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REGULATION OF ARABIDOPSIS FLOWER AND ANTHER DEVELOPMENT AT THE TRANSCRIPTIONAL AND POST-TRANSLATIONAL LEVELS

A Dissertation in

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ABSTRACT

Development of multicellular organisms is a complex process requiring orchestrated regulations at transcriptional, post-transcriptional, translational, and post-translational levels. Numerous studies have revealed many important genes for different aspects of plant development. Most of these genes encode regulatory proteins, which are involved in various biological processes including gene transcription and signal transduction. The mutations of many of these genes cause defects in morphological or physiological phenotypes. However, the molecular functions of many of them remain poorly understood. *Arabidopsis thaliana* has been used as the model organism in my projects to investigate the regulatory mechanisms controlling flower and anther development at the transcriptional and post-translational levels.

A number of transcription factor genes have been characterized to be essential for male fertility by regulating anther development at various stages. The *DYSFUNCTIONAL TAPETUM 1* (*DYT1*) and *MYB35* genes were reported to regulate the tapetum function and pollen development based on genetic and morphological evidence. The molecular regulatory functions of DYT1 and MYB35 proteins were investigated in one of my research projects and the results suggested that they probably function as transcriptional activators. Transient induction of DYT1 activated the *MYB35* transcription rapidly, suggesting that *MYB35* may be immediately downstream of *DYT1*. The DNA binding specificity of the DYT1 protein was investigated using gel-shift assays, and the results revealed that DYT1 binds to a specialized G-box motif TCACGTGA. Combined with the transcriptomic information of *dyt1* mutant anthers from our lab, this study consolidated the function of DYT1 in controlling a large number of anther genes, which may explain its importance in tapetum and pollen development.

The ubiquitin-proteasome system (UPS) is involved in almost every biological process. *Arabidopsis* E3 ubiquitin ligases have been demonstrated to regulate various plant physiological

and developmental processes. The *ARABIDOPSIS SKP1-LIKE 1* (*ASK1*) gene, which encodes a key subunit of Skp1-Cul1-F-box (SCF)-type E3 ubiquitin ligases, has been shown to be involved in plant development and reproduction, especially in flower development and meiosis. Despite many lines of genetic evidence, the role of ASK1 in regulating the proteome and transcriptome is still not clear. In my second project, the flower bud proteomes and transcriptomes were compared between an *ask1* mutant and wild-type to identify proteins that are putative substrates of the ASK1-containing E3 ubiquitin ligases (ASK1-E3s). Proteins that accumulate to higher levels in the flower bud proteome of the *ask1* mutant than that of wild-type, and whose cognate RNA levels are not significantly different between the two genotypes, may be regulated by ASK1-E3s at the post-translational level. The putative substrates include transcription regulators, kinases, peptidases, and interestingly ribosomal proteins, suggesting that regulatory proteins are more likely to be regulated by the UPS and that the ribosomal proteins, which are usually believed to be only involved in protein synthesis, may also be regulated by selective proteolysis mechanisms. Another project investigated the impact of *ask1* mutation on the anther transcriptome, and the results suggested that ASK1-E3s may regulate the destabilization of transcription factors to modulate the expression of genes involved in multiple signaling pathways. Therefore, the regulations at the transcriptional and post-translational levels may be coordinated by the ubiquitin system.

These studies not only provided large-scale proteomics and transcriptomics information for further investigations, but also revealed specific functions of certain transcription factors that regulate anther development. Further studies may lead to discoveries of novel functions of the regulatory proteins and the overall properties of the regulatory networks underlying flower development.

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Chapter 1

Literature Review

The flower is one of the most complex and amazing structures in plants. Flowers of the model plant *Arabidopsis thaliana* have four concentric whorls of organs: sepals, petals, stamens, and carpels. The genetics of flower development has been relatively well understood. The ABC model has been generally used to describe the development of floral organs from the floral meristem (Robles and Pelaz, 2005). *Arabidopsis* contains three groups of genes that control floral organ identities: the A-function genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the B-function genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the C-function genes *AGAMOUS* (*AG*). The A-function genes alone in the outmost whorl specify the identity of sepals. The A- and B-function genes together in the second whorl determine the petal identity. The B- and C-function genes in the third whorl cooperatively specify the stamen identity. And the C-function gene determines the carpel identity in the inner whorl and terminates the floral meristem activity. In addition to the ABC genes, another two types of genes, D- and E-function genes were also involved in the floral organ identity determination. The D-function genes are close paralogs of *AG* and are required for the ovule development (Colombo et al., 1995; Favaro et al., 2003) and the E-function genes are essential for the specification of all the four floral organs (Pelaz et al., 2000). All the genes required for controlling floral organ identities except *AP2* encode MADS-box transcription factors and are conserved among flowering plants, suggesting that a transcriptional regulatory mechanism is conserved in flowering plants with various modifications in the gene expression patterns or protein functions to give rise to diverse flower morphologies. Of course, with the increasing molecular and genetic evidence both in model plants and non-model plants, the

regulatory models for the flower development mechanisms may continue to evolve (Soltis et al., 2007; Causier et al., 2010).

The *Arabidopsis* male reproductive organ, the stamen, is consisted of a filament and an anther. The filament is a supporting structure that conducts water and nutrients to the anther and elongates to raise the anther to the height of the stigma for successful pollination. The anther is a more complex organ composed of several layers of anther wall cells (the epidermis, endothecium, middle layer, and tapetum) surrounding the locules where diploid pollen mother cells undergo meiosis to produce haploid microspores and where mature pollen grains are finally produced (Sanders et al., 1999). Among the four layers of anther wall cells, the tapetum is the cell layer that directly encircles pollen mother cells or developing microspores and is believed to provide nutrients, signals, enzymes, and other materials for microsporogenesis and pollen maturation. The formation of these anther structures is a complex process, which requires well coordinated regulations at different levels, including transcriptional regulations, post-transcriptional regulations, post-translational regulations, signal transduction, etc.

The anther is a good biological system for studying many different topics including, but not limited to, organ identify specification in the meristem, organ primordia initiation, cell fate determination, cell division (especially meiosis), cell differentiation, programmed cell death, cell wall biogenesis and degeneration, sporopollenin biogenesis and pollen wall formation, cell-cell communication, hormone signaling, and environmental effects on male fertility. Many studies have been dedicated to these research topics. In recent years, molecular evidence for the involvement of a number of genes encoding proteins that regulate anther development at transcriptional and post-translational levels has been accumulating, and a tentative regulatory network has been proposed for a better understanding of this complex process.

Transcriptional regulatory mechanisms determining stamen identity

As generalized in the typical ABC model, the B- and C-function genes in combination specify the stamen identity in the floral meristem. However, the ABC proteins alone are not sufficient to determine floral organ identities. When the E-function genes are added to the ABC model, the ABCE model can explain more about the molecular mechanisms behind the floral organ identity determination. In *Arabidopsis*, the B-function genes *AP3* and *PI*, and the C function gene *AG*, encode MADS-box proteins which form a tetrameric protein complex with the E-function SEPALLATA (SEP) proteins to bind to the CArG-box cis-elements in the regulatory regions of target genes. It was demonstrated that co-expression of SEP, AP3, PI, and AG proteins in vegetative tissues converted leaves into stamen-like organs (Honma and Goto, 2001), suggesting that the stamen identity specification involves a combinatorial regulatory mechanism.

Recently, the molecular functions of MADS-box transcription factors have been under intensive investigations. Many target genes of AP1, AP2, and AG were reported by several studies which used a variety of techniques including chromatin immunoprecipitation (ChIP) assays, microarrays, and transient induction of transcription factors fused to a hormone receptor domain named glucocorticoid receptor (GR) domain (Ito et al., 2004; Gomez-Mena et al., 2005; Sundstrom et al., 2006; Ito et al., 2007a; Kaufmann et al., 2010; Yant et al., 2010). Despite these efforts in identifying the large number of putative target genes with both high-throughput and low-throughput approaches, the detailed regulatory network of the stamen identity specification still needs to be elucidated.

Transcriptional regulation of early anther development

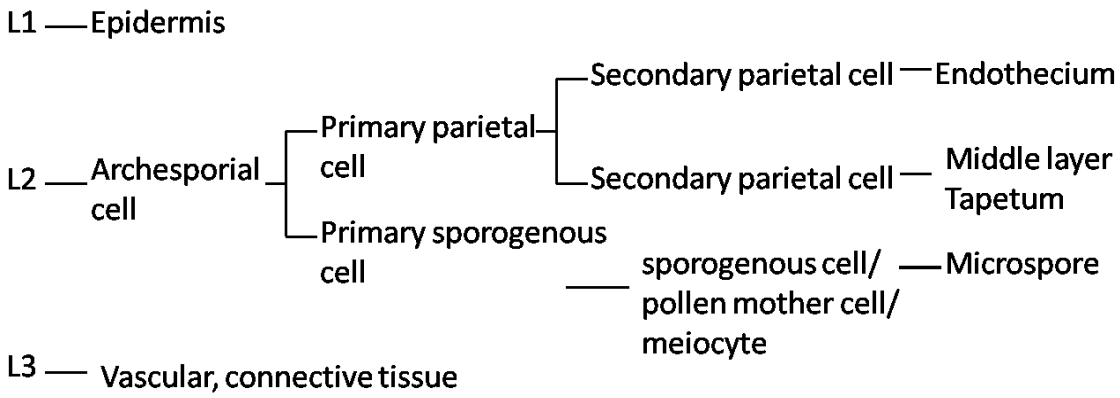

Figure 1-1. Schematic demonstration of anther cell differentiation in *Arabidopsis*. Stamen primordia are composed of L1, L2, and L3 cell layers. L1 cells form the epidermis. L3 cells develop into vascular and connective tissues. L2 cells divide and differentiate to produce germ cells and three layers of anther wall cells including the endothecium, middle layer, and the tapetum.

During early stamen stages, the anther is the first part to develop while the filament emerges later. At the early anther developmental stages before meiosis, the most prominent events are cell division, cell differentiation and cell growth, which result in the growth of the anther and formation of different cell types including the innermost pollen mother cells and concentric layers of anther wall cells surrounding the pollen mother cells (Sanders et al., 1999). When the anthers initiate from the floral meristem, they are relatively simple organs with only three cell layers, the L1 layer of epidermis cells, the L2 layer of cells from which most of the later anther cell types will be derived, and the L3 layer of cells that finally develop into vascular and connective tissues in the center of the anther to connect to the filament (Figure 1-1). The L2 cells later differentiate into archesporial cells at the four corners of the anther. The archesporial cells

then divide to form two cell layers, the primary parietal cells and the primary sporogenous cells. The primary sporogenous cells divide and differentiate into the pollen mother cells (meiocytes), which undergo meiosis to produce haploid microspores. The primary parietal cells divide to produce another two cell layers named as the secondary parietal cells. The outer secondary parietal cells divide and differentiate into the endothecium layer and the middle layer. The inner secondary parietal cells divide and differentiate to make the tapetum layer and possibly contribute to the middle layer as well. In *Arabidopsis*, the middle layer degenerates before meiosis. However the biological significance of the middle layer and its degeneration is not clear. When all the anther cell types are established before meiosis, the anther is butterfly-shaped when observed from the cross section. One anther contains four lobes and each lobe contains the anther wall cells and pollen mother cells in the center locule. The two abaxial lobes are larger than the two adaxial ones and the cell differentiation states are slightly different in the abaxial and adaxial lobes, indicating that there may be coordinated and independent mechanisms that control the development of individual anther lobes.

A putative transcription factor *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*) (Schiefthaler et al., 1999a; Yang et al., 1999b) was reported to control microsporogenesis and megasporogenesis in *Arabidopsis*. The expression of the *SPL*/*NZZ* gene is activated by AG, which binds to a CArG-box motif in the downstream intergenic region of *SPL*/*NZZ* (Ito et al., 2004), suggesting that the C-function gene *AG* relays its partial function to the *SPL*/*NZZ* gene in controlling sporocyte differentiation in both male and female reproductive organs. The SPL/NZZ protein was suggested to directly or indirectly regulate the expression of many genes: about 2000 genes showed differential expression between the *spl* mutant and the wild-type young anthers (Wijeratne et al., 2007a). Despite its important role in controlling sporogenesis, the molecular function of the *SPL*/*NZZ* gene and the regulation of its spatiotemporal expression patterns are yet to be determined.

Transcriptional regulation of tapetum development and functions

The tapetum layer has unique roles in anther development, microsporogenesis and pollen development. The tapetum layer immediately surrounds the locule containing pollen mother cells or microspores and pollen grains at later stages. The tapetum cells have been suggested to be very active in metabolism, which is required for providing materials for pollen development. It has been found that the tapetum cells have extremely active gene expression activities. Many of the anther-specific genes discovered so far are differentially expressed in the tapetum cells and the mutations of many of these genes affect tapetum development and functions, thus impairing male fertility (Hirano et al., 2008; Hobo et al., 2008; Huang et al., 2009). The dynamic gene expression activities should be required for making enzymes to catalyze biosynthetic reactions and for providing regulatory proteins for normal tapetum functions and pollen development. The tapetum cells may also mediate nutrient transport from vegetative tissues to the anther locules where the meiosis and pollen development demand a huge amount of materials and energy (Polowick and Sawhney, 1993; Mamun et al., 2006).

Several transcription factor genes have been shown to be expressed in the tapetum cells and essential for regulating gene expression in the tapetum. *DYSFUNCTIONAL TAPETUM 1* (*DYT1*), encoding a basic helix-loop-helix (bHLH) transcription factor, is specifically expressed in the tapetum cells and is essential for male fertility (Zhang et al., 2006). In the *dyt1* mutant anthers, the tapetum showed early onset of degeneration just before the meiosis stage as shown by abnormally large vacuoles; and the meiocytes could not complete cytokinesis, which finally results in complete male sterility (Zhang et al., 2006). The cell wall structures of the pollen mother cells are defectively thinner in the *dyt1* anthers than that in the wild-type, which may also contribute to the overall defects of meiosis and result in male sterility (Feng et al., 2012b). Other aspects of anther development including cell differentiation and cell division are not obviously

affected. These findings suggested a role of *DYT1* in specifically maintaining normal tapetum functions to facilitate meiosis and possibly later pollen maturation.

The *MYB33* and *MYB65* genes also function at similar stages as *DYT1* does (Millar and Gubler, 2005). The expression of the *MYB33* and *MYB65* genes is independent of *DYT1*, and the expression of *DYT1* is independent of *MYB33* and *MYB65*, suggesting that *MYB33* and *MYB65* and *DYT1* may be regulated by some common upstream transcriptional regulators (Millar and Gubler, 2005; Zhang et al., 2006). The mutations in the *MYB33* and *MYB65* genes individually do not cause visible morphological defects; but when they are both mutated, the anthers of the double mutant are defective in the tapetum and later become mostly male sterile. This suggests that the *MYB33* and *MYB65* genes function redundantly to regulate tapetum gene expression. A microRNA, miR159, whose targeting sequence was found in the coding regions of *MYB33* and *MYB65* was shown to be essential for their correct expression patterns, indicating that the miR159 confines *MYB33* and *MYB65* expression specifically within young anthers (Millar and Gubler, 2005). However, the relationships between *DYT1* and *MYB33*/*MYB65* and their upstream or downstream genes have not been uncovered.

Some other transcription factors including *ABORTED MICROSPORES* (*AMS*), *MALE STERILE1* (*MS1*), *MYB35*, *MYB80* (originally named *MYB103*), and *MYB99* are also required for normal tapetum functions in anther development and male fertility (Wilson et al., 2001a; Sorensen et al., 2003b; Alves-Ferreira et al., 2007; Zhu et al., 2008; Xu et al., 2010a; Zhu et al., 2010; Phan et al., 2011). These genes generally function in the tapetum and/or meiocytes at post-meiosis stages and may be directly or indirectly regulated by the transcription factors like DYT1, MYB33/MYB65, or other pre-meiotic regulators (Zhang et al., 2006). The null mutations of these transcriptional regulators all cause complete male sterility, indicating an absolute essentiality of these regulators for normal tapetum functions and pollen development. However, the exact

relationships between these male fertility-related genes and their regulatory networks are largely unknown.

Cell-cell signaling mechanisms controlling anther cell differentiation

As in animals, plant cells can also communicate with other cells, especially between adjacent cells, to determine cell fates or regulate cellular activities. Signaling pathways are crucial for anther development, especially during cell differentiation processes to establish multiple somatic cell types and meiocytes from the simple cell types derived from the organ primordia. Cell-cell communications usually require extracellular ligands secreted from one cell and receptors on the plasma membrane surface of another. The *BARELY ANY MERISTEM 1 (BAM1)*, *BAM2* and *BAM3* genes encode receptor-like kinases. The single mutants of the *BAM1*, *BAM2* and *BAM3* genes respectively do not show obvious defective phenotypes. The double mutant *bam1 bam2* shows pleiotropic defects in the shoot and floral meristem development. The mutation of *BAM3* within the *bam1 bam2* mutant background further enhanced the defects, suggesting redundant functions of these three genes. The *BAM* genes are positively regulated by *SPL/NZZ* (Hord et al., 2006) to control the development of anther wall cells, and later the *BAM* genes restrict the *SPL/NZZ* gene expression in the sporogenous cells. Therefore, the *BAM* genes and the *SPL*/*NZZ* gene may form a feedback regulatory loop to coordinate the anther cell differentiation by providing temporal and spatial cues.

At slightly later stages, the *EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS* (*EMS1*/*EXS*), *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1* (*SERK1*) and *SERK2* genes which encode putative leucine-rich repeat receptor-like kinases (LRR-RLKs) are crucial for tapetum cell fate determination (Zhao et al., 2002a; Albrecht et al., 2005). The *TAPETUM DETERMINANT 1* (*TPD1*) which encodes a small protein (Yang et al., 2003a; Yang

et al., 2005a; Zhang et al., 2006) may be a peptide ligand produced by the pollen mother cells and interacts with the extracellular domain of the receptor EMS1/EXS to initiate the signaling transduction for determining the tapetum cell identity. The mutations in either the receptor EMS1/EXS or the ligand TPD1 result in the loss of tapetum cells; instead, more pollen mother cells are produced. However, the downstream signaling events of the ligand-receptor interactions, e.g., phosphorylation cascades initiated by the kinase activities of the LRR-RLKs and transcriptional regulations activated/repressed by the signaling, have not been investigated. More efforts are needed to explore the intermediate regulatory steps between the initial signals and the final output for tapetum cell fate determination.

Post-translational regulation of plant development by the ubiquitin-proteasome system

The ubiquitin-proteasome system (UPS) plays important post-translational regulatory roles in selective protein degradation (Smalle and Vierstra, 2004; Schwechheimer et al., 2009; Sorokin et al., 2009). The ubiquitin proteins are usually attached to the lysine residues of protein substrates through a cascade of reactions catalyzed by E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases. Eukaryotes usually have multiple E1, E2, and E3 enzymes. In combination, they can specifically ubiquitinate a large number of protein substrates. The E3 ubiquitin ligases consist of several types: monomeric E3 ligases including HECT and RING/U-box E3 ligases; multimeric E3 ligases including anaphase-promoting complex (APC), Skp1-Cul1-F-box (SCF), and E3 ligases based on other Cullin types. Each type of E3 ubiquitin ligases are usually encoded by large gene families probably originated from gene/genome duplication events during evolution. Multiple ubiquitin molecules can be attached to the existing ubiquitin moieties on the protein substrates to form polyubiquitin chains. And since the ubiquitin itself has seven lysine residues, further ubiquitin addition to the ubiquitin

chains may form many different structures, which provide docking sites for other proteins that have different affinities or that can recognize different topologies of polyubiquitin chains. The polyubiquitinated proteins are usually targeted to the 26S proteasome for degradation. The ubiquitin molecules may be cleaved off the protein substrates and reused, and the substrates are digested by the proteases within the proteasome into short peptides or amino acids to be recycled.

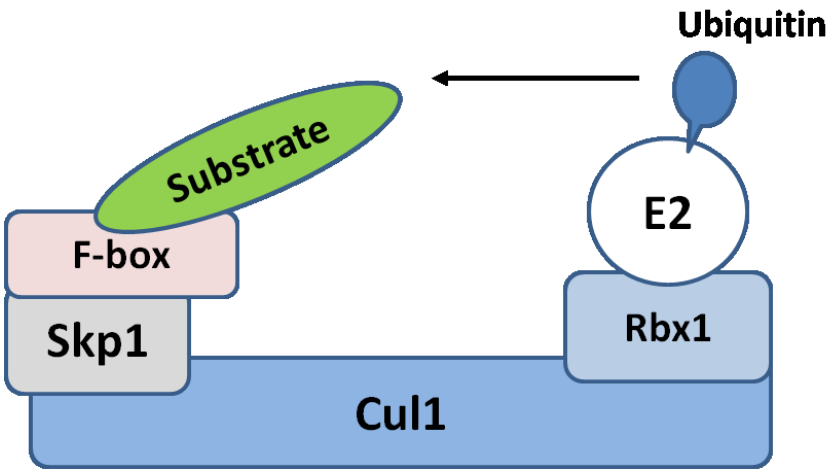

Figure 1-2. A model of Skp1-Cul1-F-box (SCF) complex-mediated protein ubiquitination. The SCF complex is composed of an F-box protein, Skp1, Cul1, and Rbx1. The F-box proteins specifically bind to substrate proteins. The SCF complexes catalyze the reaction of transferring the ubiquitin from E2 ubiquitin conjugating enzymes to the substrates.

The ubiquitin-proteasome pathway is involved in almost all processes of plant biology, including various aspects of plant physiology and development (Smalle and Vierstra, 2004; Dreher and Callis, 2007; Stone and Callis, 2007; Schwechheimer et al., 2009). This broad spectrum of functions is made possible by the large set of genes encoding components in the ubiquitin-proteasome pathway. Animals and plants usually possess only a few E1 ubiquitin activating enzymes, but tens of E2 ubiquitin conjugating enzymes and hundreds of E3 ubiquitin

ligases. Among these components, the E3 ligases determine the specificity of the substrates. Therefore, the large number of E3 ligases indicates that they can ubiquitinate numerous protein substrates. Moreover, the modular design of the SCF-type E3 ubiquitin ligases greatly expands the number of proteins to be ubiquitinated (Figure 1-2). The subunits of SCF complexes are encoded by multi-gene families, especially the F-box proteins, which are usually encoded by tens of genes in animals and hundreds of genes in plants. Thus, they can form a variety of SCF complexes to ubiquitinate numerous target proteins. Of course, some of these F-box proteins may have redundant functions, or have similar substrate specificities but different expression patterns. Tremendous efforts are needed to identify the specific protein substrates of these E3 ubiquitin ligases and the mechanisms underlying their functions.

Non-proteolytic functions of ubiquitination have also been discovered (Chen and Sun, 2009). Monoubiquitin or polyubiquitin chains of certain topologies may endow the labeled proteins with new properties, including new binding sites for protein-protein interactions or altered protein conformation, so that the ubiquitinated proteins may participate in different processes like receptor endocytosis, protein activation, protein sorting, signal transduction, DNA damage repair, etc.

On the other side, the UPS also include a number of deubiquitinases, ubiquitin-specific proteases that specifically cleave ubiquitin molecules from the polyubiquitin chains or from the protein substrates, a reaction that may modify the topologies of the polyubiquitin chains or totally remove ubiquitin molecules from protein substrates. Deubiquitinases may antagonize E3 ubiquitin ligases when determining the fate of protein substrates, which may be required for fine-tuning many biological pathways (reviewed by Komander, 2010).

F-box proteins regulate various pathways in *Arabidopsis*

As the receptors for the specific substrates of SCF-type E3 ubiquitin ligases, F-box proteins are of special interest for researchers studying the ubiquitin system. Previous studies have identified many F-box proteins in plants involved in hormone signaling pathways, self-incompatibility, development processes, light signaling, circadian regulation, etc.

Among the F-box proteins important for plant hormone signaling, the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and the AUXIN SIGNALING F-BOX (AFB) proteins are involved in the auxin signaling pathway (Gray et al., 1999; Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005); the F-box protein CORONATINE INSENSITIVE 1 (COI1) is required for regulating jasmonic acid (JA) signaling (Devoto et al., 2002; Xu et al., 2002; Thines et al., 2007); the F-box proteins SLEEPY1 (SLY1) and SNEEZY (SNE) regulate gibberellin (GA) signaling (McGinnis et al., 2003; Dill et al., 2004; Strader et al., 2004); the ETHYLENE INSENSITIVE 3 (EIN3)-BINDING F-BOX PROTEIN 1 (EBF1) and EBF2 are two F-box proteins essential for the signaling pathway of ethylene (Binder et al., 2007). Other hormone signaling pathways including abscisic acid (ABA) pathways also require E3 ubiquitin ligases but not necessary SCFs that contain F-box proteins (Stone and Callis, 2007).

F-box proteins are also discovered to regulate processes in addition to the hormone signaling pathways. The S-locus F-box proteins (SLFs) function as the pollen-specific determinants of self-incompatibility in petunia (Lai et al., 2002; Hua and Kao, 2006; Hua et al., 2007; Fields et al., 2010). The UNUSUAL FLORAL ORGANS (UFO) in *Arabidopsis* controls the meristem identity determination and petal and stamen development (Ingram et al., 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). It was reported that UFO can interact with LEAFY (LFY) to activate *AP3* expression in the regions of the floral meristem where petal and stamen primordia emerge (Lee et al., 1997; Samach et al., 1999b; Chae et al.,

2008). F-box proteins are also found to regulate late anther development. The anther-specific F-box protein REDUCED MALE FERTILITY (RMF) is essential for tapetum function (Kim et al., 2010). The SECONDARY WALL THICKENING-ASSOCIATED F-BOX 1 (SAF1) is required for secondary cell wall thickening in the endothecium cells of the anther (Kim et al., 2012).

Although the F-box proteins are responsible for determining the specificity of substrates, the ubiquitination reaction is accomplished only when F-box proteins form functional SCF complexes with other subunits. It has been reported that in the absence of protein substrates, some F-box proteins in the SCF complex can be auto-ubiquitinated for degradation (Galan, et al., 1999). This may be a feedback mechanism to dampen the F-box protein levels when the protein substrates are removed. Although the substrates of a handful of animal F-box proteins are increasingly being identified through multiple methods including yeast two-hybrid, co-immunoprecipitation, and *in vitro* reconstitution of SCF complexes for ubiquitination assays, few novel substrates of F-box proteins have been discovered in plants. Even the substrates for the genetically well-known F-box proteins like UFO are still elusive. It will be interesting to use a variety of techniques to answer the important but challenging question of what protein substrates are regulated by the hundreds of F-box proteins in plants.

ARABIDOPSIS SKP1-LIKE 1 (ASK1) regulates flower development and male meiosis

In *Arabidopsis*, the *ARABIDOPSIS SKP1-LIKE1* (*ASK1*) gene encodes a subunit of SCF E3 ubiquitin ligases (Yang et al., 1999a). ASK1 was one of the first E3 ubiquitin ligase components ever discovered in plants. The mutation of the *ASK1* gene causes defective vegetative growth, petal development, and male meiosis. Since the *ASK1* gene is expressed throughout the plant with higher levels in growing organs, its mutation is expected to cause more defects in many plant organs. However, the actual defects are milder than the expected, probably due to the

redundancy among the *ASK* gene family members. The *ASK2* gene is the most closely related gene to *ASK1*. The single mutant of *ask2* is similar to the wild-type plants. But the double mutant *ask1 ask2* is severely abnormal during embryo development and lethal soon after seed germination. This indicates that the redundancy between ASK1 and ASK2, and perhaps other ASK proteins, should have masked many aspects of the developmental role of ASK1 by complementing its functions in the tissues other than the abnormal floral organs of the *ask1* mutant. Studying these masked aspects of ASK1 functions will need tissue-specific silencing of multiple *ASK* family members, or tissue-specific *ASK1* complementation within the *ask1 ask2* double mutant or higher order mutants. From another angle, the relative weaker defects of the *ask1* mutant may be useful for dissecting floral organ-specific functions of ASK1 since the vegetative growth is only mildly affected and the floral organ identities are not dramatically impaired. The initiation of floral organ primordia from the floral meristem and the early developmental stages are generally normal. In terms of anther development, the stages before meiosis in the *ask1* mutant are not obviously different from wild-type. The predominant defects are in the meiosis and probably in the tapetum as well. Abnormal chromosome condensation and pairing are detectable in the prophase of the meiosis I stage. Later the segregation of chromosomes is defective and no viable microspores are produced, resulting in complete male sterility. These genetics and cytology observations suggested that ASK1 may regulate certain proteins that are crucial for chromatin dynamics during meiosis. However, in spite of so many studies after the discovery of ASK1, the substrates of the SCF E3 ubiquitin ligases involving ASK1 during flower development and meiosis have not been identified.

According to the crystal structures of SCF complexes, ASK1 bridges Cul1 subunit and F-box proteins to make SCF complexes in which the F-box proteins recruit specific protein substrates for ubiquitination. It has been shown that the ASK1 protein can interact with many F-box proteins, e.g., UFO (Samach et al., 1999; Takahashi et al., 2004), COI1 (Dai et al., 2002), and

TIR1 (Gray et al., 1999). Since these F-box proteins have important roles in different pathways, ASK1 as a key component in the SCFs should also have crucial functions in the regulation of many processes. This was suggested by the previous studies of the *ask1* mutant showing that it had defects in male meiosis, petals, and vegetative growth (Yang et al., 1999a). Although a few substrates of the SCFs, such as the well-studied F-box proteins described above, have been identified, a large number of other ASK1-interacting F-box proteins and their substrates remain to be identified.

Transcriptomics for studying transcriptional regulations

Gene expression levels can be tested with traditional methods including northern blotting and quantitative PCR, which are low-throughput technologies and can test only a limited number of genes at a time. Also, it is almost impossible to test the expression levels of all genes that might be affected in a mutant if no previous evidence is available. Thus large-scale technologies are needed for measuring the expression levels of a large number of genes simultaneously. Genome-wide gene expression profiling studies have been made possible and remain to be an active research area since the advent of large-scale technologies including microarrays and high throughput sequencing.

Although there are several types of microarray platforms, they are all based on the complementary base pairing between mRNA molecules and probes fixed on the surface of chips. The probes are either from cDNAs prepared from mRNAs and attached to the chips, or are *de novo* synthesized directly on the chips. Currently the commercial or customized microarray chips generally use the synthesized oligonucleotide probes for detecting mRNAs in the samples. The probes are used to hybridize to the cDNAs or cRNAs prepared from biological samples of interest under very stringent conditions and the fluorescent signals emitted from each hybridization spot

are detected to reflect gene expression levels. With the whole genome been sequenced and commercial DNA microarrays available, the *Arabidopsis thaliana* transcriptomes of many different tissues/cell types and plant materials with various biotic and abiotic treatments have been intensively investigated in the last decade by using microarrays. These studies have greatly facilitated the measurement of the expression of tens of thousands of genes in one experiment therefore making it feasible to look at many questions that were not easy to tackle before. One can discover new genes that are regulated by the transcriptional regulators under study by comparing the transcriptomes of mutants and the wild-type plants, or by transient induction of the expression of transcription factors. The transcriptomes of different plant tissues, organs or even individual cell types can be compared to find genes that are expressed in specific locations or in several or many different locations. Follow-up studies of the tissue-specific genes may identify genes that are required for specific cell or organ development; and studies of the genes that are expressed in more than one location may reveal their functions required for common processes shared by these tissues or cells. When the microarray technique is applied to study plant physiology, it can extend our insights from morphological plant physiology down to the gene expression level. Even before the phenotypic responses can be detected, the intracellular changes have occurred. The dynamics of gene expression activities can be captured by microarray assays to dissect the early responsive genes and the late responsive ones. The detection of gene expression dynamics can also be used to identify genes that regulate developmental processes. When the transcriptomes of certain organs of different developmental stages are determined, the gene expression curve for each gene can be plotted to demonstrate which genes are expressed at certain stages. Of course the applications of DNA microarrays are not limited to the ones described here.

The high-throughput sequencing or next-generation sequencing technologies are being developed rapidly and are becoming more accessible to researchers. High-throughput sequencing

technologies can sequence millions of DNA sequences in parallel thus to accelerate the speed and cut down the cost for every sequence. Most of the high-throughput sequencing technologies yield relatively shorter sequences (reads) compared with traditional Sanger sequencing. However, the read length is improving these years to satisfy both *de novo* genome sequencing and transcript sequencing.

The application of high-throughput sequencing technologies in measuring gene expression levels is particularly powerful with their high speed and large read numbers produced by each run. High-throughput sequencing can be used in any area that microarray has been applied to. But high-throughput sequencing has better performance since it produces the actual sequences of DNAs or RNAs, enabling the analysis of alternative splicing of transcripts, detection of novel transcripts, studying gene expression in species without sequenced genomes, etc. Deep sequencing also has higher dynamic range in measuring gene expression levels than microarrays. With the further development of the currently available and upcoming high-throughput sequencing technologies, we can anticipate that genome-wide transcriptome analysis will be much easier and more powerful in the near future.

Proteomics for studying post-translational regulations

Large-scale gene expression studies are mostly focused on the RNA level. However, the mRNA levels cannot truly represent the final protein levels: the transcript levels usually provide only an estimation of the protein abundance. The proteome is intrinsically more complex than the genome or transcriptome. Translational rates, protein degradation, protein modifications can all affect protein levels and functions in the cell. These aspects are impossible to be revealed by looking at the RNA level.

There have been rapid developments in proteomics technologies and thus they are becoming feasible to unravel at least part of the mystery at the protein level. Traditionally, proteomics studies used gel-based techniques, like two-dimensional PAGE, to separate protein mixtures and individual protein spots are analyzed by mass spectrometers to identify the protein content; or fluorescence two-dimensional differential gel electrophoresis (2-D DIGE) in which two protein samples are differentially labeled and separated by a 2-D gel to find proteins with different abundance. These methods are useful in identifying proteins in a complex mixture or proteins with altered levels in response to certain stimuli or gene mutations. In recent years, mass spectrometry has been improved dramatically and is becoming more affordable and accessible for many researchers for studying various proteomes. When mass spectrometry is combined with different protein/peptide pre-separation methods including 1-D gel, 2-D gel, or high performance liquid chromatography (HPLC), the throughput of protein detection can be dramatically increased. Proteomics studies of individual tissues, organs, cell types, or even cell organelles usually aim at detecting as many proteins as possible to find proteins associated with certain tissues/organs/cells or subcellular compartments. Comparative proteomics studies can be used to detect proteome-wide changes during development, biotic or abiotic treatments, or between different cells or tissues or organs. Comparison of the proteomes of the wild-type plants and mutants, in which certain proteins involved in post-translational regulations are defective, is useful in characterizing the functions of these regulators.

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Chapter 2

The Role of the Transcription Factor DYSFUNCTIONAL TAPETUM 1 (DYT1) in Controlling *Arabidopsis* Anther Development and Tapetum Function

Abstract

Several transcription factors have been shown to be essential for male fertility in plants, especially in the model plant *Arabidopsis thaliana*, suggesting that transcriptional regulation is a major mechanism controlling anther development. DYSFUNCTIONAL TAPETUM 1 (DYT1), a putative bHLH transcription factor, plays a critical role in regulating tapetum function and pollen development. The comparative transcriptomics of wild-type and *dyt1* mutant young anthers studied by our lab demonstrated that DYT1 is upstream of at least 22 genes encoding transcription factors and it regulates the expression of a large number of genes, including those involved in specific metabolic pathways. This study showed that DYT1 can bind to DNA in a sequence-specific manner *in vitro* and induction of DYT1 activity *in vivo* activated the expression of downstream transcription factor genes *MYB35* and *MS1* at different time points after treatment. Male fertility of *DYT1-SRDX* (a repressor domain) transgenic plants was dramatically reduced, implying that DYT1 probably acts as a transcriptional activator, which is consistent with its role in activating the expression of *MYB35* and *MS1*. The MYB35 was also shown to be a transcriptional activator by using a similar repressor domain fusion method. These results demonstrated the important role of DYT1 in regulating anther transcriptome and tapetum function supporting normal pollen development.

Note: This chapter presents my work that was published in our paper (Feng et al., 2012). Contributions from other authors are not included in the result section of this chapter but only used for discussion.

Introduction

Somatic tissues in reproductive organs provide germ (reproductive) cells with nutrients and signal molecules. In typical flowering plants, the male reproductive organ (the anther) contains four similarly structured lobes, each with a few somatic cell layers surrounding the germ cells, which undergo meiosis to produce microspores. The tapetum layer immediately adjacent to the germ cells is highly active metabolically and nurtures pollen development from microspores. In *Arabidopsis thaliana*, the tapetal cells are formed at anther stage 5, function at anther stage 5 to 9, and degenerate via programmed cell death starting from anther stage 10 to release their contents for pollen wall formation (Sanders et al., 1999). Defects in the tapetum usually cause abnormal pollen development, resulting in reduced fertility.

Previous results support the idea that tapetum development is controlled by a complex transcriptional regulatory network, including several key genes (Ito et al., 2007b; Yang et al., 2007; Zhang et al., 2007; Thorstensen et al., 2008; Zhu et al., 2008). In particular, *SPOROCYTELESS*/*NOZZLE* (*SPL*/*NZZ*) encodes a putative transcription factor and is required for the specification of both germ cells and somatic cell layers (including the tapetum) (Schiefthaler et al., 1999b; Yang et al., 1999c). *ABORTED MICROSPORES* (*AMS*) plays an important role in the tapetum for biosynthesis and secretion of pollen wall components (Sorensen et al., 2003a; Thorstensen et al., 2008). Other transcription factor genes important for the tapetum and pollen development include *MALE STERILITY 1* (*MS1*) (Wilson et al., 2001b; Ito and

Shinozaki, 2002a; Ito et al., 2007b; Yang et al., 2007) and *MYB* family members (Preston et al., 2004a; Millar and Gubler, 2005; Zhang et al., 2007; Zhu et al., 2008). The analysis of genes for these transcription factors and other genes suggests that tapetum development is controlled by a sophisticated transcriptional regulatory network (Zhang et al., 2006; Wijeratne et al., 2007b; Zhu et al., 2008).

Cell-cell signaling is also required to establish the tapetum identity. Previous studies revealed important roles of mitogen-activated protein kinases (MAPKs), MPK3 and MPK6, and leucine-rich repeat receptor-like kinases (LRR-RLKs), ERECTA (ER), ERECTA-LIKE1 (ERL1), and ERL2, in early anther development including tapetal cell differentiation (Ge et al.; Hord et al., 2008). A putative ligand-receptor pair encoded by *TPD1* and *EMS1* is essential for the specification of the tapetal cell fate (Zhao et al., 2002b; Yang et al., 2003a; Yang et al., 2005b; Jia et al., 2008). Mutations in either gene cause the formation of extra microsporocytes and the failure to form tapetal cells. The changes in the *ems1* transcriptome suggest that cell-cell signaling dramatically affects components of a transcriptional regulatory network in the tapetum (Wijeratne et al., 2007b). Another study showed that brassinosteroids regulate key genes for anther development via a transcription factor BRI1-EMS-SUPPRESSOR 1 (BES1), providing a link between phytohormone signaling and transcriptional regulation (Ye et al., 2010).

Recently, a bHLH transcription factor, DYT1, was discovered to be important for tapetum function (Zhang et al., 2006). In the *dyt1* mutant, the tapetal cells are formed but undergo premature vacuolation, with a reduction in the dense cytoplasm as observed in wild-type tapetal cells. Even though *dyt1* meiocytes undergo meiotic nuclear division to produce four haploid nuclei, the malfunction of the tapetum is linked to the failure to produce microspores, resulting in the absence of any developing pollen grains. Previous studies suggested that *DYT1* functions genetically downstream of *SPL* and *TPD1/EMS1*, but upstream of *AMS* and *MS1* (Zhang et al., 2006; Sun et al., 2007). Therefore, *DYT1* integrates early signaling and transcriptional pathways

and controls the expression of late genes in a transcriptional regulatory network for tapetum development. However, the extent of transcriptome regulated by DYT1 and mechanisms of DYT1 function remain to be investigated.

In this study, the DNA-binding specificity of the DYT1 protein was determined *in vitro*. DYT1 was demonstrated to function as a transcriptional activator and the transient induction of DYT1 activated the expression of downstream transcription factor genes *MYB35* and *MS1*. Also, the MYB35 was demonstrated to function as a transcriptional activator, indicating that DYT1 and its downstream transcription factors form a regulatory cascade in controlling tapetum gene expression.

Materials and methods

Plant material and growth conditions

Plants were grown under long-day conditions (16 hours under light/8 hours in dark) in a 22°C growth room. The *dyt1* mutant was genotyped as reported (Zhang *et al.,* 2006).

Recombinant DYT1 protein expression in *E.coli*

The *DYT1* coding sequence (CDS) was amplified with oMC2405 and oMC2406 using AccuPrime Pfx DNA polymerase (Invitrogen), cloned into the pENTR-D/TOPO vector yielding pMC3329, and recombined into the pDEST17 vector *via* LR reaction using Gateway LR Clonase II enzyme mix (Invitrogen). The resulting plasmid pMC3250 was transformed into the *E. coli*

BL21 (DE3) strain. Recombinant 6His-DYT1 proteins were expressed following published methods (Huang et al., 1993).

Gel-shift assays

Biotin-labeled dsDNA probes were synthesized by annealing oligonucleotide individual synthesized ssDNA oligos with Biotin-labeled primer oMC2746 and filling in with Klenow (NEB). The *in vitro* binding reaction was performed according to the published method (Huang et al., 1993). When incubated with proteins expressed from *E.coli* the free and bound probes were separated in a 6% PAGE gel in 0.5×TBE at 100V for 1 hour and transferred to Hybond-N membrane (GE Healthcare) using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad), and cross-linked to the membrane under UV light 120 mJ/cm^2 for 20 sec. The signal was detected by LightShift Chemiluminescent EMSA Kit (PIERCE) and X-film (Kodak).

Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

The SELEX protocol was adapted from a previous study (Huang *et al.,* 1993). A pool of ssDNA oMC2716 containing 30 nt random sequences flanked by 20 nt primer-annealing regions was synthesized (IDT). Biotin-labeled oMC2746 was annealed to oMC2716 to make double-stranded DNAs after Klenow (NEB) fill-in reaction. The biotin-labeled dsDNA product was diluted by 50-fold for gel-shift assays.

5 µl of a DYT1 protein extract and 10 µl of DNA probes were incubated in the binding buffer (10 mM Tris-HCI, pH7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 50 µg/ml poly(dI-dC), 100 µg/ml BSA) for 20 min at 25°C. Then the mixture was separated in a 5% polyacrylamide gel (29:1 acrylamide:bis) in 0.5×TBE running buffer. The gel slice containing

DYT1-DNA complex were cut out (position for shifted band was determined by running duplicate sample in a separate lane and visualizing with LightShift Chemiluminescent EMSA Kit) and soaked in 1×TE buffer overnight at 4°C. The eluted DNA was amplified by PCR for 20-30 cycles with oMC2718 and oMC2746, followed by DNA purification before the next round of gel-shift assay. After 6 rounds, the DNA was amplified for 30 cycles and cloned into the pGEM-T vector (Promega). Plasmids were sequenced with T7 primer to determine the insert sequences. The WebLogo tool (http://weblogo.berkeley.edu) was used to analyze the enrichment of binding sites among the cloned DNA probes.

Plasmid construction and plant transformation

A genomic region containing the *DYT1* gene (without the stop codon) and 1444 bp upstream sequence was amplified using oMC2469 and oMC2470. The GR domain was amplified from the pBI-GR plasmid with oMC2471 and oMC2472. These two fragments were cloned into pCAMBIA1300 yielding *proDYT1:DYT1-GR*. *proDYT1:DYT1-SRDX* was amplified with oMC2801 and oMC2802 and cloned into pCAMBIA1300. Constructs were transformed into *Agrobacterium tumefaciens* strain C3581, which was used to transform plants with floral dip method (Clough and Bent, 1998). *proDYT1:DYT1-GR/dyt1* plants were treated with 10 μM DEX (10 mM DEX stock solution in 100% ethanol diluted by 1000-fold in 0.015% Silwet L-77) or mock (0.1% ethanol in 0.015% Silwet L-77).

A genomic region containing the *MYB35* gene without the stop codon and 1290 bp upstream region was amplified using oMC2922 and oMC2923 and cloned into pCAMBIA1300. oMC3095 and oMC3096 were annealed to form dsDNA *SRDX* with *Pst*I and *Hind*III overhangs before being ligated to the 3' end of *proMYB35:MYB35*. To generate the *MYB35* RNAi construct,

the *MYB35* coding region was cloned into the pRR2222 vector as inverted repeats (Zhao et al., 2003). The native promoter and the inverted repeats of *MYB35* were inserted into a plant expression vector pMDC7. Plant transformation was performed using floral dip method.

Light microscopy

Arabidopsis flower buds were fixed and embedded in Spurr's resin as described previously (Zhang et al., 2006). Semi-thin (5 μm) sections were prepared using a Reichert-Jung Ultracut E microtome (Leica) at the Pennsylvania State University Microscopy and Cytometry Facility - University Park, PA. Sections were stained with 0.1% Toluidine Blue O for 30 sec at 60ºC. Images were taken using Nikon ECLIPSE E400 microscope connected to a DEI-750 CE camera head (Optronics).

Results

DYT1 binds to an E-box variant (TCACGTGA) *in vitro*

We took an unbiased approach, Systematic Evolution of Ligands by Exponential Enrichment (SELEX), to determine DYT1 binding sites *in vitro*. A consensus sequence TCACGTGA was found to be the strongest binding motif for DYT1 (Figure 2-1a). The palindromic feature of the consensus sequence suggested that DYT1 binds to DNA as homodimers. Twelve of the DNA probes enriched from SELEX were tested to determine if they could be bound by DYT1 (Figure 2-1b, c). Directed mutagenesis analysis of one half of the palindromic sequence indicated that all 8 nucleotides are essential for DYT1 binding under the *in*

vitro conditions. Changing a flanking nucleotide (p6 oMC6291) did not abolish DYT1 binding (Figure 2-1d). Sometimes two or more closely spaced sequences differing from binding consensus could bind to a transcription factor *in vitro* or *in vivo*. We also found that DYT1 could bind to probes (oMC6328, oMC6330, and oMC6338) containing two E-boxes differing from the consensus sequence at the first position (Figure 2-1b, c), suggesting that DYT1 might bind to distinct sequences with different affinity. Weak DYT1-DNA interactions that evade detection might be greatly enhanced by cooperative binding of DYT1 to multiple sites.

Microarrays of *dyt1* and wild-type anthers of stage 4-7 are available in our lab. The information of up-/down-regulated genes in *dyt1* mutant is used to determine whether these genes share common regulatory cis-elements. We found that 12 genes differentially expressed in *dyt1*, in addition to *MS1*, contain the consensus DYT1 binding sites within 1 kb from the transcription start sites, indicating that they might be DYT1 target genes. However, many other genes affected in the *dyt1* mutant do not contain the consensus DYT1-binding site, implying that they might be indirectly regulated by DYT1. If some of these genes are directly regulated by DYT1, then DYT1 could also bind to other sequences *in vivo,* perhaps by interacting with other transcription factors.

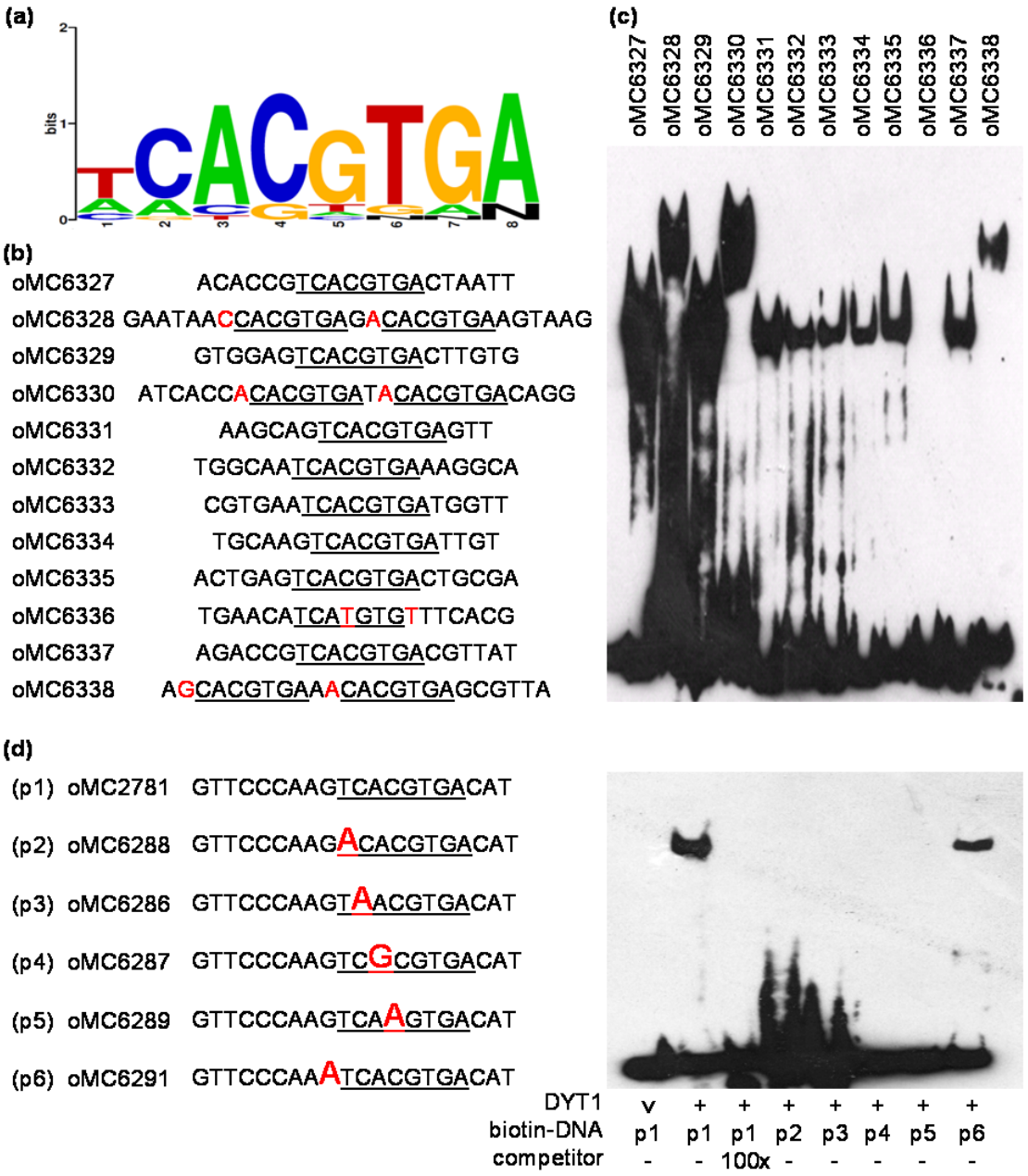

Figure 2-1. Determination of DYT1 *in vitro* binding sites.

(a) Consensus binding sequence of DYT1 determined by SELEX.

(b) Representative DNA probes enriched by SELEX (oMC6327-oMC6338). Possible binding sites are underlined. Red-colored nucleotides are different from the consensus sequence.

(c) Gel-shift assay to confirm the interaction between probes listed in (b) with DYT1.

(d) Gel-shift assay to determine DYT1 binding specificity. Probe sequences are listed on the left and gel-shift image on the right. Lysate of *E.coli* containing only the vector (v) could not bind probes. A 100-fold excess amount of unlabeled competitor probes and point mutations in one half of the palindromic consensus sequence (probe p2-p5) abolished DYT1 binding. Probe (p6) with a mutation in the -1 position could still interact with DYT1.

DYT1 activates the expression of downstream transcription factors

The observation that the *dyt1* mutation caused the reduction in expression of many genes, while increasing the expression of others suggested that DYT1 could act either as an activator or repressor of gene expression. To distinguish these possibilities, we reasoned that if DYT1 is an activator, then a fusion of DYT1 to a strong repressor domain would inhibit its function, resulting in a *dyt1* mutant-like phenotype; in contrast, if DYT1 is a repressor, then the fusion to a repressor domain would not affect male fertility. Therefore, we generated transgenic plants containing a fusion gene of the *DYT1* promoter and coding region to that encoding SRDX, which was shown to be a strong repressor (Hiratsu et al., 2003). The *proDYT1:DYT1-SRDX*/Col-0 plants produced mostly short siliques (Figure 2-2b) and sterile (Figure 2-2d) or partially fertile anthers (Figure 2-2e). The suppression of male fertility by DYT1-SRDX strongly suggests that DYT1 likely acts as an activator of gene expression.

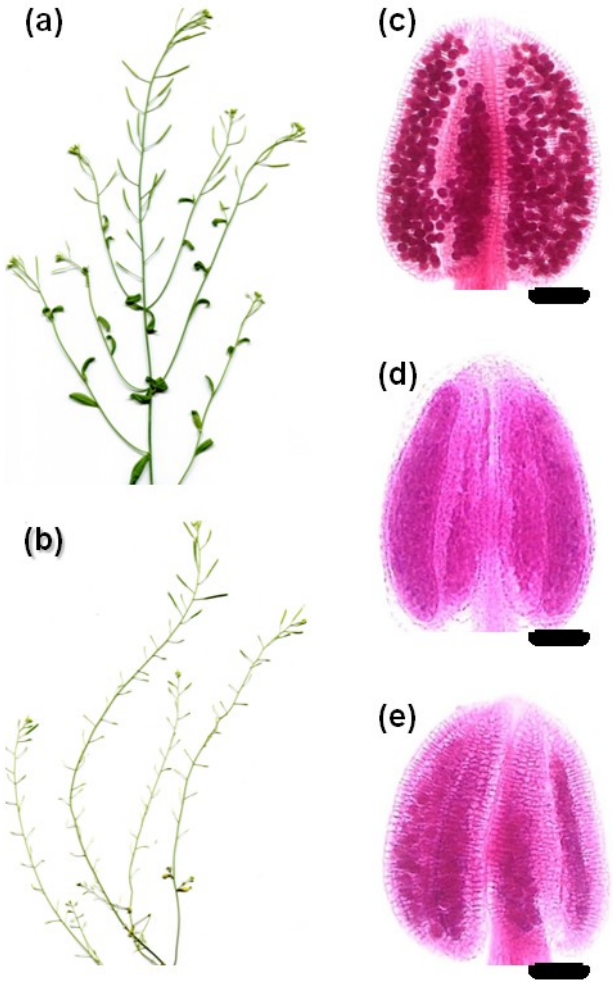

Figure 2-2. DYT1-SRDX suppresses male fertility.

(a) Col-0 plant.

(b) *DYT1-SRDX*/Col-0 plant.

(c) Col-0 anther.

(d) Completely sterile *DYT1-SRDX*/Col-0 anther. (e) Partial fertile *DYT1-SRDX*/Col-0 anther.

Scale bars: 50 μm in (c), (d) and (e).

Then we tested whether DYT1 can activate downstream gene expression by fusing DYT1 to the ligand-binding domain of the glucocorticoid receptor (GR), which was shown to regulate plant transcription factors in a ligand dependent manner (Aoyama *et al*., 1997; Gatz 1997; Ito *et al.*, 2004; Wagner *et al.,* 1999). The fusion construct *proDYT1:DYT1-GR* was introduced into the *dyt1* background. *proDYT1:DYT1-GR/dyt1* plants without Dexamethasone (DEX) treatment produced mostly short siliques (Figure 2-3a) and mainly dead pollen grains (Figure 2-3c), with few fertile siliques probably due to leaky nuclear transport of DYT1-GR proteins. A single treatment of 10 μM DEX restored male fertility of *proDYT1:DYT1-GR/dyt1* (Figure 2-3b, d). To test the regulation by DYT1, the expression levels of *MYB35* and *MS1* were determined by real-time PCR at several time points after the 10 μM DEX treatment. The *MYB35* gene expression was rapidly increased by >3-fold after 1.5 hours of 10 μM DEX treatment and its elevated expression level was maintained for several hours. After 6 hours, *MYB35* expression started to decrease with a transient increase at 12 hours. The fluctuation of *MYB35* level might result from the transient strong DEX induction of DYT1-GR and a later decline due to diminishing DEX concentration. *MS1* was induced after 12 hours of 10 μM DEX treatment. These results suggested that DYT1 might regulate the expression of different genes in temporally distinctive patterns. The expression level of *MYB35* in *dyt1* was reduced by >10-fold compared with wild-type. However, its expression did not change in *ms1*. Therefore, *MYB35* probably functions downstream of *DYT1* but upstream of *MS1*, as also suggested by a previous study (Zhu et al., 2008).

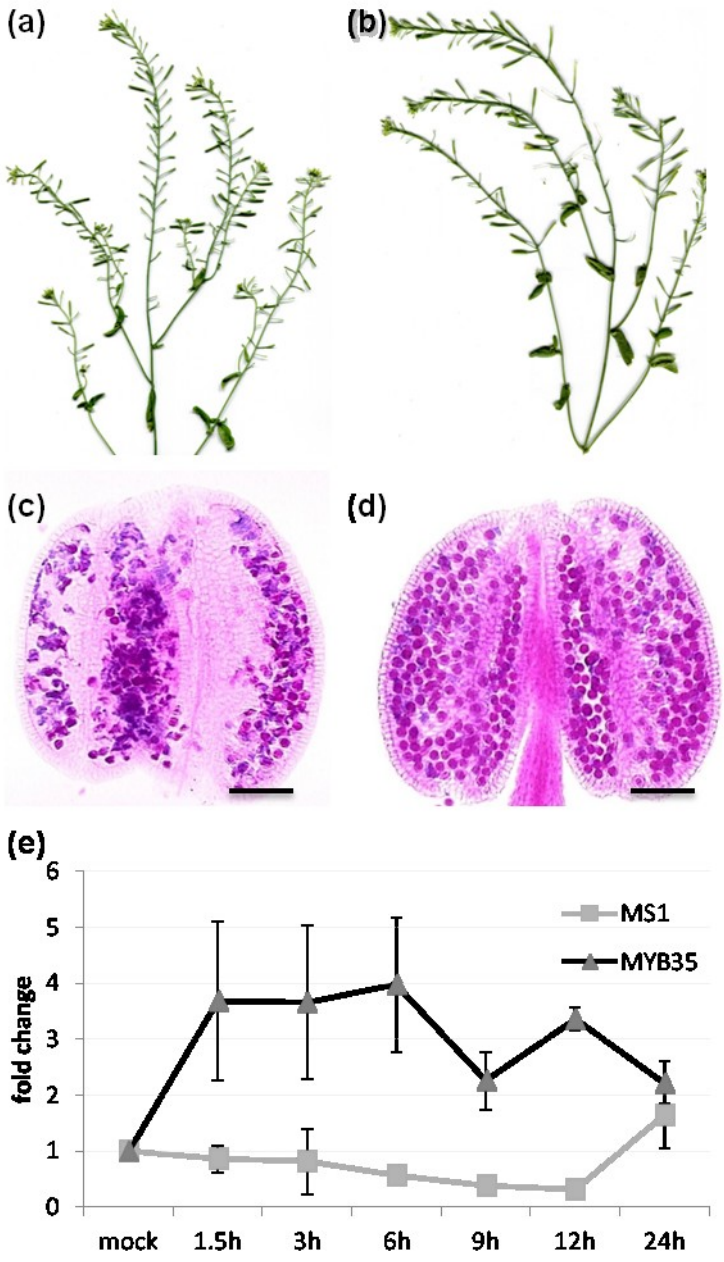

Figure 2-3. DYT1-GR induction restores male fertility in *dyt1*.

(a) DYT1-GR/*dyt1* plant without DEX treatment. Most siliques are short.

(b) DYT1-GR/*dyt1* plant 10 days after 10 μM DEX treatment. Most siliques are long.

(c) DYT1-GR/*dyt1* anther without DEX treatment contains mostly dead pollen grains.

(d) DYT1-GR/*dyt1* anther 10 days after 10 μM DEX treatment contains mostly mature pollen grains.

(e) Real-time PCR result of *MYB35* and *MS1* expression in 10 μM DEX-treated flower buds. Three biological replicates were performed for each time point. Gene expression levels in mock-treated samples were set as 1.

Scale bars: 50 μm in (c) and (d).

MYB35 functions as a transcriptional activator downstream of DYT1

Among the 22 transcription factor genes decreased in expression in *dyt1* anthers, *MYB35* expression showed the most reduction. To test whether *MYB35* acts as a transcriptional activator, we generated *proMYB35:MYB35-SRDX*/L*er* (*MYB35-SRDX* for short) transgenic plants, among which 11 out of 80 were completely male sterile (the low ratio of complete sterile plants may due to low expression of the transgene driven by the native promoter of *MYB35*). Unlike wild-type anthers (Figure 2-4a), anthers of these 11 plants could not produce pollen grains (Figure 2-4f). These plants produced seeds after being pollinated with wild-type pollen, indicating that the female fertility was not impaired. This is consistent with the fact that the native promoter of *MYB35* drives its expression only in the anther. Semi-thin sections of anthers showed that tapetal cells of *MYB35-SRDX* anthers had larger vacuoles than those in the wild-type at stage 6 (Figure 2-4b, g). At stage 7, *MYB35-SRDX* anthers completed meiosis and produced tetrads, but the tapetal cells had even larger vacuoles than those in wild-type (Figure 2-4c, h). No obvious defects of chromosome behaviors were observed in *MYB35-SRDX* meiocytes using DAPI staining (Figure 2-5p-t), indicating that MYB35 probably does not regulate meiosis. At stage 8, *MYB35-*

SRDX microspores were not released from the tetrads and the tapetum degenerated earlier than that of wild-type (Figure 2-4d, i). Finally, *MYB35-SRDX* anthers were devoid of microspores after stage 9 (Figure 2-4j), unlike the wild-type anther (Figure 2-4e). The phenotypes of *MYB35-SRDX* sterile plants were similar to those of the *MYB35* RNAi plants that were generated in our lab (Figure 2-5f-j) and those of the *myb35/tdf1* mutant previously reported (Zhu et al., 2008). These results suggested that MYB35 most likely functions as a transcriptional activator.

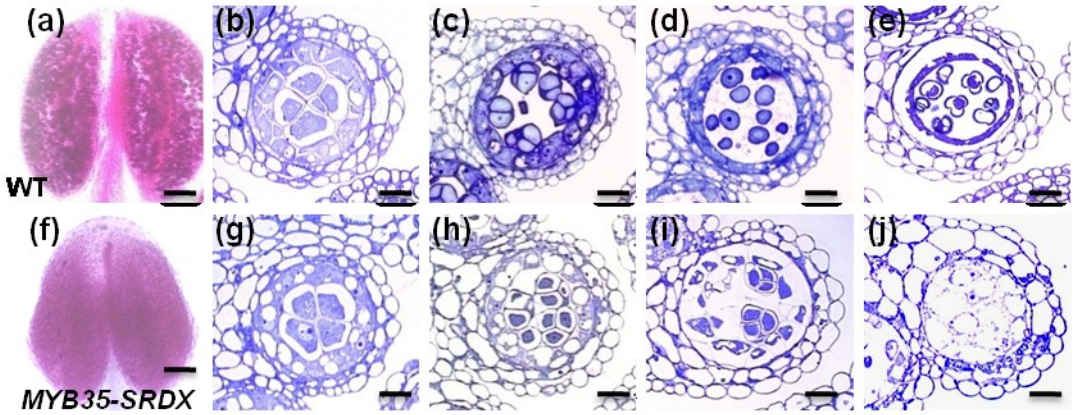

Figure 2-4. *MYB35-SRDX* transgenic plants have abnormal tapetum and microspore development.

(a-e) Wild-type (WT) anthers.

(f-j) *MYB35-SRDX* transgenic plant anthers.

(a) WT anther staining showing mature pollen grains.

(f) *MYB35-SRDX* anther staining showing no pollen grains.

(b-e) WT anthers of stage 6-9.

(g-j) *MYB35-SRDX* anthers of stage 6-9.

Scale bars: 50 μm in (a) and (f); 10 μm in (b)-(e) and (g)-(j).

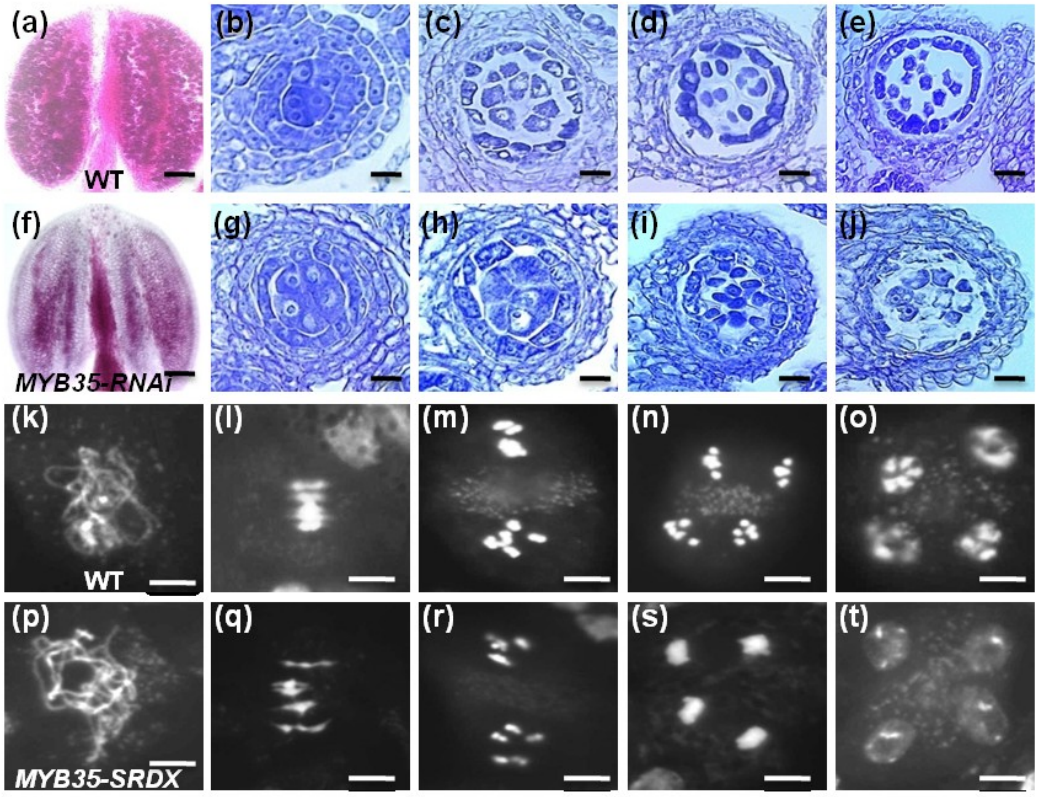

Figure 2-5. *MYB35-RNAi* abnormal anthers and *MYB35-SRDX* normal male meiosis.

(a-e) Wild-type (WT) anthers.

(f-j) Anthers from the *MYB35-RNAi* transgenic plants.

(a) WT anther showing stained mature pollen grains.

(f) *MYB35-RNAi* anther lacking stained pollen grains.

(b-e) WT anthers of stage 6-9.

(g-j) *MYB35-RNAi* anthers of stage 6-9.

(k-o) WT and (p-t) *MYB35-SRDX* male meiosis. Prophase I (k, p), metaphase I (l, q), anaphase I (m, r), anaphase II (n, s), and telophase II (o, t). *MYB35-SRDX* shows no obvious abnormal phenotype in meiosis.

Scale bars, 50 μm in (a) and (f); 10 μm in (b)-(e) and (g)-(j); 10 μm in (k)-(t).

Discussion

For successful male reproductive development in plants, proper differentiation of meiocytes and surrounding somatic tissues including the tapetum is crucial. Transcriptional regulation is a major mechanism controlling cell differentiation and function. The *dyt1* mutant is defective in the tapetum function around the time of meiosis, providing an excellent system for studying the regulation of the tapetum transcriptome. By investigating the transcriptome of anthers at stages 4-7, we were able to exclude noise signals from other stages or other floral organs. The results on the altered expression of genes in various biochemical pathways provided potential explanations for morphological defects in the *dyt1* tapetum. The tapetal cells in *dyt1* are precociously vacuolated and degenerated around meiosis stages, suggesting a role of DYT1 in promoting and maintaining the active tapetum function to facilitate meiotic cytokinesis (Zhang et al., 2006). The comparative transcriptome analysis of *dyt1* and wild-type anthers indicates that DYT1 is needed for normal expression of pathways including lipid metabolism and transport, pollen coat formation, cell wall modification, lignin and flavonoid biosynthesis, transport.

The abnormally thin callose wall of *dyt1* meiocytes indicates that either the callose synthesis in the meiocytes or the callose precursor biosynthesis and transport processes in the tapetum are impaired. The microarray data showed that the expression of known callose synthase genes was not reduced in *dyt1*, suggesting that meiocytes should have callose synthesis machineries but lacking building blocks (Ariizumi and Toriyama, 2011). Six sugar transporter genes are downregulated in *dyt1*, suggesting that the transport of callose precursors from the tapetum might be defective. Furthermore, 34 cell wall-related enzymes are downregulated in *dyt1*. In normal anthers, these enzymes might be expressed in the tapetum during stage 4-7 and secreted for meiocyte callose wall dissolution after the tetrad stage. The enzymes might also be

involved in the dynamic tapetal cell wall modifications to facilitate material transport from the tapetum to locules, as reported for the tomato anther (Polowick and Sawhney, 1993).

DYT1 also regulates the biosynthesis, storage, and transport of pollen coat materials including oleosins, arabinogalactan proteins, lipids, and flavonoids. Oleosins, lipids and flavonoids are synthesized in tapetal cells and stored in organelles called tapetosomes until being deposited onto pollen coat after the degeneration of tapetal cells (Hernandez-Pinzon et al., 1999; Hsieh and Huang, 2005, 2007). The glycine-rich protein GRP19 (oleosin–like protein), which is abundant in the pollen coat, functions as an emulsifier for lipid deposition, and is required for pollination (Mayfield et al., 2001). Defects in these pathways may affect male fertility (van der Meer et al., 1992; Aarts et al., 1995; Ariizumi et al., 2004). For example, the *ms2* mutations cause pollen wall defects and male sterility (Aarts *et al.,* 1997).

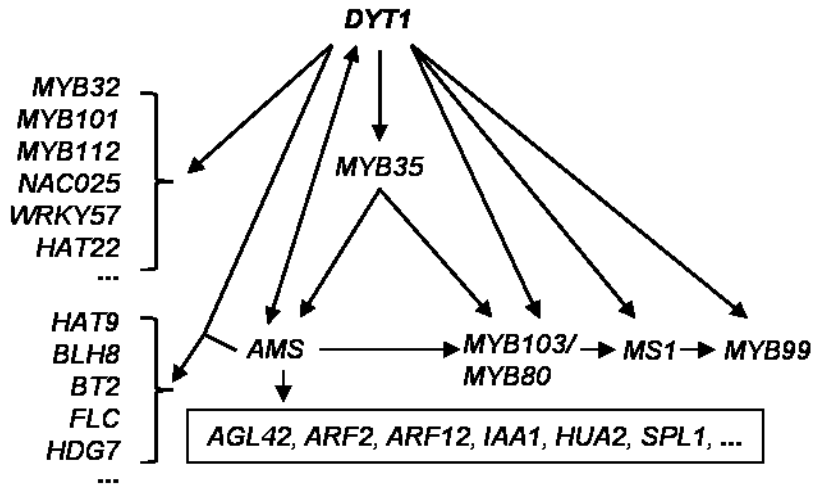

Figure 2-6. A model of the regulatory network controlled by DYT1.

DYT1, MYB35, AMS, MYB103/MYB80, and *MS1* were reported to be essential for anther development. Other genes are downstream of DYT1 but their functions in anther development are unknown. Arrows represent positive regulations. The double arrow represents physical interactions.

Previous studies suggested that the tapetum function is tightly coupled with the progress of meiosis and pollen maturation (Ma, 2005; Zhu et al., 2008). The transcriptome analysis suggested that DYT1 might function as a hub in the transcriptional regulatory network, which precisely controls the tapetum function. A putative regulatory network model was proposed in Figure 2-6, in which DYT1 might regulate downstream genes through multiple mechanisms.

In particular, DYT1 might regulate distinct temporal patterns of gene expression through feed-forward loops. The feed-forward loop is a minimally three-node network motif in which one transcription factor regulate a second transcription factor and then they together regulate a third gene (Shen-Orr et al., 2002). DYT1 can regulate a target gene either by directly binding to its promoter or by activating an intermediate transcription factor, which can also bind to the promoter of the same target gene (Figure 2-6). For example, DYT1 may bind to the *MS1* promoter, which contains the DYT1-binding site as shown by the gel-shift assay (Figure 2-1). DYT1 also positively regulates *MYB99*, which is also regulated by MS1 (Figure 2-6). Therefore, *DYT1-MS1-MYB99* might form a feed-forward loop. In another transcription cascade, DYT1 regulates *MYB35* and *MYB103/MYB80*, and *MYB35* also regulates *MYB103/MYB80* (Zhu et al., 2008). Thus, *DYT1-MYB35-MYB103/MYB80* might also form a feed-forward loop (Figure 2-6). The feed-forward loops provide a mechanism for temporal separation of different target genes of DYT1: those requiring only DYT1 can be activated as soon as the DYT1 level reaches a threshold; whereas those requiring both DYT1 and the product of another DYT1 target gene need to wait for both regulators to be expressed at sufficient levels. However, concrete evidence of *in vivo* protein-promoter interactions is required to support these regulatory loops.

Another potential regulatory mechanism of DYT1 is the direct regulation by DYT1 of a number of genes encoding enzymes and structural proteins, including cell wall proteins; such a single input motif directly transfers a regulatory input signal to an effector for specific

biochemical functions. Our microarray and *in vitro* binding site analyses suggested that DYT1 might directly regulate a group of effector genes to initiate of the tapetum function at early stages.

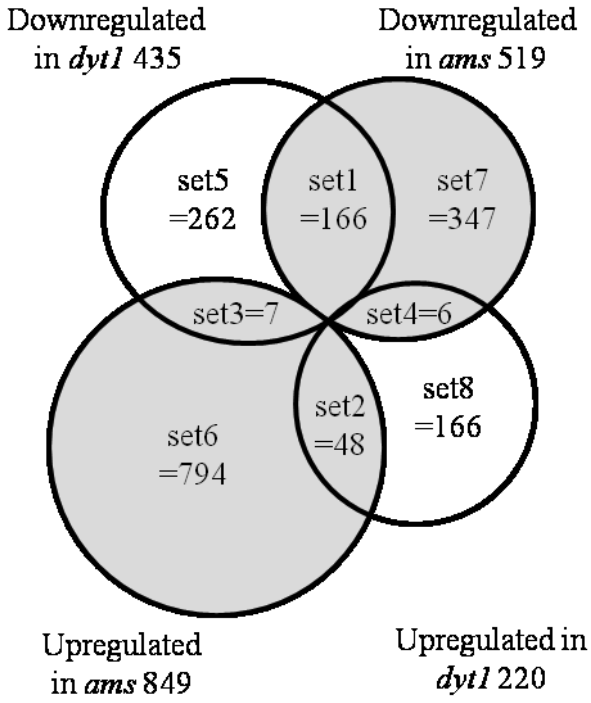

Figure 2-7. A Venn diagram showing the overlap between genes affected in *dyt1* and *ams* anthers.

All the differentially expressed genes in *dyt1* and *ams* anthers were divided into 8 sets according to their overlap. The numbers in the circles represent gene numbers of individual sets. The numbers outside the circles represent the total numbers of genes that are differentially expressed in *dyt1* or *ams*.

The microarray results from our lab showed that the expression of 166 genes was significantly reduced in both *dyt1* and *am*s (Figure 2-7, set1). These genes might be regulated by AMS alone following the linear DYT1-MYB35-AMS cascade, or by DYT1-AMS heterodimers as suggested by the yeast two-hybrid results from our paper (Feng et al., 2012). The DYT1-AMS heterodimers might regulate a distinct group of genes differing from those regulated by other complexes involving DYT1 or AMS separately. The expression of these genes requires double regulatory inputs and thus is limited to a defined time window when DYT1 and AMS are both present.

As shown in Figure 2-7, DYT1 and AMS also regulate distinct sets of genes; 428 genes were regulated by DYT1 but not by AMS (Figure 2-7, set5 and set8). In other words, the expression of these *DYT1* downstream genes does not require *AMS*. Conversely, AMS might regulate 1141 genes independent of DYT1 (Figure 2-7, set6 and set7). It is possible that the reduced AMS level in *dyt1* (not a null mutant) still allows residual AMS function, while the AMS function is abolished in *ams* (a null mutant). Formation of different transcriptional complexes might be another mechanism that differentiates target genes regulated by DYT1 and/or AMS. For example, a SET domain protein ASH1-RELATED 3 (ASHR3) was shown to interact with AMS (Thorstensen *et al*., 2008). DYT1 might interact with different proteins including three bHLH proteins (Figure 2-8). The different complexes might have different DNA binding specificities and therefore regulate different genes.

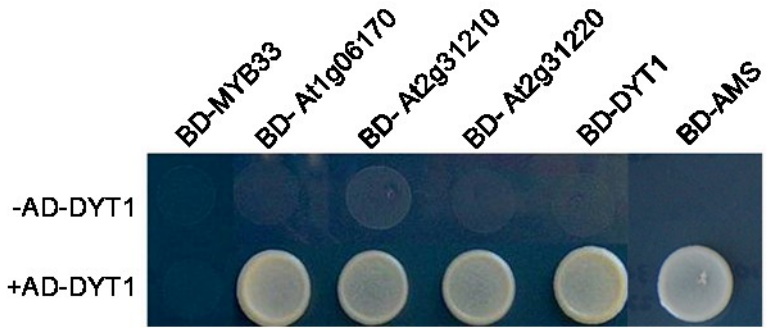

Figure 2-8. DYT1 interacts with itself, AMS and three bHLH transcription factors preferentially expressed in the anther, but not with MYB33.

Interactions were tested with proteins fused with DNA-binding domain (BD) and DYT1 fused with activation domain (AD). Yeast transformants were spotted on SD-His-Ade plates to score potential interactions.

In summary, this study highlights the function of DYT1 as a key transcriptional regulator of the tapetum function and male fertility. Further investigations of the transcriptional regulatory network are necessary to provide more insights into the mechanisms controlling anther development in *Arabidopsis*.

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Chapter 3

Post-Translational Regulation of Flower Development by ASK1-Containing E3 Ubiquitin Ligases in *Arabidopsis thaliana*

Abstract

The ARABIDOPSIS SKP1-LIKE1 (ASK1) functions as a subunit of Skp1-Cul1-F-box (SCF) E3 ubiquitin ligases. It connects the Cul1 subunit and F-box proteins, which specifically interact with substrates to be ubiquitinated. Previous studies suggested an important role of ASK1 in regulating *Arabidopsis* flower development and male meiosis. However, the impact of ASK1-containing SCF E3 ubiquitin ligases (ASK1-E3s) on the floral proteome and transcriptome is largely unknown. This study identified proteins/genes that are possibly regulated by ASK1-E3s by comparing the floral bud proteomes and transcriptomes of an *ask1* mutant and wild-type plants. Proteins that were only detected in the *ask1* mutant and those with higher levels in the *ask1* mutant than in the wild-type may be regulated at the protein level by ASK1-E3s because their RNA levels were not significantly affected by the *ask1* mutation. Integrated analyses of proteomics and transcriptomics of *ask1* and wild-type uncovered several novel aspects of ASK1-E3 functions, including regulation of transcription regulators, kinases, peptidases, and ribosomal proteins. Possible mechanisms of ASK1-E3 functions in regulating the proteome and transcriptome are discussed. The information from this study shed light on the functions of the ubiquitin-proteasome system in *Arabidopsis thaliana* and opens up many possibilities for further research.

Note: The results in this chapter are included in my submitted manuscript. The microarray experiment was performed by my co-authors., and the microarray data analysis and proteomics analysis were performed by me.

Introduction

The ubiquitin-proteasome system (UPS) plays important roles in targeted protein degradation (Smalle and Vierstra, 2004; Schwechheimer et al., 2009; Sorokin et al., 2009). Ubiquitination reactions are catalyzed by E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases. Multiple ubiquitin molecules can be attached to the existing ubiquitin moieties on the protein substrates to form polyubiquitin chains and the polyubiquitinated proteins are then degraded by the 26S proteasome.

The UPS is involved in many processes of plant biology including, but not limited to, plant development, and biotic and abiotic stress responses (Smalle and Vierstra, 2004; Dreher and Callis, 2007; Stone and Callis, 2007; Schwechheimer et al., 2009). This broad spectrum of functions is made possible by the large number of genes encoding the components in the UPS. Eukaryotes often contain a small number of E1 enzymes, dozens of E2 enzymes, and hundreds or even thousands of E3 enzymes. Among these enzymes, the E3 ubiquitin ligases determine the specificities of the substrates. Therefore, the large number of E3 ligases indicates that they can ubiquitinate many proteins. In addition, the modular design of multisubunit E3 ubiquitin ligases greatly increases the possible combinations of subunits and expands the number of substrates that can be specifically ubiquitinated. For example, the subunits of Skp1-Cullin-F-box (SCF) complexes are encoded by multi-gene families, especially the F-box proteins, which are encoded by tens to hundreds of genes in eukaryotic organisms. Thus, SCF complexes with different

specificities can be made by changing only one or few subunits without replacing the entire protein complexes.

Previous studies have identified some F-box proteins that are involved in different pathways in plants. Among the F-box proteins important for hormone signaling, TRANSPORT INHIBITOR RESPONSE 1 (TIR1) is the receptor of auxin and the SCF^{TIR1} complex ubiquitinates the transcriptional repressors (AUX/IAA proteins) for degradation to derepress auxin-responsive genes (Gray et al., 1999; Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The F-box protein CORONATINE INSENSITIVE1 (COI1) regulates jasmonic acid (JA) signaling with a mechanism similar to TIR1, in which the COI1 functions as the receptor of JA and the SCF^{COI1} complex destabilizes JAZ proteins resulting in the derepression of MYC2, a G-box-binding transcription factor that regulates JA-responsive genes (Devoto et al., 2002; Xu et al., 2002; Thines et al., 2007). Other hormone signaling pathways including ethylene, gibberellic acid (GA), and abscisic acid (ABA) pathways also require components of the UPS (Stone and Callis, 2007). Some F-box proteins have been found to regulate non-hormonal pathways. For example, S-locus F-box proteins (SLFs) function as the pollen-specific determinants of self-incompatibility (Lai et al., 2002; Hua and Kao, 2006; Hua et al., 2007; Fields et al., 2010); UNUSUAL FLORAL ORGANS (UFO) is an F-box protein involved in controlling meristem identity and floral organ development (Ingram et al., 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995).

In *Arabidopsis*, the *ASK1* gene encodes an SCF subunit which links Cul1 and F-box proteins (Yang et al., 1999a). It has been shown that ASK1 can interact with many F-box proteins, e.g., UFO (Samach et al., 1999b; Takahashi et al., 2004b), COI1 (Dai et al., 2002), and TIR1 (Gray et al., 1999). Since these F-box proteins have important roles in different pathways, ASK1, as a key component in SCFs, should have crucial functions in many processes. This was suggested by previous genetic studies of the *ask1* mutant which shows defects in male meiosis,

petal development, and vegetative growth (Yang et al., 1999a). Although a few substrates of SCFs have been identified, they are mainly specific to the well studied F-box proteins described above. A large number of other ASK1-interacting F-box proteins and their substrates as well as the biological pathways regulated by ASK1-E3s still remain elusive.

Most of the known ubiquitin ligase substrates were identified by protein-protein interaction methods. However, these methods are mostly small scale and technically challenging. Recently, mass spectrometry (MS)-based proteomics approaches have been increasingly applied in various areas including differential gene expression, post-translational modifications, disease marker discovery, etc. Several studies used proteomics approaches to identify ubiquitin ligase substrates either by identification of ubiquitinated proteins (Maor et al., 2007; Igawa et al., 2009), or by comparing proteomes of the wild-type and mutants of E3 ubiquitin ligases (Burande et al., 2009).

This study used a proteomics approach, Multidimensional Protein Identification Technology (MudPIT), to identify ASK1-regulated proteins by comparing flower bud proteomes of the *ask1* mutant and wild-type plants. A comparative transcriptomics analysis of *ask1* and wild-type flower buds was also performed to determine whether ASK1 regulates protein abundance at the transcriptional level or post-transcriptional level. The integrated proteomics and transcriptomics analysis revealed that many proteins are potentially regulated by ASK1-E3s. It is proposed that ASK1 may regulate the *Arabidopsis* proteome and transcriptome through novel mechanisms.

Materials and methods

Plant material and growth conditions

The *Arabidopsis thaliana* ecotype Landsberg *erecta* (L*er*) and the *ask1* mutant within the L*er* background (Yang et al., 1999a) were used. Seeds were planted into soil (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA, http://www.sungro.com) and kept at 4°C for 2-4 days before being moved to a growth room with a temperature of 23°C and long day conditions (16 hour light and 8 hour dark). The *ask1* mutant plants were characterized from the population of *ASK1*+/- progeny by their abnormal petals, sterile anthers, and short siliques. Unopened flower buds of the *ask1* mutant and L*er* were collected from 4-5 week old plants after a few siliques being produced.

Microarray analysis of the *ask1* mutant and L*er* flower buds

The total RNA of the L*er* and *ask1* flower buds was extracted using the NucleoSpin® RNA Plant kit (MACHEREY-NAGEL, Bethlehem, PA, http://www.mn-net.com). RNA quality analysis was performed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, http://www.genomics.agilent.com), controlled by the Agilent 2100 Expert software, using the Plant RNA Nano assay following the RNA 6000 Nano kit protocol. Microarray was performed using the GeneChip *Arabidopsis* ATH1 Genome Array (Affymetrix, Santa Clara, CA, http://www.affymetrix.com) in the Penn State Genomics Core Facility – University Park, PA. Three replicates of *ask1* and two replicates of L*er* were performed.

Data analysis was conducted as previously described with a few modifications (Feng et al., 2012b). Microarray datasets (.CEL files) were normalized by the R package RMA and

exported as Excel files. Microarray signal values were averaged from replicates of each genotype and compared between *ask1* and L*er* to find differentially expressed genes. GO categorization was conducted using the Singular Enrichment Analysis (SEA) from agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) (Du et al., 2010). The Affymetrix ATH1 Genome Array (GPL198) was selected as the background reference which contains 22479 annotated genes. The statistical test was set to Fisher and significance level set to 0.05. The raw microarray datasets were deposited to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) with the accession number GSE42841.

Flower bud protein extraction with the Trichloroacetic Acid /Acetone method

The protein extraction method was modified from a previous study (Damerval et al., 1986). Flower buds were ground thoroughly in liquid nitrogen with mortars and pestles and the powder was suspended in -20°C Acetone with 10% w/v Trichloroacetic Acid (TCA) and 0.07% v/v β-Mercaptoethanol (1 ml for 0.3 g of tissue powder). After being incubated for 2 hours (or overnight) at -20°C, the protein suspension was centrifuged for 15-20 min at 14,000 rpm. The supernatant was removed and the protein pellet was resuspended and washed with 1 ml of -20°C Acetone containing 0.07% v/v β-Mercaptoethanol followed by centrifugation for 15-20 min at 14,000 rpm. This washing step was repeated until the pellet was almost white. The protein pellet was vacuum dried for 5-10 min and stored at -20°C or immediately used for trypsin digestion.

In-solution trypsin digestion of flower bud proteins of *ask1* and L*er*

About 20-30 mg of protein extract from the TCA/Acetone method was resuspended in 1 ml of rehydration buffer [100 mM NH_4HCO_3, 10 mM Dithiothreitol (DTT), 10% v/v

Acetonitrile] and sonicated for 5 times, 20 seconds each time, duty cycle 40%, power 3 using a Branson Sonifier S-450A (Branson Ultrasonics, Danbury, CT, http://www.emersonindustrial.com/en-US/branson/Pages/home.aspx). Proteins were denatured at 60°C for 45–60 min and alkylated by 50 mM Iodoacetamide at 37 °C for 30 min in dark. 40 µl of 1 M DTT was added to quench the alkylation reaction. Alkylated proteins were digested by 20 µg of Trypsin Gold, Mass Spectrometry Grade (Promega, Madison, WI, www.promega.com) for 16-18 hours at 37°C with moderate shaking. The indigestible debris was removed by centrifugation at 12,000 rpm for 10min. The supernatant was transferred to a new 1.5 ml tube and centrifuged again to remove residual debris. The supernatant was transferred to a new 1.5 ml tube and was adjust to pH 3.0 with glacial acetic acid. The peptide solution was vacuum dried completely to evaporate off NH_4HCO_3 and acetonitrile. The pellet was resuspended in 200 µl H_2O and vacuum dried. Three repeats of resuspension and drying were performed in total. Finally the peptides were analyzed in the Proteomics and Mass Spec Core Facility, College of Medicine, Pennsylvania State University, Hershey, PA.

Mass spectrometry/MudPIT analysis of proteins from *ask1* and L*er* flower buds

Trypsin-digested protein samples were analyzed by MudPIT according to the 2D LC-MALDI separation and analysis procedures provided by the facility website (http://www.pennstatehershey.org/web/core/proteinsmassspectometry/protocols/data-analysis) and published previously (Zhao et al., 2008) except several modifications. The ProteinPilot software version 4.2 was used to perform protein identification and quantification by searching MS spectra against the protein database which included the *Arabidopsis thaliana* protein list TAIR10_pep_20101214 (downloaded from website ftp://ftp.arabidopsis.org/home/tair/Proteins/TAIR10_protein_lists/), 156 common human and lab

contaminants (ABSciex_ContaminantDB_20070711), and a reverse "decoy" version of the protein database itself (concatenated Reverse Decoy Database). Proteins with local FDR<1% were accepted as detected.

Proteomics data analysis using the spectral counting method

Since each sample was analyzed by MudPIT individually without labeling and multiplexing, the abundance of detected proteins in different samples was normalized using the spectral counting method as previously described (Baerenfaller et al., 2008). The modified formula is:

Abundance of protein K= [(measured spectra of K) / (measured spectra of all proteins in dataset)] / [(theoretical peptides of K) / (theoretical peptides of all proteins in dataset)].

The normalized protein abundance values in *ask1* and L*er* samples were averaged respectively for comparison to find proteins with higher or lower levels in the *ask1* proteome.

Results

Transcriptomics analysis of L*er* and *ask1* flower buds

L*er* and *ask1* flower bud transcriptomes were analyzed using GeneChip *Arabidopsis* ATH1 Genome Array. The average values from L*er* and *ask1* microarrays were compared to find genes whose RNA levels differ by at least two fold (p-value <0.05). We found 74 and 42 genes that were up-regulated and down-regulated, respectively, in *ask1* transcriptome compared with L*er*. We used agriGO (Du et al., 2010) to determine if certain gene categories are over-represented in the up-/down-regulated genes in *ask1*. We found that genes responsive to various

stimuli or stresses are significantly enriched (Figure 3-1). Among the 42 down-regulated genes (including *ASK1* gene) in *ask1*, 19 genes are related to biotic/abiotic signaling pathways (Table 3-1), including hormone, light/circadian, temperature, salt, and other signaling pathways. Among the 74 up-regulated genes in *ask1*, 39 genes were annotated to be involved in response to various biotic/ abiotic signals (Table 3-2). The functions of most of these genes are not well understood except for evidence from transcriptional responses to stimuli (e.g., *COLD-REGULATED 15A/15B, DARK INDUCIBLE 10, SENESCENCE 1*, etc.) and sequence homology (e.g., HAD superfamily acid phosphatase, *JUMONJI DOMAIN CONTAINING 5*, *CONSTANS-LIKE 2*, etc.). Nevertheless, a few genes have been relatively well investigated: *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1*), *JASMONATE-ZIM-DOMAIN PROTEIN 5* (*JAZ5*), etc. *CCA1* and *LHY* encode Myb-like transcription factors that synergistically regulate circadian rhythm of *Arabidopsis* (Lu et al., 2009). *JAZ* genes encode repressors, which are degraded by SCF^{COI1} and whose transcription is induced by JA through a feedback loop involving JAZ proteins and MYC2 (Chini et al., 2007). Here is a paradox: the mutation of *ASK1* gene should reduce SCF activities and JAZ proteins should be stabilized and repress MYC2 activity for transcriptional activation of JAZ genes, therefore reducing the JAZ transcript levels; however, our data show that *JAZ1* and *JAZ5* transcript levels are elevated in the *ask1* mutant than in wild-type. This paradox indicates that a novel mechanism may be involved in modulating the JA signaling pathway.

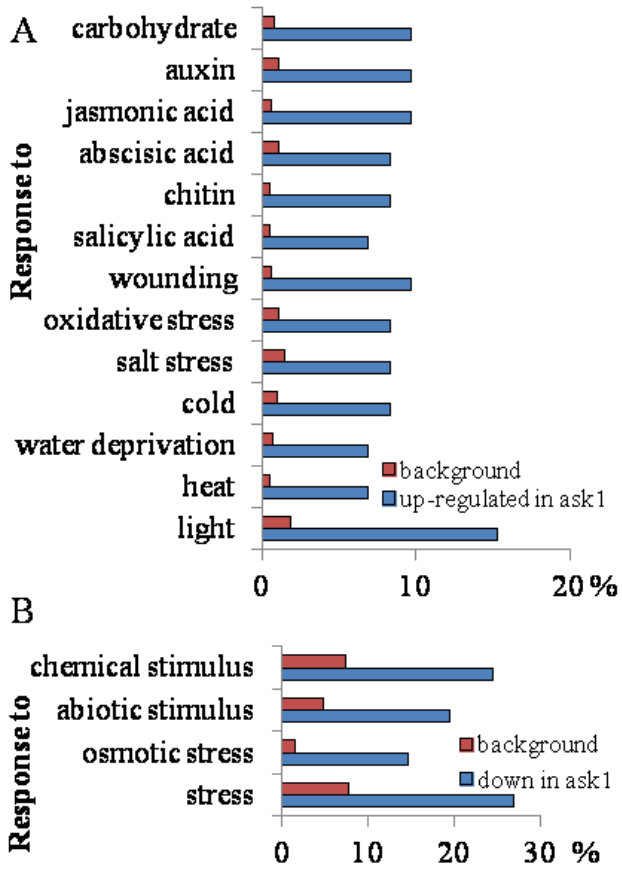

Figure 3-1. Stimulus/stress responsive genes enriched in the up-/down-regulated genes in the *ask1* transcriptome.

(A) Stimulus/stress responsive genes enriched in the up-regulated genes in the *ask1* transcriptome.

(B) Stimulus/stress responsive genes enriched in the down-regulated genes in the *ask1* transcriptome.

We analyzed possible overrepresentation of cis-elements in the putative promoter regions of these up-/down-regulated genes in the *ask1* transcriptome. The frequencies of 6-mer motifs within the 500 bp and 1000 bp putative promoter regions were determined using the Motif Analysis tool from The *Arabidopsis* Information Resource (TAIR) (Table 3-3). The G-box (CACGTG) is overrepresented in all these promoter sets of up-/down-regulated genes and of different lengths, indicating that genes with this motif may be regulated by G-box-binding transcription factors, which themselves or whose co-factors may be regulated by ASK1-E3 ligases, similar to the JAZ-MYC2 model. Some of these transcription factors/co-factors may be short-lived repressors and when they are stabilized in the absence of ASK1 their target genes are down-regulated. Others may function as unstable activators whose stabilization results in up-regulation of downstream genes. Another possibility is that certain transcription factors may have dual functions, both activation and repression, as is the case with MYC2 (Yadav et al., 2005; Dombrecht et al., 2007). The accumulation of these transcription factors up-regulates and/or down-regulates target genes as observed here. The GATAAG motif (I box), which was previously found to be enriched in promoters of light-regulated genes (Giuliano et al., 1988) and required for *Arabidopsis rbcS-1A* expression (Donald and Cashmore, 1990), is enriched in the down-regulated genes in *ask1*. Other motifs are either unknown or partially overlapped with previously characterized cis-elements. Further experiments are required to identify functional cis-elements and cognate transcription factors that connect ASK1-E3 regulation with responsive gene transcription.

Table 3-1. Responsive genes down-regulated in the *ask1* mutant transcriptome.

Gene ID	Gene name	Signaling pathways /responses
AT5G15960	*KIN1*	Cold and ABA
AT1G35720	*ANNEXIN 1* (*ANNAT1*)	Oxidative stress
AT2G42530	*COLD REGULATED 15B* (*COR15B*)	Cold
AT5G42900	*COLD REGULATED GENE 27* (*COR27*)	Cold
AT2G42540	*COLD-REGULATED 15A* (*COR15A*)	Cold
AT4G30650	Low temperature and salt responsive protein	Low temperature and salt
AT5G20250	*DARK INDUCIBLE 10* (*DIN10*)	Light, sucrose
AT1G56220	Dormancy/auxin associated	Dormancy/auxin
AT2G33830	Dormancy/auxin associated	Dormancy/auxin
AT1G28330	*DORMANCY-ASSOCIATED PROTEIN-LIKE 1*	Dormancy
AT3G20810	*JUMONJI DOMAIN CONTAINING 5* (*JMJD5*)	Circadian
AT5G37260	*CIRCADIAN 1* (*CIR1*)	Circadian
AT4G35770	*SENESCENCE 1* (*SEN1*)	Phosphate starvation
AT3G17790	*PURPLE ACID PHOSPHATASE 17* (*PAP17*)	Phosphate starvation, and hydrogen peroxide
AT1G77120	*ALCOHOL DEHYDROGENASE 1* (*ADH1*)	Anaerobic response
AT2G39920	HAD superfamily acid phosphatase	Cadmium ion
AT4G33020	*ZINC IRON PERMEASE* (*ZIP9*)	Zinc ion
AT5G06870	*POLYGALACTURONASE INHIBITING PROTEIN 2* (*PGIP2*)	Fungal infection, Methyl jasmonate
AT2G05520	*GLYCINE-RICH PROTEIN 3* (*GRP3*)	ABA, salicylic acid, ethylene, desiccation

Table 3-2. Responsive genes up-regulated in the *ask1* mutant transcriptome.

Gene ID	Gene name/description	Signaling pathways /responses
AT5G54490	*PINOID-BINDING PROTEIN 1* (*PBP1*)	Auxin
AT3G09870	SAUR-like auxin-responsive protein	Auxin
AT5G61600	*ETHYLENE RESPONSE FACTOR 104* (*ERF104*)	Ethylene
AT4G34410	*REDOX RESPONSIVE TRANSCRIPTION FACTOR 1*	Ethylene
AT1G19180	*JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1*)	Jasmonic acid
AT1G17380	*JASMONATE-ZIM-DOMAIN PROTEIN 5* (*JAZ5*)	Jasmonic acid
AT3G11480	SABATH methyltransferase	Jasmonic acid, fungus, wounding
AT4G27280	Calcium-binding EF-hand family protein	Karrikin
AT3G02380	*CONSTANS-LIKE 2* (*COL2*)	Light
AT3G22840	*EARLY LIGHT-INDUCABLE PROTEIN1* (*ELIP1*)	Light
AT4G14690	*EARLY LIGHT-INDUCIBLE PROTEIN 2* (*ELIP2*)	Light
AT3G17609	*HY5-HOMOLOG* (*HYH*)	Light
AT3G59060	*PHYTOCHROME INTERACTING FACTOR 3-LIKE 6*	Light
AT5G59820	*RESPONSIVE TO HIGH LIGHT 41* (*RHL41*)	Light
AT2G30520	*ROOT PHOTOTROPISM 2* (*RPT2*)	Light
AT2G46830	*CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*)	Circadian
AT1G01060	*LATE ELONGATED HYPOCOTYL* (*LHY*)	Circadian
AT3G09600	*REVEILLE 8* (*RVE8*)	Circadian
AT3G12580	*HEAT SHOCK PROTEIN 70* (*HSP70*)	Heat
AT5G51440	*HSP20*-like	Heat
AT2G31380	*SALT TOLERANCE HOMOLOGUE* (*STH*)	Salt
AT1G27730	*SALT TOLERANCE ZINC FINGER* (*STZ*)	Salt
AT3G55980	*SALT-INDUCIBLE ZINC FINGER 1* (*SZF1*)	Salt
AT2G33380	*RESPONSIVE TO DESICCATION 20* (*RD20*)	Desiccation
AT5G24660	*RESPONSE TO LOW SULFUR 2* (*LSU2*)	Sulfur deficiency
AT1G19640	*JASMONIC ACID CARBOXYL METHYLTRANSFERASE*	Wounding or methyljasmonate
AT5G64510	*TUNICAMYCIN INDUCED 1* (*TIN1*)	ER-stress, heat, light, hydrogen peroxide
AT5G57560	*TOUCH 4* (*TCH4*)	Mechanical stimulus

Table 3-2. Continued.

Gene ID	Gene name/description	Signaling pathways /responses
AT1G12110	*NITRATE TRANSPORTER 1.1* (*NRT1.1*)	Nitrate, water deprivation
AT1G61800	*GLUCOSE-6-PHOSPHATE/PHOSPHATE TRANSLOCATOR 2* (*GPT2*)	Glucose, sucrose, karrikin, nematode
AT2G46400	*WRKY DNA-BINDING PROTEIN 46*	Chitin
AT5G51190	Ethylene response factor	Chitin, wounding
AT3G61190	*BON ASSOCIATION PROTEIN 1* (*BAP1*)	Chitin, cold, fungus, heat, jasmonic acid, salicylic acid, wounding
AT4G11280	*ACC SYNTHASE 6* (*ACS6*)	ABA, auxin, chitin, ethylene, jasmonic acid, oxidative stress, wounding
AT5G59310	*LIPID TRANSFER PROTEIN 4* (*LTP4*)	ABA, cold, salt, water deprivation
AT4G25100	*FE SUPEROXIDE DISMUTASE 1* (*FSD1*)	Cadmium ion, copper ion, oxidative stress
AT1G02930	*GLUTATHIONE S-TRANSFERASE 6*	Cadmium ion, oxidative stress, salt, water deprivation
AT3G21890	B-box type zinc finger protein	UV-B, sucrose
AT2G37040	*PHE AMMONIA LYASE 1* (*PAL1*)	UV-B, karrikin, oxidative stress, wounding

Table 3-3. Top five enriched cis-elements in the putative promoter regions of down-/up-regulated genes in the *ask1* transcriptome.

oligomer	# in query	# in genomic set	# of promoters in query with oligoMer	# of promoters in genomic set with oligoMer	binomial distribution p-value
500 bp promoters of down-regulated genes in *ask1*					
CACGTG	30	7766	12/42	3253/33602	3.53E-04
ACACGT	23	7390	17/42	5609/33602	1.61E-04
CGCAAA	13	4569	13/42	3995/33602	6.16E-04
GCCACG	11	2914	10/42	2594/33602	8.46E-04
GATAAG	27	9179	19/42	7797/33602	9.09E-04
1000 bp promoters of down-regulated genes in *ask1*					
CACGTG	42	12404	16/42	5033/33602	1.57E-04
AACTGT	33	17175	28/42	13171/33602	2.04E-04
GATAAG	41	18464	28/42	13811/33602	4.93E-04
ATTATG	60	33774	35/42	20241/33602	8.37E-04
CGTGTA	22	7824	17/42	6717/33602	1.25E-03
500 bp promoters of up-regulated genes in *ask1*					
CACGTG	72	7766	24/74	3253/33602	4.95E-08
ACACGT	57	7390	30/74	5609/33602	7.19E-07
ACGTGG	30	5475	22/74	4404/33602	9.42E-05
AAGTGG	31	7504	27/74	6502/33602	2.63E-04
ACACTC	23	5788	23/74	5177/33602	3.37E-04
1000 bp promoters of up-regulated genes in *ask1*					
CACGTG	100	12404	30/74	5033/33602	6.83E-08
ACACGT	77	12599	38/74	9080/33602	5.14E-06
ACGTGG	46	9196	31/74	7047/33602	2.50E-05
ATATTA	177	65927	68/74	25116/33602	1.22E-04
TGAGAC	48	12304	36/74	10027/33602	2.98E-04

Proteomics analysis of L*er* and *ask1* flower buds

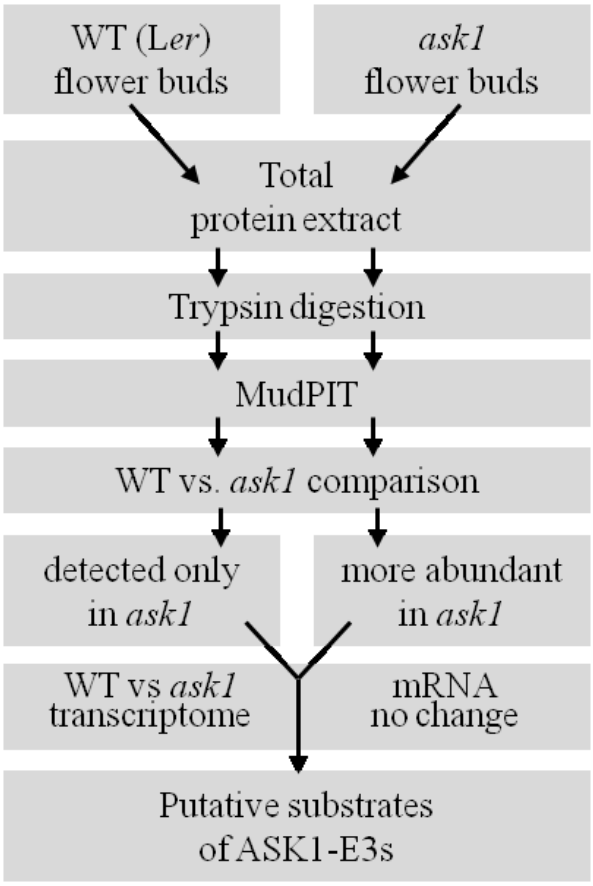

Figure 3-2. The workflow of the integrated proteomics and transcriptomics to identify putative ASK1-E3 substrates.

A label-free proteomic method, MudPIT, was used to analyze flower bud proteomes of the *ask1* mutant and L*er* (Figure 3-2). Total protein extracts of four L*er* and five *ask1* flower bud samples were in-solution digested with trypsin without pre-separation, which might enable digestion of proteins with different properties (e.g., hydrophobicity and charges) and compartmentalization (cytosol, membrane, nucleus and organelles). Four MudPIT runs of L*er* samples (L*er*-1 ~ L*er*_4) detected 2348, 2258, 1658, and 1400 proteins with false discovery rate

(FDR) <1%, respectively. When the four datasets were merged, a total of 3220 non-redundant proteins were detected with FDR<1%. Five MudPIT runs of *ask1* samples (*ask1*_1 ~ *ask1*_5) detected 1780, 1441, 1959, 1007, and 363 proteins with FDR<1%, respectively. A total of 2916 non-redundant proteins were detected with FDR<1%. The *ask1*_5 run detected fewer proteins because the protein amount was reduced to ~20% of the others to test whether a smaller amount of input protein extract could lead to different efficiency of protein detection. The test result did not show a huge difference in the detection efficiency when the amount of starting material was changed. The 363 proteins detected in this test run were included in the total *ask1* proteins, but excluded when doing comparison between individual runs with spectral counting normalization in the following sections.

Due to the stochastic sampling nature of MS-based proteomics, only a portion of the whole proteome can be covered in each experiment, and different MS runs usually detect proteins that are partially overlapped. In our L*er* and *ask1* samples, partially overlapping sets of proteins were detected (Figure 3-3): 884 (27.5%) of the 3220 total L*er* proteins (FDR<1%) were detected by all four runs, 684 (17.2%) proteins by three runs, 554 (21.2%) proteins by two runs, and 1096 (34.1%) proteins detected only once. Among the 2899 *ask1* proteins (proteins unique to *ask1*_5 not included), 568 (19.6%), 493 (17.0%), 598 (20.6%), and 1240 (42.8%) proteins were detected by four, three, two and one of the runs, respectively. Although many proteins were only detected once, the proteins have high confidence (FDR<1%) resulting from very stringent MS detection and searching criteria, and thus were considered to be detected.

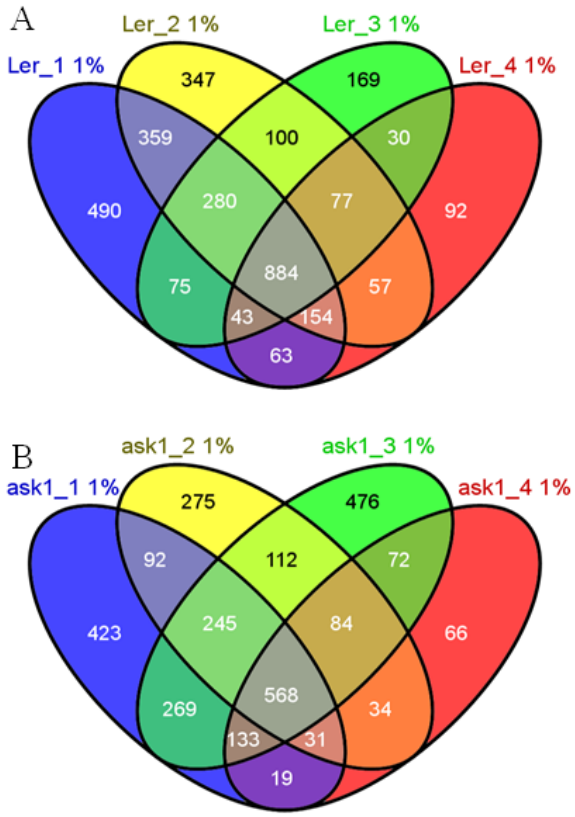

Figure 3-3. Independent proteomics samples detect partially overlapping sets of proteins.

(A) Four L*er* samples L*er*_1 to L*er*_4.

(B) Four *ask1* samples *ask1*_1 to *ask1*_4. 1% after each sample name represents FDR<1%.

Proteins of some cellular component categories (e.g., membrane proteins) that are usually considered to be difficult to be detected by MS without using detergents are well represented in our L*er* and *ask1* proteomes, indicating that our method did not cause obvious bias against proteins localized to the plasma membrane, organelle membrane, and nuclear envelope. One important improvement to minimize bias may be that total protein extracts were digested by trypsin without further separation into soluble or insoluble portions. Although insoluble proteins were often thought to be recalcitrant to enzyme digestion, extensive sonication, denaturing treatments, and prolonged tryptic digestion may improve detection efficiency.

Detection of additional floral proteins compared with previous proteomics studies

By comparing our flower bud proteomes with previously published proteomics data, we detected many additional proteins (Figure 3-4). Wild-type (WT) flower bud proteins from two previous studies (Baerenfaller et al., 2008; Feng et al., 2009) were combined into one dataset (named "previous WT") containing 5461 non-redundant proteins (FDR<1%). Compared with the previous WT, 516 proteins were only detected in our L*er* proteome and 752 proteins were detected in our L*er* + *ask1* combined proteome (3762 non-redundant proteins). The identification of these additional proteins suggests that proteomics detection is far from saturation.

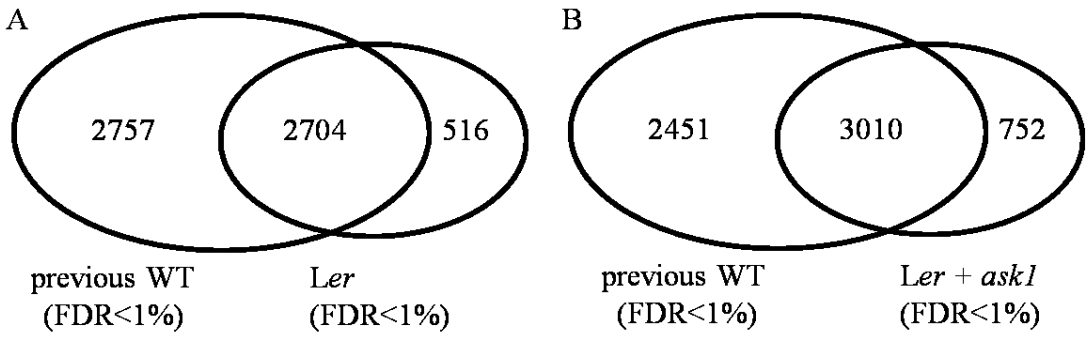

Figure 3-4. Detection of additional flower bud proteins compared with previous studies.

(A) Comparison of wild-type flower bud proteins detected in previous studies (previous WT) and L*er* from this study (FDR<1%).

(B) Comparison of flower bud proteins detected in previous WT and total proteins detected in our L*er* and *ask1* samples (FDR<1%).

Proteins detected only in *ask1* or higher in *ask1* may be regulated by ASK1-E3s

Our *ask1* and L*er* proteomes were compared and 542 proteins were found only in *ask1* (Figure 3-5). These proteins were considered to accumulate in the *ask1*, and are possible ASK1-E3 substrates. Since the limited number of MudPIT runs was not enough to saturate the proteome, we cannot rule out the possibility that some proteins detected only in *ask1* might have also been detected in L*er* if more MS runs had been done. In order to narrow down putative ASK1-E3 substrates, we combined our L*er* flower bud proteome data with the previous WT data yielding a larger wild-type flower bud proteome dataset consisting of 5977 proteins (Pooled WT). By comparing *ask1* and the Pooled WT proteomes, we found that 236 proteins were only detected in *ask1* (*ask1*-only proteins). These proteins are more likely to be ASK1-E3 substrates.

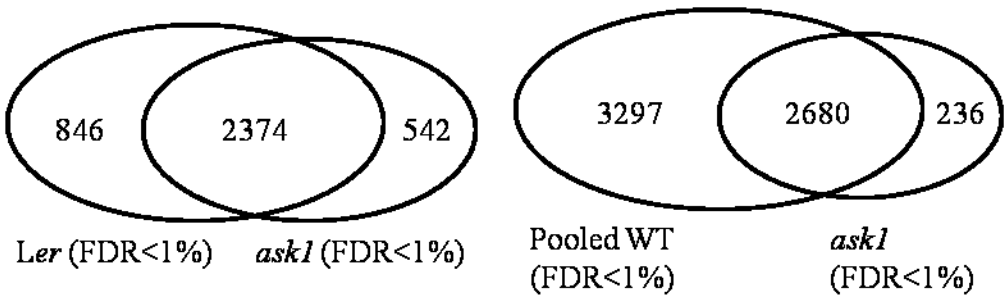

Figure 3-5. Proteins only detected in the *ask1* but not in the wild-type proteomes.
(A) Comparison of L*er* and *ask1* proteomes from this study.
(B) Comparison of the *ask1* proteome with the pooled WT, a combined WT proteome from previous studies and this study.

We examined RNA levels of these *ask1*-only proteins from microarray data to determine whether increased transcription contributes to the accumulation of these proteins. The RNA levels of all these genes are not significantly different between *ask1* and L*er* (two-fold cutoff) except 11

genes without probes on microarray and At3g11480 whose transcript level change is slightly higher than two-fold but the p-value is larger than 0.05. Therefore, these proteins are probably controlled by ASK1-E3s at the protein level.

Because we used a label-free proteomics method, protein abundance cannot be directly compared between samples. Proteomics datasets from individual samples were normalized using spectral counting method (Baerenfaller et al., 2008) and the average values were compared between L*er* and *ask1* to find proteins with higher abundance (1.5-fold cutoff) in *ask1* (*ask1*-higher). The previous WT data were not included because they were generated by different proteomics methods and difficult to be quantitatively compared with our data.

We extracted RNA values from microarray data for these *ask1*-higher proteins to determine whether their elevated protein levels were due to transcriptional activation. The RNA level of AT2G33380 was 2.2-fold higher in *ask1* than L*er*. The RNA levels of other genes were not significantly different between *ask1* and L*er*, except 12 genes which have no probes on microarray, indicating that most *ask1*-higher proteins are probably regulated at the protein level.

ASK1-E3s may regulate multiple categories of proteins

Categorization of the *ask1*-only and *ask1*-higher proteins shows that some molecular functional categories are overrepresented (p-value<0.05) (Figure 3-6). Since regulatory proteins are usually regulated by the UPS, certain categories are of particular interest including transcription regulators (Table 3-4), kinases (Table 3-5), and peptidases/proteases (Table 3-6). Interestingly, many ribosomal proteins were found to accumulate in *ask1* (Table 3-7) indicating a new role of the UPS in translational regulation or novel functions of ribosomal proteins.

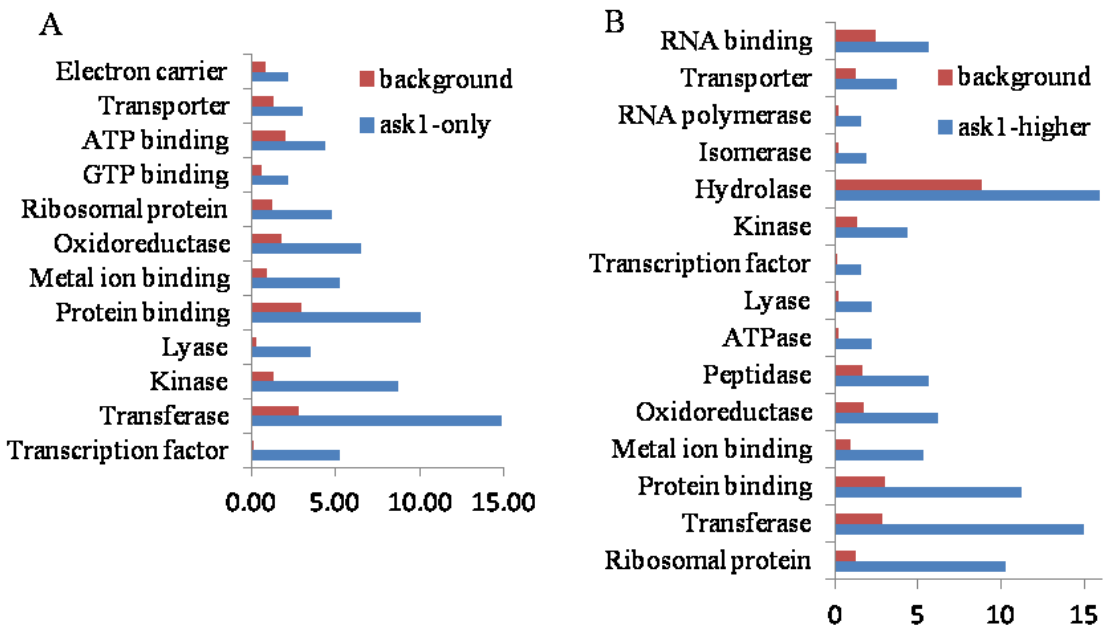

Figure 3-6. Overrepresented categories in the proteins accumulated in the *ask1* proteome.

(A) Overrepresented categories in the *ask1*-only proteins.

(B) Overrepresented categories in the *ask1*-higher proteins.

ASK1-E3s may regulate transcription regulators

The transcription regulators possibly regulated by ASK1-E3s include a few with some functional information (Table 3-4). A basic helix-loop-helix (bHLH) transcription factor MYC3 was shown to interact with JAZ proteins and functions with MYC2 and MYC4 to activate JA responses (Cheng et al., 2011; Fernandez-Calvo et al., 2011). MYC3 binds to G-box sequences (Fernandez-Calvo et al., 2011), indicating that MYC3 may regulate genes with G-boxes in their promoters. This may explain the observation that G-box is enriched in the promoters of the up-/down-regulated genes in *ask1* transcriptome as described above. A Polycomb group protein SWINGER (SWN) was shown to interact with other Polycomb group proteins in *FLOWERING LOCUS C* (*FLC*) epigenetic repression during vernalization and in controlling the initiation of

endosperm development (Chanvivattana et al., 2004; Wang et al., 2006; Wood et al., 2006). The RELATIVE OF EARLY FLOWERING 6 (REF6), a histone H3 lysine 27 demethylase (Lu et al., 2011), positively regulates flowering and brassinosteroid signaling (Yu et al., 2008). The accumulation of these transcription regulators in *ask1* suggests that they may be normally destabilized by ASK1-E3s to control gene transcription during plant development (e.g., flowering) and signal responses (e.g., hormones).

Table 3-4. Transcription regulators enriched in the *ask1*-only or *ask1*-higher proteins.

Transcription factors	
Enriched in ask1-only proteins	
AT5G46760	MYC3, basic helix-loop-helix (bHLH) transcription factor
AT1G32360	Zinc finger (CCCH-type) family protein
AT2G24500	Zinc finger (C2H2-type) protein FZF
AT5G60850	Zinc finger OBF BINDING PROTEIN 4 (OBP4)
AT3G61850	Zinc finger DOF AFFECTING GERMINATION 1 (DAG1)
AT4G36620	Zinc finger GATA TRANSCRIPTION FACTOR 19 (GATA19)
AT2G02540	ZINC FINGER HOMEODOMAIN 3 (ZHD3)
AT5G15210	ZINC FINGER HOMEODOMAIN 8 (ZHD8)
AT1G54830	NUCLEAR FACTOR Y, SUBUNIT C3 (NF-YC3)
AT1G58100	TCP DOMAIN PROTEIN 8 (TCP8)
AT3G10490	NAC DOMAIN CONTAINING PROTEIN 52 (ANAC052)
AT4G02020	Polycomb group protein SWINGER (SWN)
Enriched in ask1-higher proteins	
AT1G49480	RELATED TO VERNALIZATION1 1 (RTV1)
AT1G76880	Duplicated homeodomain-like superfamily protein
AT3G28920	ZINC FINGER HOMEODOMAIN 9 (ZHD9)
AT3G48430	RELATIVE OF EARLY FLOWERING 6 (REF6); JUMONJI DOMAIN-CONTAINING PROTEIN 12 (JMJ12)
AT4G35570	HIGH MOBILITY GROUP B5 (HMGB5)
AT4G38130	HISTONE DEACETYLASE 1 (HD1);HISTONE DEACETYLASE19

ASK1-E3s may regulate kinases

Among the kinases that accumulated in *ask1* (Table 3-5), CALCIUM-DEPENDENT PROTEIN KINASE 6 (CPK6) is a positive regulator in salt/drought stress tolerance (Xu et al., 2010b), methyl jasmonate signaling in guard cells (Munemasa et al., 2011), and ABA regulation of guard cell ion channels (Mori et al., 2006). The Cyclin T partner CYCT1;5 is a subunit of cyclin-dependent kinase C complexes involved in cauliflower mosaic virus infection, plant growth and development (Cui et al., 2007). A cyclin-dependent kinase CELL DIVISION CONTROL 2 (CDC2) is required for male gametogenesis (Iwakawa et al., 2006). LYSM DOMAIN RECEPTOR-LIKE KINASE 1 (LYSM RLK1) is involved in chitin-mediated plant innate immunity (Gimenez-Ibanez et al., 2009; Iizasa et al., 2010). MAP KINASE KINASE 2 (MKK2) regulates cold and salt stress signaling and innate immunity (Teige et al., 2004; Brader et al., 2007; Gao et al., 2008). SNF1-RELATED PROTEIN KINASE 1.2 (SnRK1.2)/ SNF1 KINASE HOMOLOG 11 (ATKIN11) was demonstrated to interact with ASK1 (Farras et al., 2001) and is degraded during phosphate starvation (Fragoso et al., 2009). BR-SIGNALING KINASE 1 (BSK1) mediates brassinosteroid signal transduction (Tang et al., 2008). Other kinases are largely unknown but may have important functions in signal perception and transduction. For example AT5G43020 and AT3G14350 contain transmembrane domains and could be membrane receptor kinases. ASK1-E3s may regulate these kinases to control cell cycle, plant immunity, hormone signaling, and other processes.

Table 3-5. Kinases enriched in the *ask1*-only and *ask1*-higher proteins.

Kinases	
Enriched in ask1-only proteins	
AT2G17290	CALCIUM-DEPENDENT PROTEIN KINASE 6 (CPK6)
AT4G21940	CALCIUM-DEPENDENT PROTEIN KINASE 15 (CPK15)
AT5G45190	Cyclin T partner CYCT1;5
AT3G48750	Cyclin-dependent kinase CELL DIVISION CONTROL 2 (CDC2)
AT4G29810	MAP KINASE KINASE 2 (MKK2)
AT3G29160	SNF1-RELATED PROTEIN KINASE 1.2 (SnRK1.2)
AT5G63650	SNF1-RELATED PROTEIN KINASE 2.5 (SNRK2.5)
AT4G26100	CASEIN KINASE 1 (CK1)
AT4G35780	ACT-like protein tyrosine kinase
AT5G49470	PAS domain-containing protein tyrosine kinase
AT5G11020	Protein kinase superfamily protein
AT5G24010	Protein kinase superfamily protein
AT5G57610	Protein kinase superfamily protein
AT5G43020	Leucine-rich repeat protein kinase family protein
AT3G21630	LYSM DOMAIN RECEPTOR-LIKE KINASE 1 (LYSM RLK1)
AT3G14350	STRUBBELIG-RECEPTOR FAMILY 7 (SRF7)
AT4G33240	1-phosphatidylinositol-3-phosphate (PtdIns3P) 5-kinase
Enriched in ask1-higher proteins	
AT1G31910	GHMP kinase family protein
AT2G18170	MAP KINASE 7 (ATMPK7)
AT2G27970	CDK-SUBUNIT 2 (CKS2)
AT3G02880	Leucine-rich repeat protein kinase family protein
AT4G21210	PPDK REGULATORY PROTEIN (RP1)
AT4G35230	BR-SIGNALING KINASE 1 (BSK1)

ASK1-E3 may regulate peptidases/proteases

The peptidase category is enriched in the *ask1*-higher proteins (Table 3-6). Four peptidases (AT1G53850, AT5G66140, AT1G77440, and AT3G60820) are isoforms of 20S proteasome alpha/beta subunits indicating that the proteasome core complex may also be regulated by UPS. Two ubiquitin-specific proteases UBIQUITIN-SPECIFIC PROTEASE 5 (UBP5) and UBIQUITIN-SPECIFIC PROTEASE 6 (UBP6) were also detected in *ask1*-higher proteins indicating that deubiquitinases, which antagonize protein ubiquitination, may also be regulated by the UPS. The BRI1 SUPPRESSOR 1 (BRS1), a secreted serine carboxypeptidase, is involved in brassinosteroid signaling possibly by processing some proteins (Li et al., 2001). Other peptidases are largely unknown except information from expression and homology. Peptidases/proteases may normally be subject to regulation by ASK1-E3s, thus coupling peptidase-mediated protein processing or degradation with the UPS.

Table 3-6. Peptidases enriched in the *ask1*-higher proteins.

Peptidases	
AT1G01300	Eukaryotic aspartyl protease family protein
AT1G79720	Eukaryotic aspartyl protease family protein
AT1G02305	Cysteine proteinases superfamily protein
AT3G62940	Cysteine proteinases superfamily protein
AT5G43060	Granulin repeat cysteine protease family protein, ESPONSIVE TO DEHYDRATION 21B (RD21B)
AT4G30610	SERINE CARBOXYPEPTIDASE 24 PRECURSOR (SCPL24); BRI1 SUPPRESSOR 1 (BRS1)
AT4G30810	SERINE CARBOXYPEPTIDASE-LIKE 29 (SCPL29)
AT1G13270	METHIONINE AMINOPEPTIDASE 1B (MAP1C)
AT3G14067	Subtilase family protein
AT5G04710	Zn-dependent exopeptidases superfamily protein
AT5G05740	S2P-like putative metalloprotease, ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW-GREEN-LIKE 2 (EGY2)
AT2G40930	UBIQUITIN-SPECIFIC PROTEASE 5 (UBP5)
AT1G51710	UBIQUITIN-SPECIFIC PROTEASE 6 (UBP6)
AT1G53850	20S PROTEASOME ALPHA SUBUNIT E1 (PAE1)
AT5G66140	20S PROTEASOME ALPHA SUBUNIT D2 (PAD2)
AT1G77440	20S PROTEASOME BETA SUBUNIT C2 (PBC2)
AT3G60820	20S PROTEASOME BETA SUBUNIT F1 (PBF1)

ASK1-E3 may regulate ribosomal proteins

Interestingly, many ribosomal proteins were identified only or with higher levels in *ask1* (Table 3-7), indicating that ASK1-E3s may also have a role in translational regulations. Several genes have been genetically studied: NUCLEAR FUSION DEFECTIVE 3 is a mitochondrial ribosomal protein and required for polar nuclei fusion during female gametophyte development (Portereiko et al., 2006); PIGGYBACK1 influences leaf vascular patterning (Pinon et al., 2008); OLIGOCELLULA 7 is involved in ribosome biogenesis and organ size control (Fujikura et al., 2009); POINTED FIRST LEAF 2 plays a role in early leaf development (Ito et al., 2000). Ribosomal proteins may be regulated by ASK1-E3s either for ribosome turnover or for extraribosomal regulatory purposes. It was suggested that ribosomal proteins can be ubiquitinated for selective degradation of ribosomes by autophagy (Tagwerker et al., 2006; Kraft et al., 2008). So, the accumulation of ribosomal proteins in *ask1* may result from the failure of ubiquitination by ASK1-E3s and autophagy subsequently. Alternatively, ribosomal proteins may have extraribosomal functions, which are exemplified by the findings that several ribosomal proteins can block the ubiquitination of the tumor suppressor p53 upon ribosomal stress (Zhou et al., 2012). Therefore, the ribosomal proteins accumulated in *ask1* may function as regulatory proteins which themselves may be regulated by ubiquitin-mediated proteolysis.

Table 3-7. Ribosomal proteins enriched in the *ask1*-only and *ask1*-higher proteins.

Ribosomal proteins	
Enriched in ask1-only proteins	
AT5G02610	Ribosomal L29
AT1G26880	Ribosomal protein L34e
AT4G25890	60S acidic ribosomal protein family
AT5G67510	Translation protein SH3-like family protein, large ribosomal subunit
AT5G39850	Ribosomal protein S4
AT5G43640	Ribosomal protein S19
AT4G34555	Ribosomal protein S25
AT3G61110	Ribosomal protein S27
AT1G31817	Mitochondrial 50S ribosomal L21, NUCLEAR FUSION DEFECTIVE 3
AT2G38140	Plastid-specific ribosomal protein 4 (PSRP4)
Enriched in ask1-only proteins	
AT1G07830	Ribosomal protein L29 family protein
AT1G15930	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
AT1G26910	Ribosomal protein L16p/L10e family protein
AT1G27400	Ribosomal protein L22p/L17e family protein
AT1G41880	Ribosomal protein L35Ae family protein
AT1G67430	Ribosomal protein L22p/L17e family protein
AT1G69620	RIBOSOMAL PROTEIN L34 (RPL34)
AT1G78630	Ribosomal protein L13 family protein, EMBRYO DEFECTIVE 1473 (EMB1473)
AT2G27530	Ribosomal protein L10aP, PIGGYBACK1 (PGY1)
AT3G07110	Ribosomal protein L13 family protein
AT3G54210	Ribosomal protein L17 family protein
AT3G59540	Ribosomal L38e protein family
AT5G23900	Ribosomal protein L13e family protein
AT5G27850	Ribosomal protein L18e/L15 superfamily protein
AT5G39740	RIBOSOMAL PROTEIN L5B (RPL5B); OLIGOCELLULA 7 (OLI7)
AT2G28830	PLANT U-BOX 12 (PUB12) with ribosomal protein L10e/L16 domain
AT1G74970	RIBOSOMAL PROTEIN S9 (RPS9)
AT2G40510	Ribosomal protein S26e family protein
AT2G40590	Ribosomal protein S26e family protein
AT3G04920	Ribosomal protein S24e family protein
AT3G13120	Ribosomal protein S10p/S20e family protein
AT3G56340	Ribosomal protein S26e family protein
AT4G00100	RIBOSOMAL PROTEIN S13A (RPS13A); POINTED FIRST LEAF 2 (PFL2)
AT4G33865	Ribosomal protein S14p/S29e family protein
AT4G39200	Ribosomal protein S14p/S29e family protein
AT5G04800	Ribosomal S17 family protein
AT5G15200	Ribosomal protein S4
AT5G28060	Ribosomal protein S24e family protein
AT5G52650	RNA binding Plectin/S10 domain-containing protein
AT3G16080	Zinc-binding ribosomal protein family protein
ATCG00800	Chloroplast ribosomal protein S3, RESISTANCE TO PSEUDOMONAS SYRINGAE 3
ATCG01240	30S chloroplast ribosomal protein S7, RIBOSOMAL PROTEIN S7 (RPS7.2)
AT1G07320	Plastid RIBOSOMAL PROTEIN L4 (RPL4); EMBRYO DEFECTIVE 2784 (EMB2784)

Possible mechanisms underlying the transcriptomic and proteomic regulations by ASK1-E3s

By integrating transcriptome and proteome data, we hypothesize that ASK1-E3s may destabilize transcription repressors or activators to derepress or inactivate gene transcription, respectively (Figure 3-7A). In the absence of ASK1, the abnormal accumulation of these transcriptional repressors or activators results in down-regulation or up-regulation of gene transcription, respectively. Two mechanisms may exist in wild-type: some transcriptional regulators may be constitutively removed by ASK1-E3s until been stabilized by certain signals; others may be generally stable but degraded upon signal induction (e.g., hormone signaling). The former mechanism may be useful for restraining signal responses in the absence of signals but launch responses rapidly upon signal perception. For example, heat, cold or salt stress responses may be suppressed in normal flower development by constantly removing key regulators; only when these stresses are perceived are these regulators stabilized to boost downstream responses. The later mechanism is also important for fast responses to signals like hormones or developmental transitions. For example, degradation of regulators including the Polycomb group protein SWN may lead to epigenetic activation of gene expression.

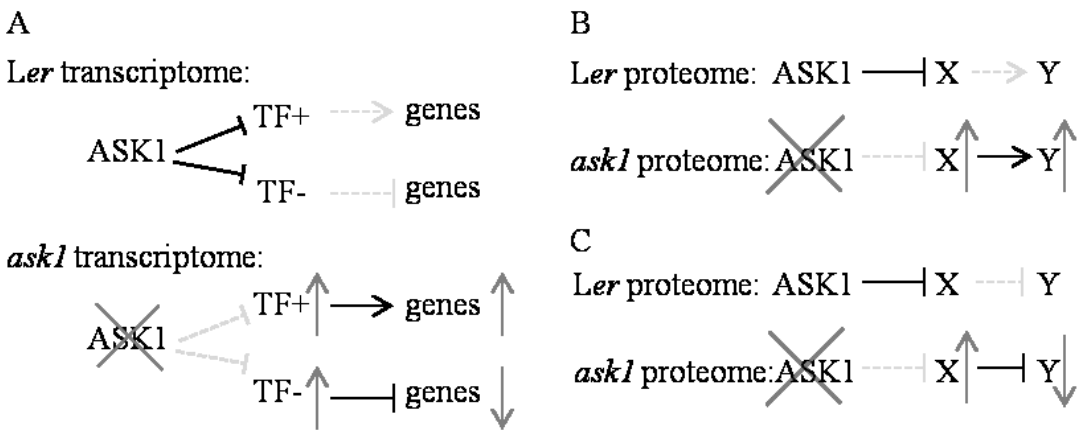

Figure 3-7. Possible mechanisms of the transcriptome and proteome regulations by ASK1-E3s.

(A) ASK1-E3s may regulate gene transcription by destabilizing transcription factors in L*er*. The transcription factors are stabilized in *ask1* mutant and activate or repress downstream gene transcription. TF+, transcriptional activators; TF-, transcriptional repressors.

(B) ASK1-E3s may destabilize substrates (X) which positively regulate the abundance of other proteins (Y). In the *ask1* mutant proteome, ASK1-E3 substrates (X) and their target proteins (Y) accumulate.

(C) ASK1-E3s may destabilize substrates (X) which negatively regulate the abundance of other proteins (Y). In the *ask1* mutant proteome, ASK1-E3 substrates (X) accumulate and target proteins decrease (Y).

Bars, negative regulation; horizontal arrows, positive regulation; dashed gray bars and horizontal arrows, missing regulations; upward arrows, increase in abundance; downward arrows, decrease in abundance.

The proteins accumulated in *ask1* may be direct substrates of ASK1-E3s, or stabilized by ASK1-E3 substrates (Figure 3-7B). For example, ubiquitin-specific proteases UBP5 and UBP6, which accumulate in the *ask1* proteome (Table 3-6), may be substrates of ASK1-E3s; UBP5 and UBP6 may deubiquitinate and prevent degradation of ubiquitinated proteins whose protein levels are then increased in *ask1*. One of the reported examples is that the human herpesvirus-associated ubiquitin-specific protease (HAUSP) stabilizes a tumor suppressor p53 by deubiquitination (Li et al., 2002). Ribosomal proteins may share a similar mechanism: accumulation of ribosomal proteins in *ask1* may increase protein synthesis; or if ribosomal proteins have extraribosomal regulatory functions, they may stabilize some proteins in a similar way as those stabilizing p53 in human (Zhou et al., 2012).

In another possible scenario, ASK1-E3s may destabilize some proteolytic enzymes (e.g., E3 ubiquitin ligases or peptidases), which can degrade other proteins (Figure 3-7C), forming a double negative regulation cascade. The accumulation of such proteolytic enzymes in *ask1* may cause reduced levels of their proteolytic substrates. Proteasome subunits and peptidases that accumulate in *ask1* may be involved in degradation of their substrate proteins, which could be detected with lower levels in *ask1* proteome. However, it remains difficult to identify these proteolytic substrates due to lack of functional information of the proteolytic enzymes. In principle, a cascade with an even number of negative regulations has the final effect of positive regulations; and the final output of odd numbers of negative regulations is still negative. More evidence is required for elucidating these possible mechanisms.

Discussion

Different methods and more MS runs increase proteome coverage

It has been well known that proteomic methods are being developed very rapidly. However, all proteomic methods are facing several challenges: huge dynamic range of proteins, low coverage of the whole proteome, and poor reproducibility. Even individual MS runs of the same starting protein sample may result in discovery of partially overlapping proteins. Ideally, it may increase the proteome coverage using multiple sample preparation methods (e.g., with or without detergents), different protein pre-separation methods, different proteomics platforms (gel-based or gel-free), different MS instruments and searching engines. Due to the limitation of resources most proteomics studies focus on improving one or a few of these aspects.

Here, we used a gel-free proteomic approach with in-solution tryptic protein digestion and HPLC-based two-dimensional peptide separation, followed by tandem MS. Proteins of many cell component categories were detected without obvious under-representation, indicating that our approach was not biased against certain cellular proteins. This could partly due to the in-solution digestion of the total protein extracts and extensive two-dimensional liquid chromatography separation of peptides. In addition, we used Trichloroacetic Acid (TCA) extraction method to obtain as many proteins from tissues as possible without additional purification steps to avoid biased protein loss. Although the crude protein extract was not absolutely soluble in the tryptic digestion buffer, ultrasonic resuspension and prolonged tryptic digestion may facilitate cleavage and solubilization of peptides from recalcitrant proteins including membrane proteins. Many proteins in our datasets have not been detected by previous larger-scale proteomics studies, suggesting that there is still much room for improvement in proteomics.

The L*er* floral proteome analysis adds information to *Arabidopsis* floral proteins

Since our plants are in the L*er* background and the previous WT plants are Col, some of the newly detected proteins may result from differences between L*er* and Col. However, there is little evidence showing dramatic differences between proteomes of the two ecotypes. It is reasonable to assume that L*er* and Col share major molecular mechanisms controlling flower development; thus their proteomes are generally comparable if we focus on the conserved aspects instead of polymorphisms. The L*er* proteome data may provide valuable information to understand flower development since L*er* has been widely used for genetic studies, through which numerous mutants have been generated within the L*er* background.

Novel aspects of ASK1 functions in regulating the *Arabidopsis* proteome and transcriptome

It is well known that E3 ubiquitin ligases usually ubiquitinate proteins for degradation, which is the logical basis of comparative proteomics between wild-type and *ask1* to find putative ASK1-E3 substrates. We found many candidates with higher protein levels in *ask1* than in wild-type and with similar transcript levels between the two. The large number of the putative substrates may not be surprising because ASK1 is highly expressed throughout the plant and can interact with many F-box proteins, which may recognize many substrates. Although the mutation of *ask1* did not cause very severe defects probably due to redundancy, the perturbation at the protein level seems to be dramatic based on the proteomic analysis. Several novel aspects of ASK1 functions have not been discovered before.

Regulatory proteins including transcription factors are often believed to be regulated by the UPS. One of the well known examples is that JAZ proteins are ubiquitinated and degraded upon JA perception (Devoto et al., 2002; Xu et al., 2002; Thines et al., 2007). However, JAZ

proteins are co-regulators that inhibit MYC transcription factors. Examples are rare for ubiquitination-mediated degradation of transcription regulators that directly regulate transcription in plants. Here, we found a putative ASK1-E3 substrate MYC3, which may function with MYC2 in regulating JA responsive genes (Cheng et al., 2011; Fernandez-Calvo et al., 2011). Our study raises the possibility that MYC3 and its homologs may be regulated by the UPS, a mechanism that has not been reported. More investigation is needed to elucidate the mechanism of MYC3 functions and regulation of its stability. The regulation of chromatin remodeling proteins, e.g., the Polycomb group protein SWN and the histone H3 lysine 27 demethylase REF6, by the ubiquitin pathway may also play an important role in controlling gene expression. Transcription regulators that were not detected in this study possibly due to low protein levels may also be under regulation by proteolysis. Many transcription factors have been identified to be important for *Arabidopsis* development. For example, the anther-specific transcriptional factor DYSFUNCTIONAL TAPETUM1 (DYT1) is only expressed in the tapetum for a limited time period around the meiosis stage (Feng et al., 2012b). The precise temporal regulation of its RNA and protein levels may be a prerequisite for successful meiosis and pollen development. However, evidence for its regulation by UPS is lacking. Studying the regulation of transcription regulators by the ubiquitin pathway may connect transcriptomics and proteomics, two research areas that are mostly explored independently so far.

Another new aspect of ASK1 functions uncovered here is that ribosomal proteins may be regulated by the ubiquitin pathway. Ribosomal proteins are traditionally thought to be structural components in ribosomes. However, increasing evidence has started to challenge this view. Some ribosomal proteins were found to be involved in translation of specific mRNAs and/or essential for specific development processes (Byrne, 2009; Warner and McIntosh, 2009; Kondrashov et al., 2011). It is not surprising that such complex molecular machines as ribosomes are subject to sophisticated regulation of their biosynthesis, functioning, and turnover. A connection between

autophagy and ubiquitin pathways has been reported to be involved in selective turnover of ribosomes (Kraft et al., 2008). However, little information is available for the E3 ubiquitin ligases that ubiquitinate ribosomal proteins. Here, we detected many ribosomal proteins that accumulate in the *ask1* proteome, indicating that they may be regulated by ASK1-E3s. Further studies of ribosomal protein ubiquitination and responsible E3 ubiquitin ligases are needed.

There are probably more proteins regulated by ASK1-E3s than suggested here. Proteins with low expression levels and restricted spatiotemporal expression are difficult to be uncovered, especially if they are subject to degradation. Moreover, in the *ask1* mutant, other ASK family members like ASK2 may have partially redundant functions with ASK1 and hence compensate *ask1* mutation to some extent. In other words, some ASK1-E3 substrates may be ubiquitinated by SCFs containing other ASK proteins and thus would be unable to accumulate in the *ask1* proteome. Knockout or knockdown of multiple *ASK* genes may help discover more substrates. In addition, characterization of the ubiquitinated proteome may pinpoint substrates of E3 ubiquitin ligases and ubiquitination sites within each protein.

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Chapter 4

ASK1 Functions as a Connection between the Regulations of Protein Degradation and Gene Transcription in the *Arabidopsis* Anther

Abstract

Gene expression is usually regulated at multiple steps including transcriptional, post-transcriptional, translational and post-translational levels. Numerous studies have investigated regulators involved in each of these steps, especially transcriptional regulators, small RNAs and components of the ubiquitin-proteasome system (UPS). However, few studies have looked at the connections between regulations at different levels. In *Arabidopsis thaliana*, the *ARABIDOPSIS SKP1-LIKE 1* (*ASK1*) gene encodes a core component of Skp1-Cul1-F-box (SCF)-type E3 ubiquitin ligases and has been reported to play important roles in post-translational regulations. But whether the regulation of protein degradation by the ASK1-containing SCF E3 ubiquitin ligases (ASK1-E3s) may affect gene transcription is not clear. In this study, we compared the transcriptomes of the *ask1* mutant and wild-type anthers at meiosis stages and found the mutation in *ASK1* indeed perturbs the transcription of many genes. As expected, a few meiosis-related genes are affected in *ask1*. Moreover, the misregulation of some tapetum-specific genes in *ask1* suggests a new role of ASK1 in regulating tapetum functions. The expression levels of many genes that response to various biotic and abiotic stimuli are decreased in *ask1*, indicating that transcriptional repressors may be normally destabilized by ASK1-E3s constitutively or upon signal perception. The expression levels of eleven transposable element genes are elevated in the *ask1* anther, indicating that ASK1-E3s may be involved in suppressing transposable elements during reproduction to prevent genome damages. Possible mechanisms underlying ASK1

regulatory functions are discussed. This study shed light on the role of ASK1-E3s in connecting regulations at the post-translational and transcriptional levels.

Note: The microarray experiments were performed by Yujin Sun and Wei Zhang. I analyzed the microarray data and prepared the manuscript.

Introduction

Development of every plant organ requires coordinated cellular processes including signal transduction, gene transcription, post-transcriptional regulation, translational and post-translational regulations, etc. The anther is one of the plant organs that serve as good model systems for studying various processes such as cell-cell communication, transcriptional regulation, post-translational regulation, and hormone signaling, among which transcriptional regulation is one of the most extensively investigated aspects.

Many transcription factor genes have been characterized to be essential for male fertility by regulating anther development at different stages in *Arabidopsis thaliana* (reviewed by Ge et al., 2010). *SPOROCYTELESS*/*NOZZLE* (*SPL*/*NZZ*) is required for the sporogenous cell fate determination and further differentiation of anther wall cells as well as pollen mother cells (Schiefthaler et al., 1999a; Yang et al., 1999b). At stages around meiosis, *DYSFUNCTIONAL TAPETUM 1* (*DYT1*), *MYB33*, *MYB65*, *MYB35*, *MYB80*, *ABORTED MICROSPORES* (*AMS*), and *MALE STERILITY 1* (*MS1*) are involved in regulating tapetum functions to support successful meiosis and pollen development (Wilson et al., 2001a; Ito and Shinozaki, 2002b; Sorensen et al., 2003b; Millar and Gubler, 2005; Zhang et al., 2006; Zhu et al., 2010; Phan et al., 2011; Feng et al., 2012a). *MYB21* and *MYB24* are required for male fertility and interact with Jasmonate-ZIM domain (JAZ) proteins (Song et al., 2011).

Many studies have examined anther transcriptomes of wild-type plants or male sterile mutants to discover genes expressed at different anther stages, in different anther cells, or affected by certain mutations (Wijeratne et al., 2007a; Hirano et al., 2008; Hobo et al., 2008; Ma et al., 2008; Aya et al., 2009; Huang et al., 2009; Ye et al., 2010; Zhu et al., 2010; Aya et al., 2011; Deveshwar et al., 2011; Ma et al., 2012). Gene expression profiling studies of transcription factor mutants are useful for identifying their putative target genes. However, many male sterility-related genes encode proteins that do not directly regulate gene transcription but are involved in metabolism, protein degradation, signal transduction, etc. One example is the ubiquitin-proteasome system (UPS) which has been reported to be important for male fertility. The *ARABIDOPSIS SKP1-LIKE 1* (*ASK1*) is one of the first UPS components discovered in plants (Yang et al., 1999a). The ASK1 protein is a subunit of Skp1-Cul1-F-box (SCF)-type multimeric E3 ubiquitin ligases in which ASK1 links the substrate receptor F-box proteins to the Cul1 subunit (Zheng et al., 2002). Many genetic and molecular studies have demonstrated the role of ASK1 in a spectrum of processes especially anther development and meiosis (Yang et al., 1999a).

Several F-box proteins have also been found to be required for anther development. The F-box protein REDUCED MALE FERTILITY (RMF) was shown to regulate tapetum degeneration (Kim et al., 2010). SECONDARY WALL THICKENING-ASSOCIATED F-BOX 1 (SAF1) negatively regulates endothecium cell wall thickening, which is required for anther dehiscence (Kim et al., 2012). The F-box protein UNUSUAL FLORAL ORGANS (UFO) is essential for floral meristem, petal and anther development and the *ufo* null mutant lacks petals and stamens (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Although ASK1 interacts with many F-box proteins including UFO (Takahashi et al., 2004) and is predicted to regulate many substrate proteins, the *ask1* mutant still produces some petals and wild-type-like pre-meiotic stamens and thus has weaker floral defects than the *ufo* null mutant. However, the simultaneous knockout of the *ASK1* gene and its closely related *ASK2* gene causes lethality (Liu

et al., 2004). These lines of evidence suggest that the weaker defects in *ask1* are probably due to redundancy between *ASK* gene family members. Nevertheless, the weak developmental defects in *ask1* may benefit the dissection of some aspects of ASK1 functions in anther development and meiosis, which is impossible if the entire stamen development is missing.

Although the UPS probably does not directly regulate gene transcription, it may indirectly affect gene transcription by regulating the stability of transcription regulators, kinases, and other types of signaling proteins. Some transcriptional regulatory mechanisms involving the UPS have been well established: transcriptional repressors including AUX/IAA proteins, DELLA proteins, and JAZ proteins are destabilized by the UPS upon hormone perception, releasing the suppression of transcription activators to regulate hormone-responsive genes (Gray et al., 2001; Zenser et al., 2001; Dill et al., 2004; Chini et al., 2007; Thines et al., 2007). In spite of these well known examples of hormone signaling, the connection between the regulation of protein degradation by the UPS and its effects on the transcriptomic dynamics is largely unknown.

In this study, we compared the transcriptomes of the wild-type and *ask1* mutant anthers at meiosis stages to investigate the role of ASK1 in controlling early anther development. Genes involved in many pathways including meiosis-related genes and signal-responsive genes are affected in the *ask1* mutant anther transcriptome, indicating that ASK1 may regulate multiple cellular processes by modulating gene transcription.

Materials and methods

Plant growth conditions and material collection

The *Arabidopsis thaliana* ecotype Landsberg *erecta* (L*er*) and *ask1* mutant were used in this study. Seeds were stratified at 4ºC for 2-4 days and sown into soil. Plants were grown under

23ºC and long-day conditions (16 hour light and 8 hour dark) in a growth room. One week after bolting, anthers of stage 4 to 6 were collected under a dissecting microscope (Nikon SMZ645) and immediately frozen in liquid nitrogen. Two biological replicates for both L*er* and *ask1* anthers were prepared for RNA extraction.

Microarray Analysis

The RNeasy Plant Kit (Qiagen, Valencia, CA) was used to extract the total RNA from the L*er* and *ask1* anther samples. 10 µg of total RNA was used for microarray analysis using the GeneChip *Arabidopsis* ATH1 Genome Array (Affymetrix, Santa Clara, CA) at the Penn State Genomics Core Facility - University Park, PA. The microarray results were normalized using the R package RMA and exported as Microsoft Excel files, in which expression values of each gene were averaged from replicates of each genotype. The averaged expression values were compared between L*er* and *ask1* to find the up-regulated and down-regulated genes with at least two-fold changes and t-test p-value<0.05.

Results

Comparison of the wild-type and *ask1* anther transcriptomes

Comparative transcriptomics analysis of L*er* and *ask1* anthers of stage 4-6 (meiosis stages) was performed by using Affymetrix GeneChip *Arabidopsis* ATH1 Genome Array to investigate how the regulation of protein degradation by ASK1-containing E3 ubiquitin ligases (ASK1-E3s) affects the transcriptomic dynamics in young anthers. Since the mutation of *ASK1* mainly affects meiosis without changing cell numbers and cell types at meiotic stages, the

differentially expressed genes more likely reflect the true transcriptional perturbations caused by the *ask1* mutation than the side effects of altered cell types and cell numbers at later stages.

Two biological replicates of each genotype were performed and all microarray datasets were normalized before the values were averaged between the two replicates of each genotype. Genes with microarray values lower than 50 in both L*er* and *ask1* were removed as background noise signals. Microarray values higher than 50 in at least one genotype were used for comparison to find genes with at least a two-fold difference. Totally 295 genes were down-regulated and 119 genes were up-regulated in the *ask1* anther transcriptome (two-fold cutoff and t-test p-value<0.05). We analyzed the biological process and molecular function categories of the up-regulated and down-regulated genes in the *ask1* anther transcriptome using the agriGO tool to determine whether ASK1 preferentially modulate certain pathways. The biological process categories enriched in the down-regulated genes include response to stimulus, pollen development, carbohydrate metabolic process, cellular macromolecule catabolic process, etc (Table 4-1).

ASK1 modulates the expression of signal-responsive genes

The category of "response to stimulus" can be divided into several sub-categories: response to hormones including jasmonic acid (JA), gibberellic acid (GA), abscisic acid (ABA), and salicylic acid (SA); response to stresses including water deprivation, wounding, and fungus; and others such as response to metal ion (Table 4-1). The discovery of such a large number of responsive genes down-regulated in the *ask1* mutant anther suggests that they are normally positively regulated by ASK1, possibly by destabilizing transcriptional repressors. The implication of this finding is that endogenous and environmental signals may trigger responses in the anther rapidly to facilitate normal development.

Table 4-1. Gene categories enriched in the down-regulated genes in the *ask1* anther.

Categories	query item	query total	background item	background total	p-value
Biological process categories					
response to JA	8	294	146	22479	0.00079
response to GA	6	294	116	22479	0.0046
response to ABA	8	294	255	22479	0.021
response to SA	5	294	122	22479	0.023
response to fungus	5	294	120	22479	0.022
response to wounding	6	294	132	22479	0.0084
response to abiotic stimulus	24	294	1083	22479	0.012
response to water deprivation	7	294	155	22479	0.0048
response to metal ion	10	294	345	22479	0.018
pollen development	7	294	110	22479	0.00069
carbohydrate metabolic process	20	294	795	22479	0.0059
cellular macromolecule catabolic process	11	294	400	22479	0.019
Molecular function categories					
hydrolase activity, acting on glycosyl bonds	15	294	399	22479	0.00038
oxidoreductase activity	28	294	1302	22479	0.011
coenzyme binding	7	294	189	22479	0.013
cofactor binding	8	294	254	22479	0.021
copper ion binding	5	294	123	22479	0.024
Cellular compartment categories					
endomembrane system	75	294	2768	22479	9.10E-08

The mechanism of signal-induced degradation of transcription repressors is well known in some hormone signaling pathways (e.g., JA and GA). Then the observation that JA- and GA-regulated genes were down-regulated in *ask1* anther suggests that these pathways are active in anther development, which is consistent with previous discoveries (Hirano et al., 2008; Cheng et al., 2009; Mandaokar and Browse, 2009; Song et al., 2011). A few E3 ubiquitin ligases have been

discovered to regulate certain steps of the ABA and SA signaling pathways (Lee et al., 2010; Liu and Stone, 2010; Fu et al., 2012; Li et al., 2012), raising the possibility that other hormone signaling pathways may also involve UPS components. Similar mechanisms of signal-induced transcription factor degradation have not been discovered in other processes including response to water deprivation, wounding, fungus, metal ion, etc. Although the possibility that these stimuli may affect gene transcription through crosstalk with hormone signaling pathways cannot be ruled out, it will be interesting to test whether these non-hormone signaling pathways also require UPS components.

Table 4-2. Anther development and meiosis-related genes down-regulated in *ask1*.

AGI	Gene	*ask1* microarray value	wild-type microarray value
AT1G66170	*MALE MEIOCYTE DEATH 1 (MMD1)*	10.92	83.01
AT1G10710	*POOR HOMOLOGOUS SYNAPSIS 1* (*PHS1*)	61.37	122.78
AT3G55580	*Regulator of chromosome condensation (RCC1) family protein*	41.40	98.74
AT3G11980	*MALE STERILITY 2* (*MS2*)	8.45	690.78
AT3G61730	*REDUCED MALE FERTILITY* (*RMF*)	75.95	157.10
AT4G20050	*QUARTET 3* (*QRT3*)	101.19	524.30
AT1G69500	*cytochrome P450 CYP704B1*	128.71	1636.47
AT3G06100	*NOD26-LIKE INTRINSIC PROTEIN 7;1* (*NIP7;1*)	297.79	896.18
AT3G42960	*TAPETUM1*	379.30	2090.38
AT4G28395	*ANTHER 7*	19.77	283.16

Anther development and meiosis-related genes are positively modulated by ASK1

Ten genes that are preferentially expressed in the anther and essential for anther development or meiosis are down-regulated in *ask1* (Table 4-2). The *MALE MEIOCYTE DEATH 1* (*MMD1*) gene which encode a plant homeodomain (PHD) protein was demonstrated to be essential for male meiosis (Yang et al., 2003b). The *POOR HOMOLOGOUS SYNAPSIS 1* (*PHS1*) gene is conserved at least in *Arabidopsis*, maize and wheat, and was reported to regulate homologous chromosome pairing and meiotic recombination (Ronceret et al., 2009; Khoo et al., 2012). The *AT3G55580* gene, which is predicted to encode a REGULATOR OF CHROMOSOME CONDENSATION 1 (RCC1) family protein, although without experimental evidence, might be involved in chromosome dynamics during anther cell mitosis or meiosis. *MALE STERILITY 2* (*MS2*) encodes a fatty acid reductase required for pollen exine formation (Aarts et al., 1997; Chen et al., 2011). *RMF* encodes a nuclear-localized F-box protein that regulates tapetum degeneration and pollen development (Kim et al., 2010). The *QUARTET3* (*QRT3*) gene encodes a polygalacturonase, which is specifically expressed in the tapetum and its protein product is secreted into anther locules to degrade pollen mother cell wall to release individual microspores from tetrads (Rhee et al., 2003). A cytochrome P450 gene, *CYP704B1*, encodes a long-chain fatty acid omega-hydroxylase essential for sporopollenin synthesis in the anther and thus is required for normal male fertility (Dobritsa et al., 2009). The *TAPETUM1* which encodes a short-chain dehydrogenase (LebelHardenack et al., 1997), the *ARABIDOPSIS THALIANA ANTHER 7* (*ATA7*) which is predicted to encode a protein related to lipid transfer proteins (Rubinelli et al., 1998), and the *NOD26-LIKE INTRINSIC PROTEIN 7;1* (*NIP7;1*) which encodes a boric acid transporter (Li et al., 2011) are specifically expressed in the anther and predicted to be required for pollen development.

The down-regulation of these meiosis- or pollen development-related genes in the *ask1* anther indicates that ASK1 is not only essential for male meiosis as demonstrated by previous studies (Yang et al., 1999a; Wang and Yang, 2006; Yang et al., 2006; Zhao et al., 2006), but is also involved in regulating metabolic activities in the anther, especially in the tapetum, for normal pollen development. ASK1-E3s may destabilize certain unidentified transcriptional repressors which directly or indirectly repress the expression of the anther-specific genes described above.

Carbohydrate metabolism is affected by the *ask1* mutation

Table 4-3. Carbohydrate metabolism-related genes down-regulated in *ask1*.

AGI	Gene name	*ask1* microarray value	wild-type microarray value
AT1G75940	*BETA GLUCOSIDASE 20 (BGLU20)/ATA27*	5.51235	155.80421
AT2G44490	*BETA GLUCOSIDASE 26 (BGLU26)/PENETRATION 2 (PEN2)*	43.83002	95.66224
AT5G54570	*BETA GLUCOSIDASE 41 (BGLU41)*	20.91892	97.86722
AT1G77410	*BETA-GALACTOSIDASE 16 (BGAL16)*	255.88012	860.53661
AT5G56870	*BETA-GALACTOSIDASE 4 (BGAL4)*	160.32518	749.07167
AT5G09730	*BETA-XYLOSIDASE 3 (BXL3)*	130.26733	346.47989
AT2G36190	*CELL WALL INVERTASE 4 (cwINV4)*	29.92453	66.10161
AT3G26140	*Cellulase (glycosyl hydrolase family 5) protein*	63.36905	142.82872
AT5G44680	*DNA glycosylase superfamily protein*	36.82797	79.99714
AT5G07830	*GLUCURONIDASE 2 (GUS2)*	228.84827	892.90829
AT3G55780	*Glycosyl hydrolase superfamily protein*	67.23377	252.19081
AT3G61810	*Glycosyl hydrolase family 17 protein*	3.52452	104.93431
AT3G23770	*Glycosyl hydrolases family 17 protein*	34.20104	497.53180
AT3G07850	*Pectin lyase-like superfamily protein*	8.18536	124.20375
AT4G20050	*QUARTET 3 (QRT3), polygalacturonase*	101.19457	524.30410

Fifteen carbohydrate metabolism-related genes are down-regulated in *ask1* (Table 4-3). *PENETRATION 2* (*PEN2*) encodes a glycosyl hydrolase, which was shown to be involved in a preinvasion resistance mechanism (Lipka et al., 2005). *ATA27* is an anther gene encoding a beta-glucosidase, which is predicted to be located in the endoplasmic reticulum (ER) lumen (Rubinelli et al., 1998). *GLUCURONIDASE 2* (*GUS2*) was reported to be involved in cell elongation (Eudes et al., 2008). The *QRT3* gene is a polygalacturonase gene highly expressed in the tapetum (Rhee et al., 2003). Although the exact functions of most of these enzyme genes in the anther are not clear so far, since they are expressed to various levels in the wild-type anther and are affected in the *ask1* mutant, they may play some roles in either cell wall modification, or intracellular carbohydrate metabolism. The down-regulation of these enzyme genes in *ask1* implies another previously unknown aspect of ASK1 functions in modulating carbohydrate metabolic pathways.

ASK1 is required for redox homeostasis in the anther

Oxidoreductase activities have been demonstrated to be required for anther development (Xing and Zachgo, 2008; Li et al., 2009b; Kelliher and Walbot, 2012). Twenty-eight oxidoreductase genes are down-regulated in *ask1* (Table 4-4) indicating that ASK1 may also modulate cellular oxidoreductive status through either biosynthetic pathways or regulation of protein redox status during anther development. Two of the oxidoreductases may post-translationally modify proteins: THIOREDOXIN H-TYPE 5/LOCUS OF INSENSITIVITY TO VICTORIN 1 (TRX5/LIV1) is involved in defense pathways by reducing disulfide bridges of target proteins (Sweat and Wolpert, 2007); PROLYL 4-HYDROXYLASE 2 (P4H2) can hydroxylate proline residues of target proteins (Tiainen et al., 2005). Other oxidoreductases are involved in catalyzing reactions in various biochemical pathways. For example, ALCOHOL DEHYDROGENASE 1 (ADH1) responses to hypoxia (Ferl and Laughner, 1989) and catalyze

the reduction of acetaldehyde using NADH as reductant. Several anther-specific genes, *MS2*, *CYP704B1*, and *TAPETUM1* also encode oxidoreductases and *MS2* and *CYP704B1* are essential for normal male fertility, indicating an important role of redox reactions in normal anther development.

Table 4-4. Oxidoreductase genes down-regulated in *ask1*.

AGI	Gene name	*ask1* microarray value	wild-type microarray value
AT4G03050	*2-oxoglutarate-dependent dioxygenase*	144.71	328.01
AT1G62570	*FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 4* (*FMO GS-OX4*)	72.22	650.32
AT1G77120	*ALCOHOL DEHYDROGENASE 1* (*ADH1*)	43.27	106.26
AT3G48000	*ALDEHYDE DEHYDROGENASE 2B4* (*ALDH2B4*)	1447.64	3030.33
AT3G11980	*MALE STERILITY 2* (*MS2*)	8.45	690.78
AT1G73680	*ALPHA DIOXYGENASE* (*ALPHA DOX2*)	59.82	140.01
AT1G69500	*CYP704B1*	128.71	1636.47
AT3G45140	*LIPOXYGENASE 2* (*LOX2*)	110.74	471.42
AT4G19230	*CYP707A1*	104.95	410.01
AT1G13080	*CYP71B2*; cytochrome P450 monooxygenase	25.38	76.50
AT4G37980	*ELICITOR-ACTIVATED GENE 3-1* (*ELI3-1*)	144.44	296.25
AT1G30760	*FAD-binding Berberine family protein*	19.66	208.75
AT1G20020	*FERREDOXIN-NADP(+)-OXIDOREDUCTASE 2*	310.84	632.98
AT5G35790	*GLUCOSE-6-PHOSPHATE DEHYDROGENASE 1*	204.94	413.22
AT1G12570	*Glucose-methanol-choline (GMC) oxidoreductase*	44.23	355.74
AT4G30470	*NAD(P)-binding Rossmann-fold superfamily protein*	105.55	301.50
AT1G76470	*NAD(P)-binding Rossmann-fold superfamily protein*	42.81	416.73
AT5G65205	*NAD(P)-binding Rossmann-fold superfamily protein*	49.11	121.30
AT1G68540	*NAD(P)-binding Rossmann-fold superfamily protein*	495.89	2230.62
AT1G37130	*NITRATE REDUCTASE 2* (*NIA2*)	143.06	401.55
AT2G24800	*Peroxidase*	115.38	486.46
AT1G19230	*Ferredoxin reductase-type FAD-binding domain*	83.83	381.85
AT3G13400	*SKU5 SIMILAR 13* (*SKS13*)	23.40	124.16
AT1G55560	*SKU5 SIMILAR 14* (*SKS14*)	6.79	51.14
AT3G42960	*TAPETUM 1* (*ATA1*), short-chain dehydrogenase	379.30	2090.38
AT1G45145	*THIOREDOXIN H-TYPE 5* (*TRX5*)	133.13	616.25
AT3G06300	*PROLYL 4-HYDROXYLASE 2* (*P4H2*)	314.80	689.19
AT5G39320	*UDP-glucose 6-dehydrogenase*	334.23	678.11

Transcription regulators down-regulated in *ask1*

Table 4-5. Transcriptional regulators down-regulated in *ask1*.

AGI	*Gene name*	*ask1* microarray value	wild-type microarray value
AT3G04620	*D NUCLDUO1-ACTIVATEEIC ACID BINDING PROTEIN 1 (DAN1)*	69.28	456.42
AT5G25190	*ETHYLENE AND SALT INDUCIBLE 3 (ESE3)*	62.00	137.96
AT5G61590	*Ethylene response factor*	193.02	482.73
AT5G19260	*FANTASTIC FOUR 3*	131.31	483.69
AT5G10140	*FLOWERING LOCUS C*	68.10	251.54
AT4G37790	*Homeobox, HAT22*	96.92	259.72
AT2G22800	*Homeobox, HAT9*	94.50	228.52
AT3G61890	*Homeodomain leucine zipper HOMEOBOX 12 (HB-12)*	26.45	83.42
AT5G52170	*Homeodomain leucine zipper HOMEODOMAIN GLABROUS 7 (HDG7)*	15.98	54.86
AT5G49450	*BASIC LEUCINE-ZIPPER 1*	87.76	229.42
AT2G16720	*MYB7*	47.74	105.09
AT4G34990	*MYB32*	98.36	300.93
AT2G36890	*MYB38/REGULATOR OF AXILLARY MERISTEMS 2 (RAX2)/BLUE INSENSITIVE TRAIT 1 (BIT1)*	30.27	87.66
AT4G37780	*MYB87*	3.18	50.91
AT5G62320	*MYB99*	11.65	77.25
AT1G60240	*NAC protein*	1.54	72.49
AT2G33480	*NAC041*	29.99	65.75
AT4G27410	*NAC072/RESPONSIVE TO DESICCATION 26 (RD26)*	44.85	106.85
AT5G39610	*NAC092*	59.31	241.28
AT5G41090	*NAZ095*	20.20	84.28
AT4G20330	*Transcription initiation factor TFIIE*	237.93	481.52
AT4G08360	*Global transcription factor*	234.55	484.09
AT5G35600	*HISTONE DEACETYLASE7 (HDA7)*	14.32	85.91
AT1G54230	*Winged helix-turn-helix transcription repressor*	20.36	103.92
AT3G56400	*WRKY DNA-BINDING PROTEIN 70 (WRKY70)*	35.10	77.55
AT3G19580	*ZINC-FINGER PROTEIN 2 (ZF2)*	59.40	135.30
AT5G15480	*Zinc finger family protein,C2H2-type*	12.94	53.08

Among the down-regulated genes in the *ask1* mutant, 27 genes encode transcription regulators (Table 4-5). Most of these genes have not been functionally studied except a few with functions or expression regulations characterized to some degree. Five NAC domain-containing transcription factors are down-regulated in *ask1*. Some functional information is available for two of them, *NAC072*/*RESPONSIVE TO DESICCATION 26* (*RD26*) and *NAC092*. *NAC072*/*RD26* is induced by drought and ABA and its overexpression leads to hypersensitivity to ABA (Fujita et al., 2004; Tran et al., 2004). NAC092 is responsive to salt stress, ABA, ethylene and auxin, and positively regulates aging-induced cell death (He et al., 2005; Kim et al., 2009). HOMEOBOX 12 (HB12) was reported to be rapidly induced by ABA and water deficit stress and negatively regulates expression of an enzyme required for GA synthesis (Olsson et al., 2004; Son et al., 2010). HB12 was recently shown to have negative feedback effects on ABA signaling by activating PP2C genes which encode negative regulators of ABA signaling and by repressing the expression of ABA receptor genes (Valdes et al., 2012). *ZINC-FINGER PROTEIN 2* (*ZF2*) responses to ABA and functions as a repressor of the ABA signaling pathway (Drechsel et al., 2010; Kodaira et al., 2011). The down-regulation of the above ABA-responsive transcription factors indicates that the ABA signaling pathway may be defective in the *ask1* anther and therefore, ASK1 is normally required for ABA signaling probably by destabilizing certain negative regulators.

Several other transcriptional regulators that are reported to be involved in various processes are also affected in *ask1*. Four out of the five *MYB* genes down-regulated in the *ask1* anther transcriptome have been functionally studied: *MYB99* and *MYB32* are required for tapetum function and pollen development (Preston et al., 2004b; Alves-Ferreira et al., 2007); *MYB38*/*REGULATOR OF AXILLARY MERISTEMS 2* (*RAX2*)/*BLUE INSENSITIVE TRAIT 1* (*BIT1*) and *MYB87* encodes positive regulators of blue light signaling and axillary meristem initiation (Muller et al., 2006; Hong et al., 2008). WRKY DNA-BINDING PROTEIN 70

(WRKY70) acts as an activator of SA-responsive genes and a repressor of JA-regulated genes (Hu et al., 2012). The role of FLOWERING LOCUS C (FLC) as a repressor of flowering has been well documented (Sanda and Amasino, 1996; Caicedo et al., 2004). In spite of their previously reported functions, the roles of these transcriptional regulators in anther development are largely unknown. Since ASK1 may indirectly modulate gene transcription by regulating the protein stability of transcription factors, the down-regulation of the above transcription factor genes indicate a cascade of transcriptional regulations where transcriptional repressors accumulate in *ask1* to suppress target transcription factor gene expression, or may result from a feedback mechanism in which transcription factors stabilization in *ask1* negatively regulate their own expression.

Protein kinase genes down-regulated in *ask1*

Table 4-6. Protein kinase genes down-regulated in *ask1*.

AGI	Gene name	*ask1* microarray value	wild-type microarray value
AT1G23700	*Protein kinase*	55.95	201.25
AT1G56720	*Protein kinase*	87.78	187.29
AT1G66460	*Protein kinase*	8.70	71.33
AT1G69790	*Protein kinase*	50.59	103.52
AT2G17170	*Protein kinase*	14.22	80.91
AT3G07700	*Protein kinase*	39.39	103.69
AT4G35500	*Protein kinase*	75.90	163.00
AT5G20050	*Protein kinase*	67.06	144.16
AT5G28080	*WITH NO LYSINE (K) KINASE 9 (WNK9)*	48.14	262.74
AT5G41990	*WITH NO LYSINE (K) KINASE 8 (WNK8)*	119.63	437.10
AT2G02220	*PHYTOSULFOKIN RECEPTOR 1 (PSKR1)*	30.58	74.79

Eleven protein kinase genes are down-regulated in the *ask1* anther (Table 4-6). WITH NO LYSINE (K) KINASE 8 (WNK8) protein interacts with and phosphorylates the V-ATPase subunit C (Hong-Hermesdorf et al., 2006) and is also involved in flowering time regulation (Wang et al., 2008). A paralogous gene of *WNK8*, *WNK9*, is also down-regulated in *ask1* indicating that these two homologous genes might be co-regulated by certain transcription factors and may have redundant functions. *PHYTOSULFOKIN RECEPTOR 1* (*PSKR1*) encodes a leucine-rich repeat receptor kinase that positively regulates cell longevity and growth (Matsubayashi et al., 2006; Amano et al., 2007; Loivamaki et al., 2010; Kwezi et al., 2011; Stuehrwohldt et al., 2011). Eight protein kinase super family proteins are also down-regulated in *ask1* but their functions are unknown.

Peptidase genes down-regulated in *ask1*

Table 4-7. Peptidase genes down-regulated in *ask1*.

AGI	Gene name	*ask1* microarray value	wild-type microarray value
AT5G50260	*Cysteine proteinase*	7.37	165.39
AT1G02300	*Cysteine proteinases*	49.25	113.60
AT2G21430	*Papain family cysteine protease*	356.87	1789.39
AT1G05840	*Eukaryotic aspartyl protease*	119.46	254.13
AT2G03200	*Eukaryotic aspartyl protease*	23.57	166.83
AT5G24820	*Eukaryotic aspartyl protease*	213.13	1009.11
AT4G04460	*Saposin-like aspartyl protease*	34.32	296.24
AT3G45010	*Serine carboxypeptidase-like 48 (scpl48)*	161.47	323.64
AT1G52430	*Ubiquitin carboxyl-terminal hydrolase*	10.45	96.60

Nine peptidase/protease genes are down-regulated in *ask1* (Table 4-7). Little functional information is available for these peptidases except for sequence homology. Four of these genes encode aspartyl proteases; three encode cysteine proteases; one encodes a serine carboxypeptidase-like protein; and one encodes an ubiquitin carboxyl-terminal hydrolase/deubiquitinase. These peptidases may be required for normal anther development and may be regulated by regulators that are substrates of ASK1-E3s.

Ubiquitin pathway component genes down-regulated in *ask1*

Table 4-8. Ubiquitin pathway component genes down-regulated in *ask1*.

AGI	Gene name	*ask1* microarray value	wild-type microarray value
AT3G61730	*F-box, REDUCED MALE FERTILITY (RMF)*	75.95	157.10
AT1G80630	*F-box*	89.57	187.83
AT2G29830	*F-box*	19.82	54.28
AT3G23880	*F-box*	79.72	498.54
AT3G61340	*F-box*	37.20	124.26
AT3G62430	*F-box*	68.51	140.67
AT4G34470	*ASK11/ASK12*	29.48	112.25
AT3G60010	*ASK13*	50.18	124.82
AT1G05690	*BTB AND TAZ DOMAIN PROTEIN 3 (BT3)*	39.76	123.97
AT1G52430	*Ubiquitin carboxyl-terminal hydrolase*	10.45	96.60
AT4G03360	*Ubiquitin family protein*	3.07	91.53

Interestingly, eleven genes that encode ubiquitin pathway components are also down-regulated in *ask1* (Table 4-8) indicating that the UPS has regulatory mechanisms in controlling its own functions. Six of these genes encode F-box proteins, one of which encodes REDUCED MALE FERTILITY (RMF) has been reported to interact with ASK1 and is involved in tapetum

cell degeneration (Kim et al., 2010). BTB AND TAZ DOMAIN PROTEIN 3 (BT3) is a subunit of Cullin3-based E3 ubiquitin ligases and, together with its close homologs BT1 and BT2, regulates male and female gametophyte development (Figueroa et al., 2005; Robert et al., 2009). Intriguingly, two of the *ASK* gene family members, *ASK11* (indistinguishable from *ASK12* based on the microarray probe 253271_s_at) and *ASK13* are also down-regulated in *ask1* indicating that the expression of some *ASK* genes may be dependent of ASK1.

Genes up-regulated in the *ask1* anther

Among the up-regulated genes in *ask1*, several categories are enriched, including genes required for meiosis and cell cycle regulation, transposable element genes, and RNA-binding proteins. The up-regulation of these genes in *ask1* indicates that they are normally suppressed by ASK1, possibly to timely control cell cycle progression and to prevent transposable element movement during meiosis.

Meiosis and cell cycle-related genes up-regulated in *ask1*

Table 4-9. Meiosis and cell cycle-related genes up-regulated in *ask1*.

AGI	Gene name	*ask1* microarray value	wild-type microarray value
AT1G07370	*PROLIFERATING CELLULAR NUCLEAR ANTIGEN 1 (PCNA1)*	1332.33	656.99
AT1G72440	*SLOW WALKER2 (SWA2)*	324.25	157.35
AT2G17620	*CYCLIN B2;1 (CYCB2;1)*	286.02	105.91
AT2G29680	*CELL DIVISION CONTROL 6 (CDC6)*	142.08	67.39
AT3G07050	*NUCLEOSTEMIN-LIKE 1 (NSN1)*	619.95	214.49
AT5G01630	*BRCA2-LIKE B (BRCA2B)*	106.23	49.96

Six cell cycle-related genes are up-regulated in *ask1* (Table 4-9) and most of them have been investigated to some extent. *NUCLEOSTEMIN-LIKE 1* (*NSN1*) encodes a nucleolar GTPase required for shoot apical meristem and floral meristem development (Wang et al., 2012a; Wang et al., 2012b). The up-regulation of *NSN1* may reflect the changes in the cell cycle progression in the *ask1* anther. BRCA2-LIKE B (BRCA2B) is important for both somatic and meiotic homologous recombination (Siaud et al., 2004; Seeliger et al., 2012). CELL DIVISION CONTROL 6 (CDC6) is expressed during S-phase of the cell cycle and its function is associated with endoreplication (Castellano et al., 2001; Ramos et al., 2001). CDC6 was reported to be possibly regulated by the E2F1 transcription factor at the G1/S transition (de Jager et al., 2001). The up-regulation of CDC6 indicates that its transcriptional regulators like E2F1 which are normally degraded by the UPS may have accumulated in the *ask1*. A recent study indeed demonstrated that E2F transcription factors are degraded by the 26S proteasome (Hirano et al., 2011). Thus, our gene expression data and previous results suggest that E2F transcription factors may be substrates of ASK1-E3s. SLOW WALKER2 is essential for coordinated cell cycle progression in female gametophyte development (Li et al., 2009a). PROLIFERATING CELLULAR NUCLEAR ANTIGEN 1 (PCNA1) is involved in cell cycle regulation and interacts with two cell cycle-regulated SET-domain proteins whose overexpression causes male sterility (Raynaud et al., 2006). This raises the possibility that the elevated expression level of PCNA1 may increase the amount of functional protein complexes with these SET-domain proteins, which may be one of the possible causes of male sterility in *ask1*. It was reported that E2F transcription factors positively regulate PCNA proteins and their interacting SET-domain proteins (Egelkrout et al., 2002; Kosugi and Ohashi, 2002). Again, this suggests that ASK1-E3s may destabilize E2F transcription factors to prevent ectopic expression of cell cycle genes. Since PCNA proteins have been reported to function in DNA replication and repair (Essers et al., 2005), the relatively high expression of *PCNA1* in the wild-type anther (microarray value 656) of meiosis stages is

consistent with the active DNA synthesis and DNA repair during meiosis. The elevated expression of *PCNA1* in the *ask1* anther (microarray value 1332) suggests that either DNA damages may accumulate due to defects in meiocyte cell cycle regulation; or DNA replication activity may persist due to retarded S phase or increased endoreplication which is indicated by the up-regulation of the S phase gene *CDC6* described above.

RNA-binding protein genes up-regulated in *ask1*

Table 4-10. RNA-binding protein genes up-regulated in *ask1*.

AGI	Gene name	*ask1* microarray value	wild-type microarray value
AT5G58130	*REPRESSOR OF SILENCING 3 (ROS3)*	546	240
AT3G58510	*DEA(D/H)-box RNA helicase*	689	305
AT1G77050	*DEAD-box ATP-dependent RNA helicase 29*	186	41
AT3G22330	*DEAD-box protein PUTATIVE MITOCHONDRIAL RNA HELICASE 2 (PMH2)*	1740	837
AT2G42520	*DEAD-box RNA helicase*	366	135
AT1G20220	*DNA/RNA-binding protein*	420	176
AT1G18630	*GLYCINE-RICH RNA-BINDING PROTEIN 6*	319	145
AT4G39260	*GLYCINE-RICH RNA-BINDING PROTEIN 8*	2346	728
AT5G27140	*Pre-mRNA processing ribonucleoprotein*	348	122
AT2G32415	*ribonuclease H*	80	29
AT5G02530	*RNA-binding*	1645	616

RNA-binding proteins provide post-transcriptional regulations of gene expression. Eleven RNA-binding proteins are up-regulated in *ask1* (Table 4-10) indicating that RNA metabolism or regulation is abnormal in the *ask1* anther. Four DEAD-box RNA helicase genes are up-regulated in *ask1*, among which PUTATIVE MITOCHONDRIAL RNA HELICASE 2

(PMH2) is required for efficient intron splicing in mitochondria (Kohler et al., 2010). REPRESSOR OF SILENCING 3 (ROS3) is an RNA-binding protein required for DNA demethylation (Zheng et al., 2008). ROS3 binds to small RNAs and is located with its homologous protein ROS1 to nuclear foci to prevent DNA hypermethylation and transcriptional gene silencing (Zheng et al., 2008). ASK1-E3s may normally destabilize certain positive regulators of *ROS3* expression to constrain transposable element motility during meiosis. The up-regulation of *ROS3* is expected to activate the transcription of its target genomic loci. The following observation of transposable element gene up-regulation is consistent with the expected outcome of *ROS3* elevation.

Transposable element genes up-regulated in *ask1*

Table 4-11. Transposable element genes up-regulated in *ask1*.

AGI	Gene description	*ask1* microarray value	wild-type microarray value
AT4G10580	gypsy-like retrotransposon family	107	47
AT1G42365	gypsy-like retrotransposon family	83	36
AT2G06160	gypsy-like retrotransposon family	55	19
AT4G04010	transposable element gene; similar to Ulp1 protease family protein	88	26
AT3G29210	transposable element gene; similar to Ulp1 protease family protein	84	37
AT2G15870	copia-like retrotransposon family	91	26
AT3G21020	copia-like retrotransposon family	101	46
AT3G21040	copia-like retrotransposon family	226	88
AT1G42705	hAT-like transposase family	186	30
AT3G32270	transposable element gene; similar to unknown protein	105	50
AT1G44935	Sadhu non-coding retrotransposon family member	90	38

Eleven transposable element genes are up-regulated in the *ask1* anther (Table 4-11). These genes all have low levels in the wild-type anther (microarray values <100) and nine of them are even lower than 50, a threshold set as detection of gene expression, suggesting that most of these transposable element genes are inactive in young anthers of meiosis stages. The transcript levels of these transposable element genes are all increased by more than two-fold in *ask1* compared with that in wild-type. These transposable elements may be normally epigenetically silenced during meiosis and ASK1-E3s add another layer of repression by negatively modulating the expression of certain regulators such as ROS3 to prevent genomic damages caused by transposable element transposition.

Discussion

Gene expression phenotypes reveals new aspects of ASK1 functions

ASK1 has been suggested to be involved in regulating male meiosis based on genetic and histocytological evidence (Yang et al., 1999a; Wang and Yang, 2006; Yang et al., 2006; Zhao et al., 2006). Although the effects of the *ask1* mutation on chromosome morphology and movement during male meiosis were reported in these studies, little is known about molecular changes in the mutant anther. In this study, several meiosis-related genes were found to be affected in *ask1*. *MMD1* encodes a plant homeodomain protein and may participate in chromatin remodeling or transcription (Yang et al., 2003b). The misregulation of *MMD1* indicates that ASK1 functions at stages at least earlier than diakinesis, the last stage of prophase when meiotic defects start to appear in the *mmd1* mutant. Several cell cycle-related genes are up-regulated in *ask1* including *BRCA2B*, *CDC6*, *PCNA1* and *SLOW WALKER2*. BRCA2B controls single-strand invasion steps during homologous recombination by interacting with two strand-exchange proteins RAD51 and

DMC1 (Siaud et al., 2004; Seeliger et al., 2012). *CDC6* and *PCNA1* are expressed during S phase and their dysregulation suggests an even earlier stage of ASK1 function. From these gene expression phenotypes, we can extend ASK1 functions to as early as interphase stages where ASK1-E3s may post-translationally regulate cell cycle regulators. The meiotic defects observed at later stages may be the consequences of undetected interphase perturbations or may result from misregulation of proteins that function at prophase or later phases of meiosis.

In addition to meiosis-related genes, several tapetum genes, *MS2*, *RMF*, *QRT3*, *CYP704B1*, and *TAPETUM1*, are also affected in *ask1*, indicating a possible role of ASK1 in regulating tapetum functions, which has not been suggested by previous histocytological evidence. Since ASK1 is expressed in a wide array of tissues especially in dividing cells, it is not surprising that ASK1 also functions in the tapetum cells which usually undergo mitosis as other somatic cells and endomitosis to produce binucleate cells (Weiss and Maluszynska, 2001). Most of the binucleate tapetum cells undergo endoreplication yielding two tetraploid nuclei. Interestingly, CDC6 which functions in endoreplication (Castellano et al., 2001; Ramos et al., 2001) is up-regulated in *ask1*, implicating that ASK1 precisely controls cell cycle progression not only in the meiocytes but also possibly in the tapetum cells. Another possible cause of tapetum gene misregulation is that cell-cell communications between the tapetum cells and meiocytes may be affected in *ask1*, resulting in tapetum transcriptome alteration. Further studies are required to test these possibilities.

Possible mechanisms of ASK1 regulatory functions

There could be many proteins that can be ubiquitinated by ASK1-E3s for proteasome-mediated degradation. Depending on the molecular functions of these substrates, their degradation may result in many different cellular effects. However, the outcomes of the

degradation of these substrate proteins are mostly unknown except for those involved in a few well-investigated signaling pathways (e.g., JA signaling transduction). The comparison of the *ask1* and wild-type young anthers of meiotic stages enables us to draw possible connections between regulations of protein degradation and gene transcription.

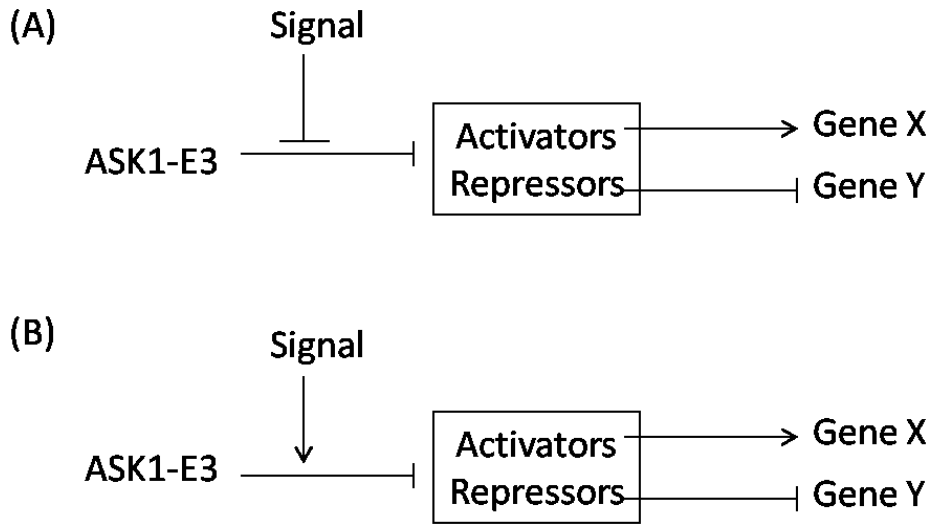

Figure 4-1. Possible mechanisms of ASK1 functions in regulating transcriptional regulators to modulate gene transcription.

(A) The transcriptional activator/repressors are constitutively destabilized by ASK1-E3s but stabilized by the signals.
(B) The destabilization of transcriptional regulators by ASK1-E3s is dependent of signal perception.

Transcriptional repressors or activators may be regulated by ASK1-E3s at the protein level to modulate downstream gene transcription (Figure 4-1). Stabilization of transcriptional repressors in the *ask1* anther may down-regulate their target genes. Accordingly, 295 genes were down-regulated in *ask1* including many signal-responsive genes, transcriptional regulators, kinases, peptidases, oxidoreductases, UPS component genes, carbohydrate metabolism-related genes, and anther development/ meiosis-related genes. Degradation of transcriptional repressors

may be a predominant mechanism of ASK1-E3 functions because many more genes are down-regulated (295 genes) in *ask1* than those being up-regulated (119 genes); and more gene categories are enriched in the down-regulated genes, indicating possible co-regulations by common or related repressors. For example, six F-box protein genes that are down-regulated in *ask1* might be regulated by common transcription repressors. However, due to lack of comprehensive transcription factor binding site information, it is hard to predict which transcription factor(s) may regulate these genes. Further experiments are required to find the short-lived transcriptional regulators between the ASK1 regulations of protein stability and gene transcription.

ASK1 may modulate gene transcription through different possible mechanisms (Figure 4-1). First, ASK1-E3s may constitutively destabilize transcriptional regulators to suppress responses in the absence of stimuli; signal perception stabilizes these transcriptional regulators to induce fast responses by activating or repressing target gene transcription (Figure 4-1A). Second, transcriptional regulators may be stable without stimuli; signals may activate ASK1-E3s to remove transcriptional repressors or activators to derepress or turn off their target gene expression, respectively (Figure 4-1B). These mechanisms have been reported to be involved in several hormone signaling pathways. For example, the first mechanism underlies the ethylene signaling pathway: the ETHYLENE INSENSITIVE3 (EIN3) protein, which is a key transcriptional regulator of ethylene-responsive genes, is constitutively ubiquitinated for degradation in the absence of ethylene; after ethylene perception EIN3 is stabilized to regulate target genes (Guo and Ecker, 2003; Potuschak et al., 2003). The second mechanism is deployed in the JA signaling pathway: the JAZ proteins which function as repressors are stable without JA hormone; upon JA perception JAZ proteins are ubiquitinated and degraded to release active MYC transcriptional factors to regulate JA-responsive genes (Chini et al., 2007; Thines et al., 2007).

These two mechanisms may not be mutually exclusive. Plants may have evolved different mechanisms to launch responses rapidly upon sensing different signals, as exemplified by the ethylene and JA signaling pathways. Or, the two modes of responses may be used in combination, either simultaneously to coordinate different pathways, or sequentially to dampen the initial responses with a negative feedback mechanism. One recent study found that JAZ repressors interact with EIN3 physically and attenuate the EIN3 function in regulating ethylene-responsive genes (Zhu et al., 2011). Thus, this protein-protein interaction establishes the crosstalk between two hormone signaling pathways that use different mechanisms of UPS-mediated transcriptional regulations.

Despite the many studies of ASK1 functions from various angles, information of the molecular processes regulated by ASK1-E3s is still scarce. This study opens many possibilities for future studies by providing clues that ASK1 may modulate gene expression through destabilizing transcriptional regulators that are involved in many signaling pathways and cell cycle progression.

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Chapter 5

Conclusions and Perspectives

Transcriptional regulations of the *Arabidopsis* flower and anther development

Plant reproduction is an intriguing biological process that is extensively investigated by plant biologists with a variety of specific interests including evolution, plant physiology, developmental biology, cytology, molecular biology, plant breeding, horticulture, ecology, etc. The projects described above investigated *Arabidopsis* flower and anther development from several different angles.

In Chapter 2, the functional study of the transcription factor DYT1 reveals that it acts as an activator in regulating downstream gene transcription. The comparison of the *dyt1* mutant and wild-type anther transcriptomes performed in our lab uncovered hundreds of genes that are affected by the *dyt1* mutation. This finding is helpful for determining downstream genes and pathways controlled by DYT1 during anther development. However, one can speculate that only a portion of these downstream genes are directly regulated by DYT1 and the direct target genes should respond earlier than the further downstream genes when DYT1 is induced. The changes in expression levels of two genes downstream from *DYT1*, *MYB35* and *MS1,* were observed upon induction of *DYT1*, and the results showed that the *MYB35* gene was induced very rapidly whereas the *MS1* gene was induced much later. MYB35 was also determined to function as a transcriptional activator in this study, suggesting that *DYT1* and *MYB35* may form a transcriptional activation cascade in regulating expression of further downstream genes.

Although some aspects of *DYT1* functions were revealed in this study, much more awaits further investigation, for example, determination of direct target genes of DYT1 and regulatory

cascades/networks. Currently, I am using the DYT1-GR *dyt1* transgenic plants to determine DYT1 direct target genes in a genome-wide manner. The transcriptomes of inflorescences treated respectively by the mock solution, the inducer Dexamethasone, Dexamethasone plus the translational inhibitor Cycloheximide, and Cycloheximide alone, have already been determined by the next-generation sequencing technology. The RNAseq data are under analysis to find genes that are induced by dexamethasone with or without the translational inhibitor Cycloheximide, but not induced by the mock or Cycloheximide alone. These genes should be directly regulated by DYT1 without *de novo* protein synthesis. However, one caveat is that this approach may miss some genes that are regulated by a protein complex involving DYT1 and *de novo* synthesized proteins. So, in addition to the induction assays, chromatin-immunoprecipitation followed by sequencing (ChIP-seq) experiment is needed to determine the *in vivo* binding sites and target genes of DYT1.

Similar approaches used for studying DYT1 functions can be extended to other transcription factors. Since many transcription factors have been characterized to be important for flower and anther development, determining their binding sites and target genes is crucial for elucidating the transcriptional regulatory networks which then will serve as a fine map for a better understanding of plant reproduction and for improving plant breeding.

Post-translational regulations of the *Arabidopsis* flower development

Proteins are regulated by various post-translational modifications including ubiquitination, phosphorylation, glycosylation, methylation, acetylation, proteolytic cleavage, etc. Several of these post-translational modifications such as phosphorylation and ubiquitination have been extensively investigated because of their ubiquitous roles in regulating signaling pathways. In the project presented in Chapter 3, the role of ASK1-E3 ligases in regulating *Arabidopsis*

flower protein degradation was studied by comparing the floral proteomes of the *ask1* mutant and wild-type. In the absence of functional ASK1-E3s, substrates that are normally ubiquitinated and degraded should accumulate to higher protein levels. Therefore, the proteins detected only in the *ask1* mutant proteome or at higher levels in *ask1* than in wild-type may be potential ASK1-E3 substrates. However, it is possible that the accumulation of these proteins may due to increased gene transcription. The flower bud transcriptomes was compared between *ask1* and wild-type, and the transcript levels of these candidate ASK1-E3 substrates are not significantly different, indicating that these proteins are probably not regulated by ASK1 at the transcriptional level.

Among the potential ASK1-E3s substrates, regulatory proteins are highly enriched, suggesting that ASK1-E3s may ubiquitinate a large number of regulatory proteins including transcription regulators, kinases, peptidases, etc. One interesting finding is that many ribosomal proteins are also putative substrates of ASK1-E3s, implying that the translation machinery may also be a target of UPS regulation either to control mRNA translation or for ribosome turnover. Although it is possible that the ubiquitinated ribosomal proteins may be degraded by autophagy, the potential involvement of ASK1-E3s in the ubiquitination of ribosomal proteins has several implications: the ribosome-associated ribosomal proteins may be selectively ubiquitinated for degradation; or certain ribosomal proteins may dissociate from ribosomes and participate in non-ribosomal regulatory processes. It will be interesting to identify the F-box proteins that recognize specific regulatory proteins including ribosomal proteins.

Identification of specific substrates of certain E3 ubiquitin ligases or identification of E3 ligases that specifically ubiquitinate certain proteins is an active research area but is also very challenging. The key to match pairs of E3 ligases and their substrates is to find protein-protein interactions. However, different from other protein-protein interactions, the interactions between E3 ligases and substrates are supposed to cause fast turnover of the substrates, hence making it difficult to detect sufficient amounts of static protein complexes. In future studies of E3-substrate

interactions, proteasome inhibitors can be used to block the degradation of ubiquitinated substrates to increase the chance of detecting the E3-substrate protein complexes by using co-immunoprecipitation (co-IP) and mass spectrometry. Another challenge is that certain biotic/abiotic stimuli and post-translational modifications such as phosphorylation of substrate proteins are often required for the E3-substrate interactions. Thus traditional yeast two-hybrid assays may not uncover these interactions. Co-IP and mass spectrometry can be used to characterize the components of E3-substrate protein complexes extracted from plant cells. Putative protein-protein interactions can be validated by using *in vivo* bimolecular florescence complementation (BiFC) assays in which the N-/C-terminal portions of the split YFP protein are fused with candidate interactors whose interactions in cells can reconstitute the functional YFP.

Thanks to the sequenced genome of *Arabidopsis thaliana* and relatively well annotated protein-coding genes, it is easy to find large families of E3 ubiquitin ligases. Experimentally identification of specific substrates for each of them is a daunting task. However, one can speculate that there should be much redundancy between closely related E3 ligases. Therefore, future studies can start from grouping the E3 ligases into closely related clades and test one or a few representative proteins without spreading efforts into all of them. Even with this phylogeny-based approach, we still need to prioritize E3 ligases that have been reported to be important for plant development and physiology.

Links between the transcriptional and post-translational regulations of plant development

The regulatory pathways in a cell or in an organism should be a subset of an interconnected network which, however, has only been partially revealed. Individual components or pathways can be investigated separately. For example, numerous studies have identified many transcriptional regulators (e.g., ABC genes) that play important roles in flower development.

However, whether these transcription factors are regulated at the post-translational level (e.g., proteasome-mediated degradation) is largely unknown. In the Chapter 4, the transcriptomes of the *ask1* mutant and the wild-type anthers were compared to answer the question whether E3 ubiquitin ligases may modulate gene transcription by post-translationally regulate transcription factors. Hundreds of genes are affected by the *ask1* mutation, indicating that ASK1-E3s are required for their normal expression. ASK1-E3s may destabilize transcriptional repressors to release the suppression of downstream gene transcription, or destabilize transcriptional activators to turn off/attenuate target gene transcription. The expression of several meiosis- and cell cycle-related genes is affected in the *ask1* anther, indicating that ASK1-E3s may regulate meiosis both by directly ubiquitinating substrate proteins and by indirectly modulate meiotic gene expression by destabilizing transcription factors.

Although this study only provided indirect evidence that the regulation of protein stabilities may modulate gene transcription, it shed light on the links between regulations at different levels. It will be interesting to identify the transcription factors that are ubiquitinated by E3 ligases and the target genes of these transcription factors, thus connecting the regulation of protein degradation with gene transcription. Future studies to identify E3 ligases that specifically ubiquitinate a certain transcription factor may start from determination its protein turnover rate with/without the addition of proteasome inhibitors. If a transcription factor has a short half-life and proteasome inhibitors block or slow down its degradation, it is likely to be degraded by the UPS. Then protein-protein interactions can be investigated using various methods including, but not limited to, co-IP and mass spectrometry.

Much more work is required to reveal the regulatory networks controlling flower development. Not only the function of individual genes awaits detailed investigations, but also the interrelationships between biomolecules need to be elucidated. Only when the evidence from

both large-scale and small-scale studies accumulates to a sufficient level can we start to understand the properties of complex biological networks with a systems biology perspective.

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