; 2, aldose 1-epimerase family protein; 3, oxygen-evolving complex-related; 4, 17.8 kDa class I heat shock protein; 5, glycine-rich RNA-binding protein (GRP7), BetVI allergen family protein, and nascent polypeptide-associated protein; 6, aspartyl protease family protein; and 7, expressed protein with unknown function. The protein features were summarized in Table 1. One of them (spot 3) exhibits experimental molecular mass smaller than the theoretically predicted. It might represent the proteolyzed fragments of the precursor form. For spot 5, three proteins were detected, suggesting that the level(s) of one,

Table 1 Proteins with higher abundance in the *ask1* flowers compared to wild type flowers

Spot No.	Gene ID	Protein identification (synonym)	Predicted molecular mass (kDa)/ <i>pI</i>	Apparent molecular mass (kDa)/ <i>pI</i>	Sequence coverage (%)	Gene-chip signal intensity in the <i>ask1</i> inflorescence	Gene-chip signal intensity in the WT inflorescence	Gene chip signal intensity in the WT stage 12 flowers
1	At2g26670	heme oxygenase 1 (HY1)	32/6.96	28.1/5.4	9.6	441±40	415±22	837±87
2	At5g66530	aldose 1-epimerase family protein	34/5.7	34/5.1	12.1	1171±20	1280±28	930±74
3	At1g76450	oxygen-evolving complex-related	27/6.8	14/5.2	7.7	329±21	331±29	537±63
4	At1g07400	17.8 kDa class I heat shock protein	17.8/6.3	19.6/5.7	29.9	Not available	Not available	Not available
5	At2g21660	glycine-rich RNA-binding protein (GRP7)	16.9/5.9	16/5.6	15.3	7082±2021	11016±1357	8394±1819
	At1g23130	Bet v I allergen family protein	17.9/6.1	16/5.6	27.3	20.4±0.7	21.2±0.3	226±21
	At1g73230	nascent polypeptide-associated complex (NAC) domain-containing protein	18.0/6.0	16/5.6	13.3	2931±119	3219±145	2344±61
6	At5g07030	aspartyl protease family protein	47/10	47/7.6	14.4	2633±216	2458±153	1755±120
7	At5g26730	expressed protein	31/9.7	23.7/7.0	14.9	Not available	Not available	Not available

two or all of the three proteins could be affected by the *ask1* mutation; all three proteins have a good score and match the observed *pI* and MW. The technical limitation of 2-DE in the range of pH3–10 also makes the extremely alkaline proteins difficult to focus in IPG strips (Bio-Rad 2-DE technical manual), thus appearing to have a lower *pI* value than the theoretically predicted value, such as spot 6 and 7. The protein spots identified can be classified into several types based on their predicted physiological functions. They are proteins related to phytochrome-mediated responses, circadian oscillations, post-translation modification and stress responses, which is consistent with the observation that ASK1 is involved in multiple physiological processes through interacting with different F-box proteins.

Proteins involved in phytochrome-mediated responses and circadian oscillations The proteomic approach identified one protein that is involved in phytochrome-mediated responses, HY1 (heme oxygenase, spot 1 in Figure2). HY1 catalyzes the conversion of heme to biliverdin (a precursor of phytychromobilin), thus playing an important role in red/far-red light perception [24]. Our results indicate that HY1 is normally present at lower level in the developing flower and that ASK1 function is necessary for this reduced level. Because HY1 is a key enzyme for the production of the precursor of phytychromobilin, its up-regulation in the *ask1* plants might make the perception of environmental light signals abnormal. Although phytychromes play important roles in regulating flowering, it is possible that a reduction of phytychrome function is needed for normal flower development. This idea is supported by the observation that a *hyl1* mutation was able to suppress the floral reversion of flowers to shoots in an *ag* mutant [25], indicating that the loss of *HY1* function promotes flower development. Simon *et al.* [26] reported that the *HY1* gene negatively regulates *CONSTANS (CO)*. The *CO* function is required for the long-day dependent promotion of flowering [27]. The high levels of HY1 protein in the *ask1* mutant flowers may result in lower levels of *CO*; it is possible that *CO* is also important for normal flower development after the flowering transition, as supported by a positive role of *CO* in controlling the floral meristem determinacy [28].

Spot 5 that was detected at a higher level in the *ask1* mutant flower than in the wild type corresponds to three proteins: a glycine-rich RNA-binding protein (GRP7: At2g21660); Bet v I allergen family protein (At1g23130) and a nascent polypeptide-associated complex (NAC) domain-containing protein (At1g73230) (Table 1). All three proteins have a matching score that is more than 50, and they all have similar predicted masses and *pI*s. It is possible that one or two or all three of the proteins are accumulated in the *ask1* mutant, although GRP7 has the closest predicted

mass and *pl*. The microarray data showed that the mRNA of At1g23130 is below the detection limit in the young inflorescences. It is expressed at very low level in the stage 12 flowers, while the mRNA level of At1g73230 (nascent polypeptide-associated complex (AtNAC) domain-containing protein) is much higher than that of At1g23130, and the level of of At2g21660 (AtGRP7) mRNA is the highest among three proteins (Table 1). AtGRP7 has been characterized as a component of a negative-feedback loop through which the circadian oscillations of its own transcript is regulated. The AtGRP7 protein accumulates with a certain delay and represses further accumulation of its transcript, presumably at the post-transcriptional level [29]. Microarray data suggest that, in the *ask1* mutant, the *AtGRP7* transcript level declines moderately compared to the wild type (table 1), consistent with the negative feedback loop. The F-box proteins (ZTL, FKF, LKP2) were demonstrated to regulate the circadian clock and cooperate with ASK1 in the regulation of circadian oscillations [17, 18]. It is possible that ASK1 participates in this negative feedback loop by targeting AtGRP7 for degradation, and the accumulation of AtGRP7 protein in the *ask1* mutant cause the down-regulation of its own transcript. For other two proteins there is no reported functional characterization about them in plants. The nascent polypeptide-associated complex (NAC) domain-containing protein (At1g73230) is similar to a human RNA polymerase B transcription factor 3 and its mRNA level is very similar in all tissues (flowers, leaves, stems and roots) based on the gene chip microarray data [23]. It is likely a housekeeping gene.

Proteins related to post-translation modification An aspartyl protease (At5g07030) was identified to accumulate at a higher than normal level in the *ask1* mutant. Some aspartyl proteases can cleave signal peptides within hydrophobic domains, and therefore, were named signal peptide peptidase (SPP) [30]. Intramembrane-cleaving proteases have become important regulators of cell growth, signaling, and pattern formation during development [31]. An aspartyl protease called PCS1 (At5g02190) in *Arabidopsis* was shown to be essential for early embryogenesis, and important but not essential for female and male gametogenesis. Knock-out of the aspartyl protease gene causes excessive cell death in reproductive organs and over-expression causes anther dehiscence failure [32]. There are several aspartyl protease genes expressed predominantly in *Arabidopsis* flower organs and their functions could be partly redundant in some of the reproductive tissues [32]. Therefore, aspartyl protease in flowers may act as an important tool to remove or activate a downstream molecule in the reproductive developmental processes. The abnormal high accumulation of the aspartyl protease in the *ask1* mutant is

consistent with the previous report that the *ASK1* and *ASK2* genes play a vital role in *Arabidopsis* embryogenesis [19], which may function by affecting the levels of the proteins essential for embryogenesis.

Stress inducible proteins We have also identified two stress-inducible proteins that are increased in the *ask1* mutant flowers. One of them, a 17.8 kDa class I heat shock protein, is a typical stress induced protein that confers cytoprotection against oxidative injury. It could play an important protective role in maintaining normal development when *ASK1* gene is disrupted. Another protein, an oxygen-evolving complex-related protein, encoded by At1g76450, is also increased in the *ask1* mutant. Mutation of *ASK1* gene may directly or indirectly cause an oxidative stress in flower organs but this still remains to be investigated further. This is not surprising because several signaling pathways are altered in the *ask1* mutant, including responses to auxin [11, 12], GA [21], and jasmonic acid [11-14]. Therefore, the

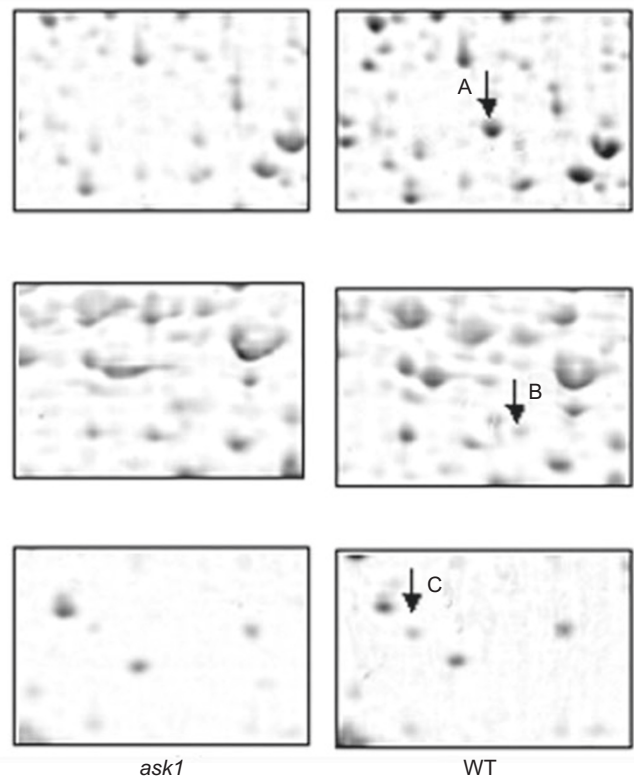


Figure 3 Proteins with higher abundance in the wild type flowers compared to the *ask1* mutant ones. The enlarged images are spot images after digitalization and spot matching. The proteins with different abundance are marked with arrows and numbered. A, xyloglucan endotransglycosylase; B, SC35-like splicing factor SCL30a; C, carbonic anhydrase;

ask1 mutant may be abnormal in some stress responses.

Proteins with decreased levels in the ask1 mutant

Although the initial goal of this study is to identify proteins that have increased abundance in the *ask1* mutant, we also found several proteins that are dramatically decreased in the mutant (Figure 3). Since it may also help to elucidate the roles of ASK1 protein plays *in vivo*, we discuss them in this paper.

Dramatic decrease of two proteins associated with hormone responses during floral development were observed in the *ask1* mutant. They are XTH3 protein that belongs to xyloglucan endotransglycosylase (XET) family and SCL30a protein. In our experiments, the protein spot of XTH3 had an obvious decrease in the *ask1* mutant flowers and could be easily identified each time. *XET* genes are predicted to encode cell wall enzymes involved in the construction and modification of the cellulose/xyloglucan framework, which is required for the normal organ growth. The transcript of *XTH3* is induced by IAA [33]. The microarray data also suggests that *XTH3* gene is specifically expressed at late stages of floral development (Table 2), indicating that XTH3 protein is probably important for IAA induced floral organ elongation at later stages, which is consistent with a reduced auxin signaling in the *ask1* mutant. Another member of the XTH family, TCH4, is capable of modifying cell wall xyloglucans [34]. This protein is dramatically up regulated in response to the growth hormones auxin and brassinosteroids [34, 35]. Furthermore, the TCH4 expression was restricted to expanding tissues and organs that undergo cell wall modification, especially at the site of future bulge formation [36]. Akamatsu *et al.* [37] reported a mutant named *acl5* that has a severe defect in the process of cell elongation after transition to the reproductive stage. RNA blotting showed that the *acl5* mutation causes a striking reduction in the transcript level of an *XET* (EXGT-A1) [38]. The *ask1* mutant phenotypes of shorter stature, reduced number and size of petal and stamen filament length are consistent with the functions of the ASK1 protein in promoting cell expansion and division [1], which is realized by the expression of proteins responsible for the cell expansion and division. The down regulation of XTH3 protein in the *ask1* mutant may result from the abnormal hormone signaling in the *ask1* mutant flowers. From the early inflorescence (floral stage 1-9) to the stage-12 flower, the transcription level of XTH3 increased 100-fold in the wild type *Arabidopsis* (from 32 to 3713). This strongly implies that XET not only affects root hair bulge formation, but also is important for normal floral development.

SCL30a is a component of spliceosomal machinery that is responsible for the processing of nuclear RNA [39, 40].

Table 2 Proteins with lower abundance in the *ask1* flowers compared to wild type flowers

Spot No.	Gene ID	Protein identification (synonym)	Predicted molecular mass (kDa)/pI	Apparent molecular mass (kDa)/pI	Sequence coverage (%)	Gene-chip signal intensity in the <i>ask1</i> inflorescence	Gene-chip signal intensity in the WT inflorescence	Gene-chip signal intensity in the WT stage 12 flowers
A	At3g25050	xyloglucan endotransglycosylase	33.5/6.39	30/5.5	17.2	32.4±3.2	33.1±2.6	3713±284
B	At3g13570	SC35-like splicing factor SCL30a	30.2/6.08	40.8/5.2	12.2	1019±29	1060±59	648±29
C	At1g23730	carbonic anhydrase	28.8/6.99	20.3/5.4	46.1	Not available	Not available	Not available

Using AtSCL30a as the bait in a yeast two-hybrid screen, Lopato *et al.* [40] found that it interacts with AtRSZ33, which is a phosphoprotein that concentrates in nuclear speckles and is predominantly present in roots and flowers. Furthermore, increased AtRSZ33 expression promotes cell expansion and changes polarization of cell elongation and division [39], suggesting that the levels of SCL30a may be important for regulating cell expansion during flower development. This is also consistent with the previous observation of Zhao *et al.* [2] that *ask1-1* mutant has reduced growth of flower organs. *ASK1* may regulate cell expansion by modulating the levels of proteins such as SCL30a, although likely indirectly.

This paper is the first proteomic analysis of the *ask1* mutant. It is of interest to note that although the protein levels of the identified proteins showed very strikingly difference in the 2-D gel, the mRNA level did not show obvious difference at early stages for most of the proteins. Considering that the targets of ASK1-containing SCF complexes may have very important functions in floral organ identity, it is more important to study the early stages of flowers than the late stages. The non-correlation of protein levels and mRNA levels indicates the need to analyze protein levels, and not simply draw conclusions based on mRNA levels. Our results strongly demonstrate that the protein level can be regulated separately from the mRNA level and it is important to study protein level.

ASK1 can potentially regulate the level of a protein (call it X) in at least three different ways: (1) regulates the degradation of the protein X; (2) regulates the level and/or activity of a transcription factor (like AP3) responsible for the mRNA level of the protein X, but does not affect the degradation of X; (3) change the development of the flower, such that the number of cells that express protein X is increased or decreased. Among the above possibilities, number 2 and 3 predict a correlation between mRNA and protein levels, barring more complex regulations. In contrast, for the first possibility, the mRNA levels might be similar between wild type and the *ask1* mutant, but the protein levels would be different. In our experiments, six out of seven protein spots (if the other two proteins are excluded for spot5) with higher abundance in the *ask1* mutant have very similar mRNA levels, indicating that the levels of these proteins do not reflect their mRNA levels. Therefore, these proteins are likely degraded by an ASK1-dependent proteolysis pathway, and thus are good candidate targets of ASK1-containing SCF complexes. For AtGRP7, the higher mRNA level in the wild type than in the *ask1* mutant is consistent with the previous report that it is a component of a negative-feedback loop through which the circadian oscillations of its own transcript is regulated [29]; so it can also be a candidate for the ASK1-containing

SCF complex.

In *Arabidopsis*, 694 potential F-box genes have been identified [20]. At least 18 out of the 21 *Arabidopsis* ASK genes were expressed under normal growth condition [8, 9]. Among the ASK genes, *ASK1* may play a major role in most fundamental processes regulated by SCF complexes, and ASK1 interacts with many F-box proteins in a yeast-two hybrid assay [7, 9, 19, 20]. Therefore, numerous biological processes regulated by SCF complexes may be abnormal in the *ask1* mutant. In the meantime, the relative weak phenotypes in the *ask1* mutant compared to loss of function mutation in other core SCF component AtCUL1 and Rbx1 make the *ask1* mutant a unique material to study potential SCF complex target proteins involved in later stages of plant development such as floral development [41, 42]. The weak *ask1* phenotype also has an advantage for proteomic analysis in that the protein difference between the *ask1* mutant and wild type are not due to major tissue structure change, which is confirmed by the similar RNA level between the mutant and wild type. The proteomic analysis in this study provides an important initial view of the proteins affected by the *ASK1* gene in the floral development process of *Arabidopsis*. The identified proteins could be the protein targets for ASK1-mediated proteolysis pathway. For the down-regulated proteins in the *ask1* mutant flowers, such as xyloglucan endotransglycosylase, it is of interest to determine its function in floral development. For those proteins with elevated levels in the *ask1* mutant flowers, further investigations are needed to ascertain whether they were directly or indirectly regulated by ASK1; in other words, whether they are degraded through the ubiquitin-mediated proteolysis pathway with ubiquitination by an ASK1-containing SCF complex.

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