The Protein Phosphatases and Protein Kinases of Arabidopsis thaliana

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INTRODUCTION

Protein kinases and protein phosphatases are major post-translational regulators of numerous cellular processes. These enzymes regulate metabolic pathways and are intimately involved in cellular signaling networks. There are over 1000 genes (Wang et al., 2003) in Arabidopsis that encode protein kinases and another 112 genes (Kerk et al., 2002) that encode protein phosphatase catalytic subunits (Table 1). While Arabidopsis contains orthologs of many of the protein kinases found in other eukaryotes, Arabidopsis, and most likely plants in general, also has an unique set of protein kinases. These include the receptor-like protein kinases and related cytoplasmic protein kinases, the calcium-dependent protein kinases and several members of the putative mitogen-activated protein kinase kinase kinases (Wang et al., 2003). The Arabidopsis protein phosphatase catalytic subunits encompass orthologs of the majority of the protein phosphatases found in other eukaryotes. However, the type 2C protein phosphatase family is notably large in number in Arabidopsis (Kerk et al., 2002). The distinct representation of genes encoding protein kinases and phosphatases in the Arabidopsis genome, relative to other eukaryotes, is a reflection of the evolutionary history of plants. The understanding that plants have developed cellular communication systems and basic developmental mechanisms independently from other multicellular eukaryotes (Meyerowitz, 2002) explains why plants have evolved a unique collection of enzymes that regulate protein phosphorylation. Indeed, we pointed out a decade ago (Stone and Walker, 1995), before the exceptionality of the Arabidopsis kinome was fully appreciated, that plants have an unique repertoire of protein kinases that control the early steps in signaling pathways which is reflective of the unique developmental and environmental responses that govern plant growth and development.

Progress in understanding the role of protein phosphorylation in plant development and environmental responses has made some significant steps in the past few years. While much of the research is focused on Arabidopsis, important insights are also being made in other plant species. Indeed, as genomic and functional data becomes more complete for other plant species, we should be better equipped to answer questions about the fundamental mechanisms plants employ to control their growth, development and responses to environmental stimuli and the role that protein phosphorylation plays in these processes.

This chapter on the protein phosphatases and protein kinases of Arabidopsis takes a gene-centric approach to summarize our current understanding of the functional roles of these important mediators of cellular processes. We have tried to focus on the unique aspects of protein kinases and phosphatases.

RECEPTOR-LIKE PROTEIN KINASES IN ARABIDOPSIS

Receptor-like protein kinases (RLKs) are defined by the presence of a signal peptide, an extracellular domain, a transmembrane domain region that anchors the receptor in a cell membrane, and a carboxy-terminal Serine/Threonine (Ser/Thr) kinase domain. Analysis of the Arabidopsis genome reveals there are at least 610 members in the RLK family (Shiu and Bleecker, 2001), thereby representing more than 2% of the predicted Arabidopsis coding sequences. Due to their large numbers and their diverse functions with roles in development, pathogen resistance, and hormone perception, RLKs have become a target of many investigations. Several reports describing the function of RLKs have been released since the cloning of the Arabidopsis RLK, _ERECTA_ (ER), in 1996 (Torii et al., 1996).
### Table 1. Overview of protein kinases and phosphatases in Arabidopsis.

<table>
<thead>
<tr>
<th>Class</th>
<th>No. of predicted genes</th>
<th>Reference</th>
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<td><strong>A Protein kinases</strong></td>
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<tr>
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<td>Lectin receptor kinase (lecRK)</td>
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<td>S-locus glycoprotein-like domain (SD)</td>
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<td>Wall-associated kinase-like (WAKL)</td>
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<td>Proline extensin-like receptor kinase-like (PERKL)</td>
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<td>Catharanthus roseus receptor-like kinase-like (CrRLK1L)</td>
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<td>Crinkly4-like (CR4L)</td>
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<td>Extensin</td>
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<td>RKF3L</td>
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<td>Unclassified</td>
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<td>MAPK Group, 2002</td>
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<td>20</td>
<td>MAPK Group, 2002</td>
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<td>10</td>
<td>MAPK Group, 2002</td>
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<td>MAPKKK</td>
<td>60</td>
<td>MAPK Group, 2002</td>
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<td>GSK3/shaggy-like</td>
<td>10</td>
<td>Dornelas et al., 1998</td>
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<td>Histidine kinase-like protein</td>
<td>17</td>
<td>Schaller et al., 2002</td>
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<tr>
<td><strong>Other protein kinases</strong></td>
<td>&gt;200</td>
<td>PlantsP, 2006</td>
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</table>
In addition to their role during organ development, ER, ERL1 and ERL2 play a role in stomatal development (Figure 1 A and B) (Shpak et al., 2005). An er single mutant exhibits an increased number of stomatal neighbor cells that fail to differentiate into stomata, which results in a decreased stomatal density. This phenotype is consistent with the identification of ER as a regulator of plant transpiration efficiency by a quantitative trait loci approach (Masle et al., 2005). In addition, both ERL1 and ERL2 maintain stomata stem cell activity and prevent terminal differentiation of the meristemoid into the guard mother cell since the erl1, erl2 and erl1 erl2 mutants have fewer stomatal neighbor cells (Shpak et al., 2005). Furthermore, these three genes control the initial decision by protodermal cells to produce either pavement cells or a stomatal complex by asymmetric division. In Arabidopsis, stomatal patterning follows the “one cell spacing” rule: no two stomata can be directly adjoining each other; there will be at least one pavement cell between two adjacent stomata (Nadeau and Sack, 2002a). The “one cell spacing” rule is disrupted when the three genes are mutated, resulting in clustered stomata (Shpak et al., 2005).

This clustered stomata phenotype is similar to the too many mouths (tmm) mutant phenotype (Geisler et al., 1998; Yang and Sack, 1995). TMM encodes a receptor-like protein that lacks a cytoplasmic kinase domain (Nadeau and Sack, 2002b). From the genetic interaction between TMM and the ER genes, TMM appears to be epistatic to ER and its homologs (Shpak et al., 2005). However, the complex and organ-specific interactions preclude solid conclusions from the current data about the interaction between the ER genes and TMM. For the ER genes, the specificity of function is likely to be due to different cis-regulatory elements, since both ERL1 and ERL2 can rescue the ER phenotype when expressed under the ER promoter (Shpak et al., 2004).

Leucine Rich Repeat RLKs

ERECTA

The Arabidopsis Landsberg erecta (Ler) ecotype has been widely used for both molecular and genetic studies. Ler, which harbors the er mutation, originally was isolated from mutagenised seed populations in the 1950s (Redei, 1992). The er mutation is responsible for most of the phenotypic differences between the Ler and Columbia ecotypes; plants mutated for ER have compact inflorescences and altered organ elongation (Tori et al., 1996). ER encodes a leucine-rich repeat (LRR) RLK that is expressed in the shoot apical meristem and in the young floral organ primordia (Yokoyama et al., 1998). ER regulates the inflorescence architecture by affecting the elongation of the internode and pedicels, as well as the shape of lateral organs (Yokoyama et al., 1998, Torii et al., 1996). While these processes also are regulated by auxin (Kepinski and Sack, 2002a). The “one cell spacing” rule is disrupted when the three genes are mutated, resulting in clustered stomata (Shpak et al., 2005).

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Figure 1. Phenotype of er1 erl2 triple mutant, sub-4 and serk1-1 serk2-2 double mutant. (A and B) Cleared differential interference contrast images of the abaxial epidermis of a mature rosette leaf of wild type (wt) (A) and er1 erl2 triple mutant (B) leaves. Arrowheads indicate SLGCs. (C) Midoptical section through an early stage-4 wt ovule. (D) Midoptical section through a stage-4 ovule from a sub-4 mutant. Endothelium differentiation has occurred, an outer integument is mostly absent on the adaxial side and the embryo sac cannot be discerned. The arrow marks a spot in the distal abaxial outer integument where irregular cell divisions occurred and the normally two-layered organization is not maintained. Note the aberrant cell shape and the aberrant orientation of the planes of cell division. The group of cells indicated by * are part of an outer integument that is mostly located outside this plane of focus. es, embryo sac; et, endothelium; ii, inner integument. (E) Inflorescence of a wt plant showing normal seed pods. (F) A wt flower showing pollen grains. (G) Inflorescence of the serk1-1 serk2-2 double mutant with small seedpods and no developing seeds. (H) A double serk1-1 serk2-2 mutant flower with shortened anther filament and no pollen grain. Scale bars in (A and B) 50 mm, (C and D) 20 mm. (A and B) From Shpak et al., 2005; © 2005 by the American Association for the Advancement of Science, used with permission. (C-D) From Chevalier et al., 2005; © 2005 by the National Academy of Sciences of the USA, used with permission. (E-H) From Albrecht et al., 2005; © 2005 by the American Society of Plant Biologists, used with permission.

In addition to its role in development, ER appears to be involved in resistance to pathogens (Godiard et al., 2003; Llorente et al., 2005), suggesting multiple functions for ER.

**BRASSINOSTEROID INSENSITIVE 1**

Brassinosteroids (BRs) share similar structures with animal steroid hormones that bind to nuclear receptors (e.g., testosterone and progesterone) (Beato et al., 1995). When applied exogenously, BRs cause several developmental effects, including the promotion of cell elongation and cell division and the inhibition of root growth.

Genetic screens for mutants that do not respond to the application of exogenous BRs identified the brassinosteroid insensitive 1 (bri1) mutant (Clouse et al., 1996) and the brassinosteroid insensitive 2 (bin2) mutant (Li and Nam, 2002). BRI1 encodes a LRR-RLK (Li and Chory, 1997). Treatment of Arabidopsis seedlings with brassinolide (BL) results in the autophosphorylation of BRI1 (Friedrichsen et al., 2000; Oh et al., 2000; Wang et al., 2001). Several in vivo phosphorylation sites have been identified (Wang et al., 2005).

A functional assay was used to study the role of BRs in the BR signaling pathway (He et al., 2000). In rice, a chimeric protein that contains the BRI1 extracellular domain, the transmembrane domain, and a short stretch of the intracellular domain fused to the XA21 kinase domain (Song et al., 1995) elicits a pathogen response when BL is applied. No response is obtained when the chimeric protein is mutated in the island domain of BRI1 or in the kinase domain of XA21. These results suggest that BRI1 is directly involved in the BR signaling pathway.

The direct binding of BL to BRI1 was demonstrated with native and recombinant BRI1 proteins (Kinoshita et al., 2005). BR analogs can be cross-linked to BRI1 in microsomal preparations and in pull-down fractions highly enriched for BRI1-GFP, indicating that BRs and BRI1 directly interact. In addition, recombinant proteins that consist of the island domain and the neighboring C-terminal LRR are sufficient to bind radioactive BL with an affinity comparable to that observed for full-length BRI1 from plants, which suggests that BRI1 is one of the key factors in the perception of BRs in Arabidopsis.

Two of the three BRI1 homologs (BRL1 and BRL3) also bind BRs. In contrast to the ubiquitous expression of BRI1, the expression of both BRL1 and BRL3 is restricted to vascular tissue (Cano-Delgado et al., 2004). Both can complement bri1 when expressed under the BRI1 promoter, suggesting that BRL1 and BRL3 play a restricted and a partially redundant role in BR signaling (Zhou et al., 2004a; Cano-Delgado et al., 2004). Both the bri1 bri1 double mutants and the bri1 bri1 bri3 triple mutants enhance the vascular defect of the bri1 single mutant. In addition, an activation tagging screen identified BRL1 as a suppressor of a weak bri1 allele (Zhou et al., 2004a). The same screen also identified BRII ASSOCIATE KINASE (BAK1), a different LRR RLK, as a suppressor.

VASCULAR HIGHWAY1 (VH1, also BRL2), the third BRI1 homolog, is unable to bind BRs (Cano-Delgado et al., 2004).
BAK1, a LRR RLK in the same class as SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES1 (SERK1) and 2 (discussed below), is BRI1’s coreceptor. It was independently identified as an interacting partner with BRI1 in a yeast-two hybrid screen and as a gain-of-function suppressor of a weak bri1 allele in an activation tagging screen (Li et al., 2002; Nam and Li, 2002). In addition, elongated (elg), an unique allele of BAK1, displays an enhancement of high-light phototropism and increased sensitivity to BRs (Whippo and Hangartner, 2005). However, it is unclear how the elg allele confers hypersensitivity to BRs.

Consistent with the role of BAK1 as an interacting partner of BRI1, the BAK1 loss-of-function mutant displays a BR-insensitive phenotype (Li et al., 2002; Nam and Li, 2002). However, this phenotype is weaker than bri1 mutants, suggesting that other members of the SERK/BAK family also may be functional in BR preception, perhaps by forming heterodimers with BRI1. Indeed, BRI1 and BAK1 interact in yeast in the absence of a ligand, suggesting that they may form a pre-existing heterodimer (Li et al., 2002). Interestingly, neither overexpression nor loss of function of BAK1 influences ligand binding to BRI1 (Kinoshita et al., 2005; Wang et al., 2005). In cowpea protoplasts, the co-overexpression of BRI1 and BAK1 results in a shift of BRI1 localization toward endosomal compartments. Furthermore, FRET between BRI1/BAK1 preferentially occurs in endosomes (Russinova et al., 2004). The demonstration of BRI1-BAK1 as a receptor of BRs suggests that other RLKs may serve as receptors for phytohormones.

RECEPTOR LIKE KINASE 1

Abscisic acid (ABA) is involved in stress responses (cold, drought, high salt), seed maturation and dormancy, and stomatal closure (Finkelstein and Rock, 2002). While the ABA signaling pathway is beginning to be elucidated (Himmelbach et al., 2003; Verslues and Zhu, 2005), the receptor and the proteins involved in the perception of ABA are still being identified. RECEPTOR LIKE KINASE 1 (RPK1) is a candidate receptor for ABA signaling.

The expression of RPK1 is specifically and rapidly induced by ABA, dehydration, high salt, and cold treatments (Hong et al., 1997). Both a T-DNA knock-out and an antisense line display decreased sensitivity to ABA and down-regulation of ABA-induced genes, while overexpression results in increased ABA sensitivity (Osakabe et al., 2005). Taken together, these results strongly suggest that RPK1 is involved in an early step of ABA signaling. Future studies are needed to determine if RPK1 is an ABA receptor and to elucidate the functional mechanism of action.

HAESA

HAESA (formerly RLK5) (Walker, 1993) was one of the first Arabidopsis protein kinases to be characterized biochemically. HAESA was isolated by screening an Arabidopsis cDNA library with the catalytic domain of ZmPK1, a maize receptor protein kinase. The protein kinase domain autoprophosphorylates on Ser/Thr residues in vitro (primarily by an intermolecular mechanism), and the protein kinase activity is lost by mutation of Lys-711, which corresponds to an invariant Lys found in many protein kinases (Horn and Walker, 1994). In vivo immunoprecipitation shows that HAESA is an active protein kinase in planta. In addition, immunoprecipitation identified phosphoproteins with apparent molecular masses of 65 and 85 kDa, which may correspond either to the proteolytic products of HAESA during the immunoprecipitation, or to endogenous substrates interacting with HAESA.

Using a promoter:glucuronidase (GUS) fusion construct and in situ hybridization, HAESA expression can be observed in the floral abscission zones. Expression also can be detected in vegetative tissues at the base of the petioles. Antisense experiments showed that a reduction in the level of HAESA protein is inversely correlated with the degree of defective floral abscission. The GUS expression of HAESA is not altered in crosses with etr1-1 (the ethylene-insensitive mutation), suggesting that HAESA acts in an ethylene-independent pathway of floral abscission (Jinn et al., 2000).

CLAVATA 1

In plants, the shoot apical meristem produces lateral organs and axillary meristems throughout the life cycle. During the vegetative phase, it gives rise to leaves, and secondary meristems and, upon floral induction, produces flowers, bracts, and secondary meristems. The continuous production of lateral organs is possible due to the maintenance of a population of undifferentiated cells, called stem cells, at the tip of the meristem. These stem cells divide slowly, and their daughter cells are either used for their self-perpetuation, or they become the periphery cells and eventually form new lateral organs and the stem. Therefore, the coordination between stem cell accumulation and organ initiation is crucial for the maintenance of a functional meristem.

Three clavata (clv) mutations in independent loci (clv1, clv2, and clv3) result in a similar phenotype: over time the inflorescence and floral meristems become progressively larger and the numbers of flowers and floral organs in each whorl increases (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). CLV1 is a LRR-RLK, CLV2 a LRR receptor-like protein lacking a kinase domain, and CLV3 is a small, secreted protein (Jeong et al., 1999; Fletcher et al., 1999; Clark et al., 1997). Based on the nature of the pro-
teins and comparisons with animal and plant receptors, it has been proposed that CLV1, CLV2, and CLV3 form a receptor complex. The CLV signaling pathway limits the proliferation and/or promotes the differentiation of stem cells in the shoot apical meristem (Laufs et al., 1998; Troughton et al., 1999; Jeong et al., 1999; Rojo et al.,

An intriguing characteristic of the clv1 mutants is that different mutations result in unexpected phenotypes. For example, strong and intermediate alleles contain a missense mutation within the LRR or kinase domains, whereas the weakest alleles lack most of the kinase domain (Clark et al., 1997). A clv1 null allele displays a weak phenotype. This suggests that strong and intermediate alleles may function as dominant negative alleles and that additional receptors function in overlapping roles with CLV1 (Dievart et al., 2003). While the obvious candidates for such receptors are the CLV1 homologs - BARELY ANY MERISTEM 1 (BAM1), BAM2, and BAM3 - loss of function of these genes results in a phenotype that is opposite of the clv1 mutant phenotype (Deyoung et al., 2006). The BAMs appear to maintain the stem cell population in the shoot apical and floral meristems, and they also regulate vascular strand and male gametophyte development (Deyoung et al., 2006; Hord et al., 2006). The broader function of the BAM genes correlates with their wider expression patterns. The different expression patterns seem to explain the different roles of CLV1 and BAMs during meristem development. Indeed, when expressed under the ER promoter, CLV1 can fully rescue the bam1 bam2 double mutant, while the BAM1 and BAM2 genes can partially rescue the clv1 phenotype. Taken together, these data suggest that the BAM genes most likely do not functionally overlap with CLV1. However, STRUBBELIG (SUB), another LRR RLK, has been shown to be important for meristem development.

**STRUBBELIG/SCRAMBLED**

The SUB/SCRAMBLED (SCR) gene encodes a LRR RLK. The sub mutant was first reported as an ovule-development mutant (Schneitz et al., 1997) because sub displays altered development of the outer integument of the ovules that results in semi-sterility (Fig. 1 C and D). However, it now appears that SUB has a wider function throughout Arabidopsis development.

sub is allelic to scr (Kwak et al., 2005). The overall morphology of the root in the sub/scr mutant is indistinguishable from the wild-type. Roots from the sub/scr alleles differ from wild-type roots only by the mis-expression of some root epidermal-specific markers. Therefore, SUB/SCM appears to regulate the establishment of positional cues that allow the correct specification of the epidermis cells. But, its mechanism of action is still unknown. One possible explanation is that SUB/SCM affects the formation of organs by influencing cell morphogenesis, the orientation of the division plane, and cell proliferation (Chevalier et al., 2005). Indeed, the original sub alleles display several other defects besides the ovule phenotype; the number and shape of floral organs are affected, as well as the morphology and cell number of the inflorescence stem. In addition, in both floral and apical meristems, sub displays an irregular L2 layer and occasional periclinal divisions, suggesting a role of SUB in controlling cell division in the meristems.

One interesting aspect of SUB is a possible new biochemical mechanism of signaling. In the SUB kinase domain, the change of two strictly conserved amino acids, which are necessary for kinase activity, results in an inactive kinase in vitro. However, the sub phenotype can be rescued by different SUB mutants, including ones that were shown to abolish all kinase activity. This suggests that a functional kinase domain is not necessary for SUB function. However, the sub-4 allele carries a missense mutation at a conserved position in the kinase subdomain VIA, which suggests that the kinase domain is necessary for SUB function. It also is possible that SUB interacts with an active RLK to form a signaling complex.

While SUB emphasizes the importance of signaling during ovule development, the anther and pollen also require functional RLKs for their development. EXCESS MICROSPOROCYTES 1/EXTRA SPOROGENOUS CELLS (Exs) and SERK1 and 2 are involved in anther development.

**EXCESS MICROSPOROCYTES 1/EXTRA SPOROGENOUS CELLS**

In Arabidopsis, the development of the anther is tightly regulated with precise cell division patterns (Sanders et al., 1999). For example, the formation of the sporogenous cell (which undergo meiotic division to give rise to the male gametophyte) results from the initial division of the archeporial initials. This initial division establishes two cell lineages: one cell differentiates into the sporogenous cells, while the other undergoes an additional round of cell division to form the endothecium, middle layer, and tapetum. However, only a limited number of archeporial initials are initiated.

The ems1 and exs allelic mutants highlight the importance of signaling during anther development (Zhao et al., 2002a; Canales et al., 2002). EMS1/EXS encodes a LRR RLK that belongs to the same class of LRR RLKs as BR1. While both mutants display the same male-sterile phenotype, the authors suggest a different function for EMS1/EXS. The mutant exhibits an increased number of sporogenous cells that do not undergo cytokinesis. In addition, the tapetal cells are missing, and the middle layer appears to be either missing or abnormal. This phenotype appears to be due either to an increased number of archeporial initials resulting in an increased number of sporogenous cells (Canales et al., 2002), or a mis-specification of the tapetal cells into sporogenous cells (Zhao et al., 2002a).

The isolation of the tapetum determinant1 (tpd1) mutant favors the second hypothesis. tpd1 exhibits the same mutant phenotype as ems1/exs, and TPD1 encodes a predicted small protein with a putative signal peptide, sug-
differentiated tapetal cells, TPD1 binds to EMS1/EXS on the surface of the cells that (Fig. 2 E) (Ma, 2005). In this model, the secreted TPD1 expression increases. These observations lead to a model in which an unknown factor triggers the differentiation of male sporocytes, resulting in a decrease in the expression of TPD1 (Fig. 2 E) (Ma, 2005). In this model, the secreted TPD1 binds to EMS1/EXS on the surface of the cells that surround the newly differentiated male sporocytes. In these cells, the interaction of TPD1 with EMS1 triggers the activation of a pathway that promotes tapetum differentiation and causes a decrease of TPD1 expression. The differentiation of the tapetum occurs at the expense of the potential male sporocytes. This model is supported by the observation that the increased number of cells in carpels ectopically expressing TPD1 is dependent on EMS1/EXS (Yang et al., 2005). Interestingly, mutations in two other LRR RLKs from a different class display the similar phenotype as ems1/exs.

**SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES**

SERK1 and 2 belong to the class same class of LRR RLKs as BAK1 (Shiu and Bleecker, 2001). DcSERK1 (from Daucus carota) was the first family member to be isolated and is a marker for competence of cells to form somatic embryos (Schmidt et al., 1997). The Arabidopsis ortholog of DcSERK1 (SERK1) also appears to enhance embryogenic competence in culture and is expressed in developing embryos and ovules (Hecht et al., 2001). However, this potential function is only based on the overexpression phenotype. Only recently has the loss of function mutant of SERK1 been reported (Colcombet et al., 2005; Albrecht et al., 2005). The serk1 and serk2 single mutants show no obvious phenotype, but the serk1 serk2 double mutants are male sterile (Fig. 1 E-H) and exhibit the same phenotype as the ems1/exs mutants (i.e., missing tapetal layer and extra sporogenous cells). While both reports proposed that the tapetal cells are miss-specified into sporogenous cells, the function of SERK1 and 2 is still unclear. In addition, it is unknown whether SERK1 and 2 act in the same signaling pathway as EMS1/EXS and TPD1.

The biochemical properties of SERK1 have been extensively studied. SERK1 is a dual Ser/Thr and tyrosine kinase, featuring an inter-molecular mechanism of phosphorylation (Shah et al., 2001). FRET has shown that SERK1 localizes to the plasma membrane and forms a homodimer (Shah et al., 2001). Additional interactions have been found in a yeast two-hybrid experiment with AtCDC48 (an AAA-ATPase), GF14k (a 14-3-3 protein), and KAPP (a PP2C kinase associated protein phosphatase) interacting with SERK1 (Rienties et al., 2005). KAPP is also proposed to control the internalization of SERK1 (Shah et al., 2002).

**HAIKU 2**

Seed development is an agronomically important trait and, therefore, has been the focus of much research. The...


hiku2 (iku2) and miniseeds3 (mini3) mutants display reduced seed size associated with reduced growth and early cellularization of the endosperm (Luo et al., 2005). This is a similar phenotype to the previously characterized iku1 mutant (Garcia et al., 2003). IKU2 encodes a LRR RLK, and MINI3 encodes a WRKY class transcription factor (Luo et al., 2005); IKU1 has not been cloned yet. No mechanism of function for IKU2 has been proposed. However, decreased expression of the Iku2::GUS reporter line both in the iku1 and mini3 mutants was found. In addition, the Mini3::GUS reporter line was only expressed in the iku1 mutant suggesting the successive action of these three genes (IKU1, IKU2, MINI3) in the same pathway of seed development.

**FLAGELLIN SENSING 2**

FLAGELLIN Sensing (FLS) is a RLK with 28 LRRs. FLS2 was isolated through map-based cloning as a mutant that is insensitive to flagellin treatment. FLS2 represents an example of conservation of the innate-immunity response among animals, insects, and plants (Gomez-Gomez and Boller, 2002). Flagellin, a building block of eubacterial flagella, is an elicitor that induces a defense response through a non-host-specific mechanism. Synthetic flagellin peptides with 15 or 22 amino acid residues (flg15 or flg22) are functional elicitors in Arabidopsis of such responses as callose deposition, induction of pathogenesis-related (PR) genes, and growth inhibition of seedlings (Gomez-Gomez et al., 1999).

The expression of FLS is ubiquitous in different organs and is not altered by flg22 treatment. Two point mutations—one in the LRR domain (fls2-24) and the other in the protein kinase domain (fls2-17) show an alteration of the FLS2-mediated signaling pathway with a blocked flagellin response (Gomez-Gomez and Boller, 2000). These missense mutations demonstrate the importance of both extracellular and cytoplasmic protein kinase domains for the flagellin-mediated defense response. Flg22 treatment of wild-type plants confers resistance to bacterial infection through the enhanced expression of many defense-related genes. From microarray studies between wild-type plants and the fls2-17 mutant the expression of 966 genes were identified as up regulated by flg22 treatment. Flg22-induced resistance seems to be independent of salicylic acid, jasmonic acid, and ethylene-mediated signal pathways, which are based on the experiments using knockout mutants in transgenic plants of the previously reported signaling components (npr1, eds1, sgt1, rar1, etr1, ein2, jar1, pad2, or pad4) or overexpression of NahG (Zipfel et al., 2004). The FLS2 protein kinase recognizes flg22 and transfers the signal via the following MAPK cascade proteins: AtMEKK1 (MAPKKK), AtMKK4a/AtMKK5a (MAPKKs), and MPK3/MPK6 (MAPKs). The MAPKs are known to activate their target transcription factors (e.g., WRKY29 and FRK1) that control the expression of defense-related genes (Asai et al., 2002).

**Other RLKS**

**CRINKLY-like**

The crinkly4 (cr4) mutant was first isolated in maize (Becraft et al., 1996). The mutant plants are shorter, have crinkled leaves, and, due to a wrong cell fate specification of the endosperm, the peripheral endosperm cells develop as starchy endosperm instead of aleurone (Becraft et al., 1996; Becraft and Asuncion-Crabb, 2000). CR4 encodes a RLK with two different domains in its ectodomain. One domain is similar to the binding domain of the mammalian tumor necrosis factor receptor. The other domain consists of seven repeats, called crinkly repeats. Both domains are thought to participate in protein-protein interactions.

Arabidopsis contains a family of five RLKs related to CR4 (Shiu and Bleecker, 2001; Cao et al., 2005): ACR4 (the Arabidopsis ortholog of CR4) (Gifford et al., 2003; Tanaka et al., 2002), AtCRR1, AtCRR2, AtCRR3, and AtCRR1. AtCRR1 is the ortholog of CRK1 from tobacco, which is negatively regulated at the transcriptional level in cell cultures by exogenous cytokinin (Schafer and Schmülling, 2002). ACR4 is expressed in protodermal cells of the embryo and the shoot (Gifford et al., 2003; Tanaka et al., 2002). While the antisense ACR4 displays only moderate defects in seed formation and in embryo morphogenesis (Tanaka et al., 2002), the ACR4 T-DNA insertion alleles exhibit altered integuments and seed coat development but no defects in embryo morphology (Gifford et al., 2003). Surprisingly, no leaf phenotype is observed, which suggests that ACR4 shares a redundant function with other genes. To check for overlapping functions with other ACR4 homologs, expression patterns were checked and all the members were found to have specific but overlapping expression patterns (Cao et al., 2005). All the possible combinations of the double mutants with acr4 displayed no additional phenotypes. However, the acr4 mutation has been shown to affect the differentiation of leaf epidermal cells, suggesting a similar role for ACR4 and CR4 in the differentiation of leaf epidermis (Watanabe et al., 2004). ACR4 and ABNORMAL LEAF SHAPE1 (ALE1), which encodes a putative subtilisin-like serine protease, genetically interact since the double mutant exhibits a synergistic phenotype (Watanabe et al., 2004). However, the mechanism of that interaction is unknown.

The biochemical activities of ACR4 and its homologs are different. AtCRR1 and AtCRR2 do not have kinase activity in vitro, most likely due to a deletion in the kinase subdomain VIII, but AtCRR2 can be phosphorylated in vitro by the active ACR4 (Cao et al., 2005). However, a mutant of ACR4 that has a mutation abolishing kinase activity can rescue the acr4 phenotype, suggesting that a functional kinase domain is not necessary for the function of ACR4 (Gifford et al., 2005).
NUCLEAR SHUTTLE PROTEIN (NSP)-INTERACTING KINASES

Nuclear Shuttle Protein (NSP) of geminivirus interacts with an NSP-interacting kinase (NIK, a transmembrane receptor) from tomato (LeNIK) and from soybean (GmNIK). Based on the sequence alignments of GmNIK with the Arabidopsis genome, three genes (At5g16000, At3g25560, and At1g60800) show high similarities to GmNIK (78%, 70%, and 61% identities, respectively). The gene products are localized at the plasma membrane and autophosphorylate the cytoplasmic Ser/Thr protein kinase domain.

The NSP of geminivirus inhibits the activities of NIK1 and NIK2, but it does not inhibit the activity of AtSERK1, which has a high-sequence similarity. Loss-of-function mutants of NIKs through T-DNA insertion are highly susceptible to the viral infection, suggesting that NIKs may be the targets of the NSP for escaping the antiviral mechanism of the host (Fontes et al., 2004).

CYSTEINE-RICH REPEAT RLKs

ArlRK3 has 12 cysteines in the extracellular domain, 8 of which reside in a tandem repeat of two 25 amino acids motifs in the following pattern: C-X8-C-X2-C-X11-C (DUF26). The expression of ArlRK3 is induced by reactive oxygen species (ROS), pathogen infection (Czernic et al., 1999), and exogenous application of salicylic acid (Ohtake et al., 2000).

The Cys-rich repeat (CRR) is a novel motif that shares a distinct structure with the Cys-rich region of the S-locus glycoproteins and the SRKs. Forty-one RLKs have the novel CRR motif in their extracellular domains. Twenty RLKs of this group are located on chromosome IV as tandem repeats, suggesting that tandem duplication may be the key mechanism of CRKs’ expansion. However, the overall sequence homologies among the genes is not high, even among the 20 tandem-arrayed RLKs (Chen, 2001; Chen et al., 2004).

The overexpression of CRK5 (At4g23130) by a CaMV3SS promoter triggers rapid induction of the disease-resistance gene PR1 and, therefore, enhances resistance to bacterial infection. An induced expression, using a steroid-inducible promoter, also results in hypersensitive reaction (HR)-like cell death, even in the eds1, ndr1, and npr1 mutants and in the NahG transgenic plants (Chen et al., 2003). A yeast two-hybrid screen showed that CRK-interacting proteins (CRKIP1, 2, and 3) interact with CRK5. Phylogenetic analysis showed that CRK4 (At3g45860), CRK19 (At4g23270), and CRK20 (At4g23280) are closely related to CRK5. These genes’ expression patterns are similar to the expression pattern of CRK5 and include induction by salicylic acid treatment and pathogen infection and HR-like cell death under the steroid-inducible promoter (Chen et al., 2004).

PROLINE-RICH, EXTENSIN-LIKE RECEPTOR KINASE-1

PROLINE-RICH, EXTENSIN-LIKE RECEPTOR KINASE-1 (PERK1) was isolated from Brassica napus. It is comprised of a proline-rich extracellular domain (which shows high similarity with extensins), a single transmembrane domain, and a Ser/Thr kinase domain with catalytic activity. Gene expression is induced rapidly by wounding and by fungal infection.

There are 15 genes in the Arabidopsis PERK family (AtPERKs). The similarities of extracellular, transmembrane, and juxtamembrane domains among the PERKs are less than 70%. However, the similarities of protein kinase domains are high. For example, there is over 80% identity between PERK1 and AtPERK1. The expressions of the AtPERK family are both ubiquitous and tissue-specific. Although a single T-DNA insertion mutant of each AtPERK does not show any phenotypic changes, the simultaneous suppression of several AtPERKs, using antisense, results in various growth defects, suggesting a functional redundancy (Silva and Goring, 2002; Nakhamchik et al., 2004).

WALL-ASSOCIATED KINASES

Wall-associated kinases (WAK) and WAK-like kinases (WAKL) have unique properties among the RLKs. They physically link the extracellular matrix and the cytoplasm, and, it is proposed, serve as a signaling intermediate between both. In addition, most of the WAK/WAKL genes are found in tandem repeats (Verica et al., 2003; Verica and He, 2002).

WAKs/WAKLs carry a cytoplasmic Ser/Thr kinase domain and an extracellular domain with some similarity to vertebrate epidermal growth-factor-like domains. The association of WAK/WAKL proteins to the cell wall components was demonstrated by immunohistochemistry for several members (Verica et al., 2003; Wagner and Kohorn, 2001; He et al., 1996). The interaction between WAK1 and the cell wall pectins is dependent on a calcium-induced conformation (Decreux et al., 2005).

The Arabidopsis genome contains 26 WAKs/WAKLs, which are divided into four families (I-IV) (Verica and He, 2002). Functional information is known only about families I and II (Wagner and Kohorn, 2001; Verica et al., 2003). Family I, which is comprised of the five WAKs, is expressed predominately in green tissues (Wagner and Kohorn, 2001). Family II, which includes WAKL5-7, is highly expressed in roots and flowers (Verica et al., 2003). Expression of these two families’ members is developmentally regulated and wound inducible. In contrast to their well-characterized expression patterns, the function of these genes is still unclear.

The analysis of loss of function in the WAKs/WAKLs genes remains a challenge because of the organization of the genes in tandem repeats. To overcome this hurdle, several approaches have been undertaken. Antisense expressions of WAK2 and WAK4 showed that both genes are required for cell elongation (Wagner and Kohorn, 2001;
Lally et al., 2001), while the yeast two-hybrid method revealed that WAK1 interacts with a glycine-rich protein (Park et al., 2001). The expressions of WAK1 and WAKL4 are induced by aluminum and several minerals (copper, nickel, and zinc) (Hou et al., 2005; Sivaguru et al., 2003). The overexpression of WAK1 and the loss of function of WAKL4 suggest that both genes are involved in the mineral response. WAK1 may confer resistance to aluminum, while the role of WAKL4 may be more versatile. Indeed, the loss of function of WALK4 results in hypersensitivity to copper and zinc and an increase in tolerance to nickel.

**LECTIN DOMAIN PROTEIN KINASES**

Legume lectins are carbohydrate-binding glycoproteins (Barre et al., 2002). In Arabidopsis, *Ath.lecRK1* is an RLK that contains a lectin-like domain (Herve et al., 1996). *Ath.lecRK1* was predicted to be a membrane-spanning receptor with an extracellular lectin domain, a membrane-spanning domain, and a cytoplasmic Ser/Thr kinase domain. Subsequent work has identified additional family members; however, not all members are likely to be functional (Herve et al., 1999). Arabidopsis sequence analysis has uncovered three classes of *lecRK* genes, most of which do not appear to contain introns (Barre et al., 2002).

Although the Arabidopsis lectin domains are very similar to the legume lectins, there is little conservation of an Asp and Asn residue that is involved in the binding of two divalent cations (Ca$^{2+}$ and Mn$^{2+}$) in legume lectins (Barre et al., 2002). Since these two ions are required for monosaccharide binding in legumes, it is unlikely that Arabidopsis lectins are able to bind monosaccharides. However, a conserved hydrophobic cavity found in legume lectins also has been found in all predicted Arabidopsis *lecRK*s, suggesting an ability to bind hydrophobic ligands including some phytohormones (Barre et al., 2002).

Little functional information exists about the *lecRK*s. Expression analysis of *lecRK-a1* showed an increase in promoter activity (as assayed with a GUS reporter gene) in parallel with senescence (Riou et al., 2002). In addition, transcription of *lecRK-a1* is locally activated in response to wounding. Recent work suggests a role for a *lecRK* in protein-protein interactions involved in mediating plasma membrane-cell wall adhesions (Gouget et al., 2006). This *lecRK* contains in its extracellular domain peptide sequences that are similar to peptide sequences that bind a protein from the plant pathogen *Phytophthora infestans*. While this *lecRK* appears to have an active cytoplasmic kinase domain and seven putative glycosylation sites, it is unable to bind simple sugars. However, it is capable of binding peptides containing sequences from the *P. infestans* protein. Binding of these peptides disrupts plasma membrane-cell wall adhesions during plasmolysis, suggesting that *lecRK*s may have structural and signaling roles at the cell surface during plant defense.

**S-LOCUS RECEPTOR PROTEIN KINASE**

Many Brassica family members are obligate outcrossing plants, with the notable exception of Arabidopsis. Outcrossing in Brassica is maintained by an elaborate signaling mechanism that includes an RLK and a small cysteine-rich ligand (Kachroo et al., 2002; Kemp and Doughty, 2003; Takayama and Isogai, 2003). The *S-LOCUS RECEPTOR PROTEIN KINASE (SRK)* is a functional RLK that contains a Ser/Thr cytoplasmic kinase domain, an transmembrane domain, and an extracellular domain that is similar to the S-locus glycoprotein.

Several *SRK*-like genes have been isolated from Arabidopsis, but most have primarily vegetative patterns of expression (Tobias et al., 1992; Walker, 1993; Dwyer et al., 1994). *SRK* and its ligand (SCR) have been cloned from *A. lyrata* (Kusaba et al., 2001). Using comparative mapping, a likely candidate *SRK* gene in Arabidopsis has been isolated. While this gene initially appeared to be a functional *SRK*, subsequent analysis of its cDNA showed it to be nonfunctional due to a mispredicted intron-exon splice site. Transfer of a functional *SRK*-SCR pair from *Lyra* into Arabidopsis, however, restores a self-incompatibility response, indicating that the remaining components of the SRK signaling pathway are found in Arabidopsis (Nasrallah et al., 2002). It will be interesting to determine what role exists for the other *SRK*-like genes that were uncovered earlier.

**MAP KINASES (MAPK) IN ARABIDOPSIS**

Mitogen-activated protein kinase (MAPK) cascades are three-kinase modules that are evolutionarily conserved (MAPK group, 2002). The basic activation mechanism of MAPKs is conserved across all eukaryotes. A MKKK (MAP kinase kinase kinase) activates a MKK (MAP kinase kinase) by phosphorylating conserved Ser/Thr residues in the activation domain. MKK (a dual-specificity kinase), in turn, activates MAPK by phosphorylating tyrosine and Ser/Thr residues in an activation loop (Thr-Xaa-Tyr, tripeptide motif, Xaa can be Asp, Glu, Gly, or Pro). MAPK cascades can be activated by an array of extracellular stimuli transduced by receptors/sensors in both yeast and in animal cells (Widmann et al., 1999; Morrison and Davis, 2003).

MAPK cascades function in stress and hormonal responses, as well as in cell proliferation, differentiation, and death (Figure 3). In yeast, animals, and plants, multi-gene families have been identified that encode each of the three tiers of the MAPK cascades. Diverse combinations of the three components of a MAPK cascade allow diverse input signals to be transduced through the MAPK cascades. The specificity of the MAPK cascades can be maintained by scaffold proteins, substrate specificity, spatiotemporal colocalization, and by interactions between MAPKs (Morrison and Davis, 2003; Elion et al., 2005). In addition, MAPK cascade signals can be attenuated by Ser/Thr phosphatases and dual-specificity tyrosine phosphatases.
Figure 3. Overview of MAP kinase pathways described in Arabidopsis. Each level of the MAPK cascade is placed in colored boxes. Inputs and any genes that are involved in these inputs, if known, are placed above the colored boxes and the corresponding outputs or targets, if known, are placed below the colored boxes, vertically arranged with the inputs. Arrows and components that are in black indicate a direct connection while those in red indicate an indirect or not entirely known connection. Arrows indicate stimulation while blunted lines indicate inhibition.
In the Arabidopsis genome, about 60 MAPKKKs (MKKK), 10 MAPKs (MKK), and 20 MAPs have been identified (MAPK group, 2002). The presence of a diverse group of MKKKs suggests that a wide range of signals may activate downstream components in MAPK cascades. In contrast, the limited number of MKKs suggests that signals from MKKKs could be integrated at the MKK level (MAPK group, 2002).

Arabidopsis MAPKs can be classified into two subtypes: "TEY" MAPKs and "TDY" MAPKs. MAPK activity is controlled by the dual phosphorylation of Thr-Xxx-Tyr in the activation loop. Similar to mammalian ERK1/ERK2, TEY MAPKs have Thr-Glu-Tyr in their activation loops. In contrast, TDY MAPKs have Thr-Asp-Tyr in their activation loops. TDY MAPKs are also distinguished by a long C-terminal tail that is reminiscent of ERK5 (the mammalian MAPK); however, ERK5 has TEY in its activation loop instead of TDY. No plant MAPK has been identified that has TDY or TPY in the activation loop, which are found in the mammalian p38 MAPKs and JNK MAPKs. While the TEY subtype has been extensively studied in many plant species, limited functional information is available about the TDY subtype (He et al., 1999; Schoenbeck et al., 1999; MAPK Group, 2002; Cheeong et al., 2003).

The 10 MKKs encoded in the Arabidopsis genome can be classified into four subgroups (A, B, C, D), according to their sequence (MAPK group, 2002). With the exception of MKK10, the Arabidopsis MKKs have a consensus S/TX3S/T phosphorylation motif in their activation domain (in contrast to mammalian MKKs that have a S/TX5S/T phosphorylation motif). Arabidopsis MKKs also possess the conserved kinase interaction motif (KR2-g3X1-5L/IXL/I) in the N-terminal domain, which has been shown to be important for MAPK activation in both animals and plants (MAPK group, 2002; Jin et al., 2003; Tanoue and Nishida, 2003).

In Arabidopsis, the proposed family of MKKKs is more diverse than MKKs and MAPKs. Based on their kinase catalytic domain sequence, MKKKs can be classified into two main categories: 12 MEKK-like protein kinases that are related to animal MEKK/Ste11/Bck1 and 48 Raf-like protein kinases (MAPK group, 2002). It has not been demonstrated that the Raf-like protein kinases in Arabidopsis (represented by CR1 and ED1) can function as MKK activators. On the other hand, there is substantial evidence that the MEKK-like MKKKs (YODA, ANP1, ANP2, ANP3, AtMEKK1, NPK1 and MAPKKK3) do function as MKK activators.

**MAPK Functions in Arabidopsis**

**MAPKs in Plant Development**

The MKKK YODA has been shown to function in embryo development and in patterning (Lukowitz et al., 2004). In Arabidopsis, embryo development starts with the zygote undergoing elongation and asymmetric division that results in a small apical cell and a larger basal cell. In the yda loss-of-function mutant alleles, zygote elongation and asymmetric division are suppressed. The mutant zygote undergoes a nearly symmetrical division, and the resulting apical and basal cells are about equal in size. Like in the wild-type, the mutant apical cell maintains the normal divisions (two rounds of longitudinal divisions followed by one round of transverse division) and differentiates into the proembryo. However, unlike the wild-type, the basal cell of the yda mutant undergoes abnormal divisions and no suspensor is established; in the wild-type, the basal cell undergoes a series of transverse divisions that give rise to a file of cells forming the suspensor (Berleth and Chatfield, 2002; Lukowitz et al., 2004).

YODA belongs to the MEKK1/Ste11/Bck1 class of MKKKs. Removal of the N-terminal negative-regulation domain allows YODA to become constitutively active. In the constitutively active YODA mutant, suspensor cells are over-proliferated, which can suppress proper embryo development (Lukowitz et al., 2004).

YODA also functions in stomata development and patterning (Bergmann et al., 2004). Stomata are specialized epidermal structures formed by two guard cells surrounding a pore, through which plants absorb CO2 and release O2. Asymmetric cell divisions precede stomata cell specification in Arabidopsis. Perturbation of the frequency of asymmetric cell division, the orientation of the asymmetric division plane, and the polarity of the progeny of the asymmetric division ultimately disrupt stomata development and patterning (Nadeau and Sack, 2002a). As mentioned earlier, in Arabidopsis, stomata patterning follows the "one cell spacing" rule. yda mutations disrupt the asymmetric division of stomata precursor cells, which results in stomata excessively clustered together. In contrast, there is no stomata development in yda mutants with a deleted N-terminal domain (Bergmann et al., 2004).

Several stomata patterning mutants have been characterized in Arabidopsis. Mutations in genes encoding TOO MANY MOUTHS (TMM, a LRR receptor protein) (Nadeau and Sack, 2002b), STOMATA DENSITY AND DISTRIBUTION 1 (SDD1, a subtilisin-like serine protease) (Berger and Altmann, 2000), and ER/ERL1/ERL2 (LRR receptor kinases) (Shpak et al., 2005) disrupt stomata patterning and result in clustered stomata. It is proposed that unknown ligands processed by SDD1 bind to the TMM/ERs' receptors in the target cells, which then triggers the activation of the YODA MAPK cascade and puts stomata development in check. However, the downstream MKK and MAPK cascades need to be identified to uncover the molecular mechanism of YODA function.

**MAPKs in Cytokinesis Regulation**

Plant cytokinesis is guided by a cytoskeletal microtubule structure, called the phragmoplast. The phragmoplast arises from the spindle midzone following mitotic division. Cytoskeletal microtubules of the phragmoplast direct Golgi-originated vesicles to deposit cell wall building material at the equator of the phragmoplast. The vesicles fuse toget-
her to form the cell plate. The cell plate expands radially (by fusion of new vesicles at its periphery) until it reaches the plasma membrane (Smith, 2002; Jurgens, 2005; Kolch, 2005).

The MAPK cascade NPK1-NQK1-NRK1 has been shown to regulate cytokinesis in tobacco BY-2 cells (Takahashi et al., 2004). NPK1 belongs to the Ste11-like MKKK family. It initially was identified because it is expressed specifically during the logarithmic phase of cell division in BY-2 cells. NPK1 localizes at the periphery of the phragmoplast during radial expansion toward the plasma membrane. Overexpression of a dominant negative mutant of NPK1 suppresses cytokinesis, resulting in multi-nucleate cells with cell wall stubs between the nuclei (Nishihama et al., 2001). Virus-induced gene-silencing (VIGS) of NPK1 results in dwarf plants with defective cytokinesis (Jin et al., 2002).

ANP1, ANP2, and ANP3 are the Arabidopsis orthologs of NPK1 and share a similar function in regulating cytokinesis (Krysan et al., 2002). Single loss-of-function mutants of the ANP genes do not have a phenotype. However, the anp2 anp3 double mutants have defective cytokinesis, as evidenced by the presence of multinucleate cells and incomplete cell wall formation. The anp1 anp2 anp3 triple mutants are gametophyte lethal (Krysan et al., 2002).

Two kinesin-like proteins, NACK1 and NACK2 (NPK1-activating kinesin-like protein 1), were identified as upstream activating factors of NPK1 (Nishihama et al., 2002). Biochemical results indicated that NACK1 activates NPK1 through direct protein-protein interactions that are mediated by the coiled-coil domain of NACK1 and NPK1. The motor domain of NACK1 also functions in targeting NPK1 to the equatorial zone of the phragmoplast during anaphase and telophase. NPK1 is mis-localized when the truncated form of NACK1, that lacking the motor domain, is overexpressed. This mislocalization results in incomplete cell plate formation.

The downstream MKK and MPK of NPK1 and ANP1 have been identified through an ingenious use of yeast genetics (Soyano et al., 2003). The phosphorylation relationship within the NPK1 MAPK cascade was established by in vitro and in vivo biochemical analyses. BY-2 cells expressing a dominant negative NQK1 have cell wall formation defects. A loss-of-function mutant of ANQ1 (the Arabidopsis ortholog of NQK1) has large cells with incomplete cell wall formation, while a loss-of-function mutant of ANR1 (the Arabidopsis ortholog of NRK1) shows severe defects in cytokinesis (Soyano et al., 2002; Takahashi et al., 2004). With corroborative evidence from mutant analysis in Arabidopsis, it is clear that NQK1-NRK1 function downstream of NACK1-NPK1 in controlling cytokinesis. AtNACK1-ANP1/ANP2/ANP3-ANQ1-ANR1 is the equivalent orthologous MAPK cascade in Arabidopsis (Soyano et al., 2003). Since cytokinesis is a very dynamic process involving the initiation of the phragmoplast at the midzone spindle, vesicle transporting, vesicle fusion, and phragmoplast radial expansion, it would be interesting to determine what stage is regulated by this MAPK cascade and what is the signaling output.

Besides cytokinesis, the NPK1 (ANP1) MAPK pathway has been demonstrated to function in plant reactive oxygen species (ROS) signaling and auxin responses. Both are discussed below.

**MAPKs in Phytohormone Responses**

*Ethylene.* The phytohormone ethylene regulates diverse aspects of plant growth and development including fruit ripening, abscission, senescence, apical hook formation in dark-grown seedlings, as well as abiotic (drought, flooding) and biotic (pathogen attack) stress responses (Ecker, 2004; Guo and Ecker, 2004). In response to ethylene treatment, dark-grown Arabidopsis seedlings exhibit a triple response: an exaggerated apical hook, radial swelling of the hypocotyl and root, and inhibition of hypocotyl elongation (Guzman and Ecker, 1990).

ctr1 was isolated as a mutant with a constitutive triple response in the absence of an exogenous ethylene application (Kieber et al., 1993). CTR1 encodes a Raf-like protein kinase. Biochemical analysis demonstrated that CTR1 has intrinsic Ser/Thr kinase activity. The N-terminus of CTR1 is unique and may function as an interaction motif. Molecular analysis of a series of ctr1 mutant alleles suggested that both the kinase activity and N-terminal motif are essential for the function of CTR1 (Huang et al., 2003).

Due to the homology of CTR1 with Raf (a MKKK), it has been proposed that a MAPK cascade functions downstream of CTR1 in the ethylene signal transduction pathway. However, there are no consensus results that support this proposal. It has been shown that ACC (the ethylene precursor) activates MAPK activity of SIMK, MMK3, and MAPKK SIMKK in a *Medicago* cell suspension culture. MPK6 activity also has been reported to be activated by ACC in an Arabidopsis cell suspension culture (Ouaked et al., 2003). However, potential problems with the pharmacological approaches used to find MPK6 activity have been raised (Ecker, 2004; Liu and Zhang, 2004). Recent genetic studies of MPK6 loss-of-function alleles and RNAi transgenic lines of MPK6 do not support ethylene-activation of MPK6, and no discernable effects on ethylene responses have been observed in these mutants (Ecker, 2004; Liu and Zhang, 2004; Menke et al., 2004). Thus, the role of a MAPK cascade in ethylene signaling remains unknown.

In response to various environmental signals, plants increase their ethylene biosynthesis to help cope with various stresses (Zhang and Klessig, 2001; Guo and Ecker, 2004; Chen et al., 2005). Conditional activation of SIPK (the Arabidopsis ortholog of MPK6) through an inducible, constitutively active NtMEK2DD (the MKK upstream of SIPK) in tobacco results in a rapid increase of ethylene production (Kim et al., 2003). The activation of SIPK also coincides with the activation of ACS (ACC synthase) activity, suggesting that a MAPK cascade is involved in post-translational modification or transcriptional activation of ACS (Kim et al., 2003).
Recently, it was demonstrated unequivocally that MPK6 is required for NtMEK2DD-induced ethylene production in Arabidopsis and that MPK6-induced-stress ethylene production is associated with increased ACS activity (Liu and Zhang, 2004). In contrast to ACS activity, ACO (ACC oxidase) activity stays high even without MPK6 activation, which suggests that ACS is the rate-limiting enzyme in stress ethylene biosynthesis (Liu and Zhang, 2004). Based on transcriptional activation by various stresses, ACS6 was implicated in stress ethylene production (Vahala et al., 1998; Wang et al., 2002). Now, genetic and biochemical evidence demonstrates that MPK6 activation leads to an increase in cellular ACS6 activity as a result of direct phosphorylation of ACS6 by MPK6 (Liu and Zhang, 2004). Mutation of the Ser phosphorylation sites to Ala abolishes MPK6-induced ACS6 accumulation in vivo. Mutation of these Ser residues to Asp, ACS6SDDD, which mimics the phosphorylated form of ACS6, results in ACS6 accumulation independent of MPK6 activation. ACS6SDDD transgenic plants overproduce ethylene and show an ethylene-induced morphology phenotype (Liu and Zhang, 2004). Thus, the MPK6 signaling cascade functions upstream in regulating stress ethylene biosynthesis. More significantly, the first MAPK in vivo substrate, ACS6, has been identified, which is the beginning of unraveling the complexity of MAPK function in plants.

Auxin. Auxin functions in virtually every aspect of plant growth and development. The mechanism of auxin action has been intensely investigated for decades (Leyser, 2002; Woodward and Bartel, 2005). Recently, it was demonstrated that TIR1 (an F-box protein) is an auxin receptor. Direct interaction of auxin with SCD-TIR1 triggers SCD-TIR1 to interact with transcriptional regulators Aux/IAA and to target Aux/IAA for degradation by ubiquitin-proteasome pathway (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005b). The degradation of Aux/IAA releases ARFs (auxin-response factors), allowing auxin-responsive transcription activation by ARF. However, it remains unknown whether other auxin-signaling mechanisms exist in plants (Dharmasiri et al., 2005b).

Several studies have implicated MAPK cascades in auxin signaling and responses. Protoplast co-transfection experiments demonstrated that NPK1 negatively regulates the induction of GH3, an early auxin response gene (Kovtun et al., 1998). Additional experiments demonstrated that ANP1 shares a similar function in suppressing GH3 expression upon auxin treatment (Kovtun et al., 2000). Hydrogen peroxide (H$_2$O$_2$) activates the ANP1 MAPK cascade, and the constitutively active ANP1 induces gene expression response that mimics oxidative stress. H$_2$O$_2$ treatment also blocks auxin-inducible GH3 promoter activity. Therefore, it has been proposed that oxidative stress suppresses GH3 activity through the activation of the ANP1 MAPK cascade (Kovtun et al., 2000). However, analyses of loss-of-function alleles of ANP mutants indicate that there is no change in auxin sensitivity. Moreover, a genome-wide gene expression analysis showed no apparent change of auxin-regulated gene expression in the anp2/anp3 double mutants (Krysan et al., 2002). Thus, additional experiments are needed to address these contradictory results.

Protein degradation and transcriptional regulation do not explain the immediate auxin responses, such as membrane depolarization and calcium spikes (Woodward and Bartel, 2005). Mockaitis and Howell (2000) demonstrated that MAPK activity is activated transiently within 5 minutes of applying auxin to Arabidopsis roots, whereas auxin-inducible MAPK activity disappears in the auxin-resistant mutant (axr4), which suggests a correlation of AXR4 function and MAPK activation (Mockaitis and Howell, 2000). While it is possible that MAPK activation could account for the immediate auxin responses, further research is required to test this hypothesis.

Another level of MAPK function in auxin biology is to regulate polar auxin transport (PAT). The Arabidopsis MKK7 overexpression mutant bushy and dwarf1 (bud1) develops fewer lateral roots and has less vascular tissue differentiation. While these phenotypes indicate that bud1 could be involved in auxin synthesis, transport, or signaling, there is no difference between bud1 and the wild-type with respect to IAA content and root growth inhibition (Dai et al., 2006). This suggests that bud1 is not involved in either auxin synthesis or in auxin signaling. Direct IAA transport assays indicate that bud1 negatively regulates PAT, and increased PAT was observed in BUD1 knockdown mutants. A double-mutant analysis of bud1 axr3-3 indicates that lowered PAT can be rescued partially by elevated auxin sensitivity in an auxin hypersensitive mutant (axr3-3). However, when bud1 is crossed with an auxin transport mutant (doc-1), the double mutant (bud1doc-1) has a more severe PAT defect (Dai et al., 2006). These genetic analyses reinforce the idea that bud1 is a negative regulatory PAT mutant. Future studies should identify the whole MAPK cascade where MKK7 functions and the downstream effectors, which will help unravel the regulation mechanism of PAT in Arabidopsis.

MAPKs in Stress Responses

Plants are subject to a diverse array of biotic and abiotic stresses. However, most of the stresses are not lethal, as plants have evolved to adapt to environmental stresses. As a first step of adaptation, plants sense the stresses and activate signaling transduction pathways that initiate various defense responses. Accumulating evidence indicates that a subset of plant responses—such as the activation of early defense genes and ROS generation—are shared by responses to abiotic and biotic stresses. MAPK cascades are likely to be one of the converging “hubs” of plant stress response signaling networks.

Pathogen Responses. Plants are constantly under attack by bacteria, fungi, nematodes, and viruses. Plants employ resistance proteins (R proteins) and other receptors/sensors to detect invasion of these pathogens. The detection of pathogen-virulence proteins or elicitors by R proteins/receptors triggers a diverse array of cellular responses, such as ROS generation, synthesis of stress ethylene, ion fluxes, strengthening of cell walls, synthesis
of phytoalexins, up-regulation of pathogen-related proteins, and induction of hypersensitive response (HR) cell death (Pedley and Martin, 2005). Often, a local defense response against one pathogen can trigger the induction of a broad-spectrum resistance to different pathogens throughout the plant; this is called systemic acquired resistance (SAR).

Activation of a MAPK cascade in a defense response was proposed initially in tobacco. A salicylic-acid-induced pathogen-related gene expression is mediated by NtSIPK (a tobacco ortholog of MAPK6) (Zhang and Klessig, 1997). Following infection of resistant tobacco leaves with tobacco mosaic virus (TMV), both NtSIPK and NtWIPK (a tobacco ortholog of MAPK3) were activated in an N-resistance gene-dependent manner (Zhang and Klessig, 1998). The tobacco N gene is a member of the TIR-NBS-LRR family of resistance proteins (Whitham et al., 1994). Constitutively active NtMEK2 (an MKK) induces HR cell death, which is preceded by the activation of endogenous NtSIPK and NtWIPK. HR cell death is often associated with plant disease resistance. In addition, the phytoalexin and salicylic acid biosynthesis enzymes HGMR (3-hydroxy-3-methyl-glutaryl CoA reductase) and PAL (β-phenylalanine ammonia lyase) also can be induced by the MAPK cascade NtMEK2-NtSIPK/NtWIPK, indicating that a MAPK cascade regulates multiple defense responses upon pathogen attack (Yang et al., 2001). Gene silencing of NtMEK2, NtSIPK, and NtWIPK by VIGS results in a much compromised N gene-mediated TMV resistance, indicating that the NtMEK2-NtSIPK/NtWIPK cascade plays a positive role in TMV resistance (Jin et al., 2003). Similarly, gene silencing of MEK1 (NQK1, Arabidopsis MKK6 ortholog) and Ntβ (NRK1, Arabidopsis MPK13 ortholog) by VIGS results in attenuated N gene-mediated resistance against TMV (Liu et al., 2004). As mentioned before, NQK1-NRK1 coupled with NPK1 (MKKK) regulates the cytokinesis process in tobacco.

How the same MAPK module can modulate two different cellular outputs remains to be solved. One possible explanation is that different MKKs employ the same MKK-MPK module and produce different cellular responses. In the Arabidopsis genome, there are 60 MKKKs, 10 MKKs, and 20 MPKs, which suggests the MKK-MPK could be the convergence point. Another explanation is that the same MKKK-MKK-MPK functions in different cellular-response processes through different substrates that have different spatiotemporal expression patterns, which contributes to signaling specificity. It also is possible that the attenuated N gene-mediated resistance against TMV in the MEK1 and in the Ntβ6 silencing mutants is a non-specific effect; the defective cell wall formation in the silencing mutants may allow TMV to move freely from cell to cell.

Similar to the tobacco orthologs NtSIPK and NtWIPK, the Arabidopsis MAPK6 (AtMPK6) and MAPK3 (AtMPK3) are activated by bacterial and fungal pathogens (Tena et al., 2001; Zhang and Klessig, 2001; Pedley and Martin, 2005). Protoplast transient expression assays demonstrated that AtMPK6 and AtMPK3 are in the MAPK signaling pathway involving FLS2-MEK1-MKK4/MKK5-MPK3/MPK6-WRKY22/WRKY20. FLS2 activates the MAPK cascade and, eventually, the WRKY transcription factors.

This pathway is the most complete innate immunity signaling cascade to be characterized so far (Asai et al., 2002). In a FLS2-dependent manner, both AtMPK3 and AtMPK6 are activated by fgl22, a fungal PAMP (pathogen-associated molecular pattern). Constitutively active MKK4/MKK5 activate AtMPK3 and AtMPK6 and, thus, bypass FLS2 in activating promoter activity of the downstream early response genes (WRKY29 and FRK1) in the fgl22 signaling pathway. In contrast, the dominant negative MKK4/MKK5 can partially suppress fgl22 activation of the downstream early response genes. These results suggest that MKK4/MKK5-MPK3/MPK6 are signaling components downstream of FLS2. Constitutively active MEKK1 activates MKK4/MKK5 (which, again, can bypass fgl22 signaling and activate fgl22 early response genes WRKY29 and FRK1), indicating that MEKK1 is the upstream MKKK (Asai et al., 2002). However, genetic evidence from whole-plant studies is needed to confirm the assembly of the fgl22 signaling cascade and to identify new components.

The enhance disease resistance 1 (edr1) mutant is resistant to Pseudomonas syringae and Erysiphe cichoracearum (powdery mildew). Disease-resistant proteins (e.g., PR-1 and PR-5) are not constitutively expressed in the edr1 mutant, which indicates that the disease resistance in edr1 is not due to SAR (Frye and Innes, 1998). The EDR1 gene encodes a B3 subgroup MKK (Frye et al., 2001; MAPK group, 2002). An in vitro kinase assay showed that the EDR1 C-terminal kinase domain has Ser/Thr kinase activity (Tang and Innes, 2002). Overexpression of a kinase-defective EDR1 leads to elevated resistance to E. cichoracearum, suggesting that the EDR1 MAPK cascade may function as a negative regulator of pathogen response signaling pathways (Tang and Innes, 2002; Tang et al., 2005). However, further research is needed to clarify if EDR1, in fact, functions as an MKKK.

**Oxidative Stress Responses.** ROS generated upon stress stimulation can serve as a signaling molecule to help initiate stress responses. This has been proposed to be a general mechanism across all eukaryotes (Hancock et al., 2001; Apel and Hirt, 2004; Wagner et al., 2004). In plants, ROS generation is an early response to pathogen infection and to abiotic stresses (Zhang and Klessig, 2001; Apel and Hirt, 2004; Mittler et al., 2004). Moreover, ROS can up-regulate MAPK activity, suggesting that ROS can serve as a signaling molecule that functions upstream of the MAPK cascade. In Arabidopsis, MPK3 and MPK6 become transiently activated upon ozone (O₃) treatment (Ahlfors et al., 2004). In tobacco, ROS activates NtSIPK and NtWIPK (Kumar and Klessig, 2000; Samuel et al., 2000). These results support the idea that MAPK signaling could be the missing link between ROS generation and downstream resistance gene activation. However, there are examples where a pathogen-induced ROS burst is not
required for MAPK activation (Romeis et al., 1999), indicating that MAPK activation and the ROS burst could be parallel events.

Studies have shown that an activated MAPK cascade, in turn, can induce ROS generation, which suggests that there may be a positive feedback loop between ROS generation and MAPK activation. In Arabidopsis, transiently induced, constitutively active MKK4 or M KK5 can initiate ROS and HR cell death (Ren et al., 2002). Independently, it has been shown that HR cell death transiently induced by a constitutively active tobacco MEK2DD was attenuated by VIGS of NbrhoB. NbrhoB is a homolog of a respiratory burst oxidase that is required for H2O2 accumulation upon fungal infection (Yoshoka et al., 2003). Together, these results indicate that ROS generation induced by MAPK activity is an important regulator of the HR response.

In Arabidopsis, OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) may be one of the candidate signaling components between ROS and a MAPK cascade. Expression and protein kinase activity of OXI1 is highly induced upon H2O2 treatment and wounding. Loss-of-function oxi1 mutants are more susceptible to fungal pathogen attack. The oxi1 mutant has reduced MPK3 and MPK6 activation upon H2O2 or elicitor treatment, indicating that OXI1 is required for full activation of ROS-induced MPK3 and MPK6 activity (Rentel et al., 2004).

Salt, Drought, Cold, and Wounding Responses. MAPK cascades respond to a diverse array of abiotic stresses, including salinity, cold, drought, wounding, touching, and other osmotic stresses. AtMEKK1 is transcriptionally activated by touch, cold, and salt stresses (Mizoguchi et al., 1996). MPK4 and MPK6 are activated by cold, drought, touching, and wounding stresses (Ichimura et al., 2000). MPK3 and MPK6 have been induced by osmotic stresses in both Arabidopsis and in tobacco (Hoyos and Zhang, 2000; Droillard et al., 2002). However, genetic evidence from intact plants is needed to confirm MAPKs function in abiotic stresses.

While MPK4 is not activated by hypersmotic stress, the loss-of-function mutant mpk4 is more tolerant to hypersmotic stresses (Droillard et al., 2004). Upon hypersmotic stress, the drought-inducible gene promoter of RAB18 is induced in the mpk4 mutant but not in the wild-type control seedlings, which suggests that MPK4 may play a negative role in regulating hypersmotic-stress responses (Droillard et al., 2004). AtMEK1s immunoprecipitated from seedlings that have been subjected to drought, high salt, wounding, and cold stresses have higher kinase activity toward recombinant kinase-inactive MPK4 (Matsuoka et al., 2002). This supports that AtMEK1 (AtMKK1) functions upstream of MPK4 in a stress-response pathway. But, again, genetic evidence is needed to support this conclusion.

A yeast two-hybrid interaction analysis indicated that AtMEKK1-AtMKK1/AtMKK2-AtMPK4 may form a MAPK cascade (Ichimura et al., 1998). Yeast osmosensitive MAPK cascade mutant pbs2Δhog1Δ complementation results showed that the combination of MKK2-MPK6 specifically complements the yeast osmosensing defect (Teige et al., 2004). Cold- and salt-stress-activated MKK2 specifically phosphorylates MPK4 and MPK6 recombinant proteins. Protoplast transient co-transformation studies showed that cold and salt activation of MPK4 and MPK6 are mediated by MKK2 (Teige et al., 2004). More convincingly, the mkk2 null mutant and the M KK2ΔEE overexpression mutant do not have any observable phenotype under normal growth conditions. However, when subjected to cold or salt stress, the mkk2 mutants are hypersensitive to cold and salt stresses. Conversely, the MKK2ΔEE overexpression mutants are tolerant to freezing and cold stresses.

The impairment of the cold-stress activation of MPK4 and MPK6 in the mkk2 null mutants indicates that MKK2 mediates MPK4/MPK6 activation in cold-stress responses (Teige et al., 2004). Protoplast transient expression experiments showed that MKK2 mediates the activation of MPK4/MPK6 by the gain-of-function AtMEKK1. This indicates that AtMEKK1 could be an upstream MKKK of MKK2 during cold and salt-stress responses (Teige et al., 2004). Again, genetic evidence from null AtMEKK1 mutants is needed to support this conclusion.

Systemic Acquired Resistance (SAR). Characterization of the mpk4 mutant indicates a negative regulatory role in SAR (Petersen et al., 2000). While the mpk4 mutant has dwarf stature, small curly leaves, and reduced fertility, it has a normal response to phytohormones and to abiotic stresses. Upon pathogen challenge, the mpk4 mutant shows enhanced resistance to pathogens. In the mpk4 mutant, the disease resistant genes (PR1, PR2, PR5) that are normally induced by SAR are, instead, constitutively induced. Whole-genome expression profiling by microarrays has confirmed the up-regulation of disease resistance gene expression in mpk4, providing further evidence of a negative regulatory role of MPK4 in pathogen resistance and SAR (Petersen et al., 2000).

Salicylic acid is necessary for SAR. In the mpk4 mutant, about 9-fold and 25-fold higher salicylic acid and salicylic acid glucosides are accumulated. Double-mutant analysis showed that nahG (a salicylate hydroxylase that converts salicylic acid to catechol) can rescue the mpk4 mutant phenotype, which indicates mpk4 is epistatic to salicylic acid in SAR signaling. Surprisingly, however, jasmonate-inducible genes are suppressed in the mpk4 mutant. Removing salicylic acid by the nahG mpk4 double mutant is not sufficient to release the suppression of jasmonate-inducible gene expression (Petersen et al., 2000). This indicates that MPK4 positively regulates jasmonate responses independently of its negative role in the regulation of SAR. Future work should identify the MAPK cascade and the target genes of MPK4, which will help in understanding the molecular mechanism of SAR.
Regulation of MAPK Cascade Specificity in Arabidopsis

As discussed, many MAPK components are involved in different cascades and assume different functions. In the Arabidopsis genome, there are more than 60 predicted MKKKs but only 10 MKks and 20 MPks, suggesting that there is signaling convergence and divergence on different levels of MAPK cascades. This raises the issue of how MAPK cascade signaling specificity is maintained. Five regulation mechanisms have been proposed: substrate specificity, colocalization (scaffolding), attenuation through phosphatases and inhibitor, interaction between MAPK cascades, and spatiotemporal regulation.

Substrate Specificity

Substrate specificity may be one of the regulation mechanisms. The same MAPK module can activate different substrates (depending on the spatiotemporal expression of individual substrates) and, thus, can determine the ultimate outputs of the MAPK cascade. However, so far, only two MAPK substrates have been identified in Arabidopsis: ACS6 and MKS1. More information on MAPK substrates is needed to substantiate this mode of regulation.

ACS6 was the first MAPK substrate to be identified. ACS6 (an ACC synthase) is phosphorylated and stabilized by MPK6, which is essential for stress ethylene production, as discussed before (Liu and Zhang, 2004). MKS1 was identified as a MPK4 substrate that regulates plant defense responses (Andreasson et al., 2005). A yeast two-hybrid screen identified MKS1 as an interaction partner of MPK4. Analysis of the sequence of MKS1 indicates the existence of a potential MAPK phosphorylation site. Biochemical analysis showed that immunoprecipitated MKS1 overexpression experiments confirmed the interaction between MKS1 and recombinant MKS1 in vitro; in vivo immunoprecipitation experiments confirmed the interaction between MKS1 and MPK4. MKS1 overexpression 3SS:MK5 transgenic plants have a dwarf phenotype (similar to mpk4 null mutants), whereas the MKS1 RNAi transgenic plants have a normal growth phenotype, indicating a functional link between these two proteins. Similar to what was observed in the mpk4 mutant, PR proteins that are normally induced in SAP are up-regulated in the 3SS:MK51 transgenic plants. These transgenic plants are also more resistant to pathogen attack, which reinforces the functional interaction between MKS1 and MPK4. Since the mpk4 mutant can be rescued partially by reducing MKS1 expression, this indicates that MPK4 negatively regulates MKS1 activity.

A MAPK that negatively regulates downstream substrates of another MAPK cascade has been demonstrated in other organisms. For example, a yeast MAPK (Fus3) controls the degradation of Tec1, a substrate of an invasive growth MAPK (Kss1) (Plaschke and Gann, 2003; Elion et al., 2005). Since MAPK cascades are extremely conserved signaling modules across all the eukaryotes, information learned from other systems will help to piece together what has been learned in Arabidopsis. The converse also is true: what has been learned about MAPK cascade signaling in Arabidopsis will contribute to the understanding of MAPK cascade signaling mechanisms in other organisms.

Colocalization (Scaffolding)

Scaffolding may be another mechanism to maintain MAPK cascade signaling specificity. Scaffolding has been demonstrated best in yeast and mammalian systems, where there are multiple examples of how scaffold proteins help confer signaling specificity (Elion et al., 2005; Kolch, 2005). So far, however, no MAPK cascade scaffolding protein has been characterized in Arabidopsis. One reason for this is that the MAPK scaffolding proteins in Arabidopsis could have a significantly different identity from their mammalian counterparts. Or, it could be that MAPK cascade components mediate protein-protein interactions themselves and function as scaffolding proteins in Arabidopsis. The yeast osmotic-stress regulator Pbs2 (a MKK and a scaffolding protein) is an example of one such dual-function MAPK component. An oxidative-stress responsive MKKK (MsOMTK1) from alfalfa has been demonstrated to activate, and to interact physically with, a MAPK (MsMMK3) through a protoplast-transient transformation assay, suggesting that MKKK in plants may serve a similar dual function (Nakagami et al., 2004).

Attenuation Through Phosphatases and Inhibitor

Yeast mating and invasive growth MAPK pathways share multiple common components. One mechanism to maintain signaling specificity is to attenuate nonessential MAPKs by means of protein phosphatases. During invasive growth, for example, a yeast MAPK (Fus3) can be selectively repressed by a phosphatase (Mgl5) (Elion et al., 2005). In Arabidopsis, several phosphatases targeting specific MAPKs have been identified. AtDsPTP1 (Arabidopsis dual-specificity protein tyrosine phosphatase) dephosphorylates and inactivates AtMPK4 (Gupta et al., 1998). Another tyrosine phosphatase AtMPK1 is a mutant that is hypersensitive to genotoxic stresses (UV-C and methyl methanesulphonate) (Ulm et al., 2001). Yeast two-hybrid experiments showed that AtMPK1 interacts specifically with MPK3, MPK4, and MPK6, indicating that MPK1 may regulate stress responses through the regulation of MAPKs (Ulm et al., 2002). In addition, MP2C (a PP2C phosphatase in alfalfa) has been demonstrated to be a specific negative regulator of SIMK (salt stress-inducible MAPK) (Meschiene et al., 1998; Meschiene et al., 2003). As more MAPK-regulatory phosphatases are thoroughly characterized, more insights into how MAPK cascades are regulated in plants are likely.

Interaction Between MAPK Cascades

Interaction between MAPK cascades may be another level of regulation to maintain signaling specificity. In tobacco,
silencing SIPK can lead to increased activation of WIPK upon prolonged ozone treatment (Samuel and Ellis, 2002). WIPK transcription and protein accumulation is regulated positively by SIPK activation (Liu et al., 2003). Upon wounding, MPK3 (WIPK ortholog in Arabidopsis) activity was 2-3 fold higher in MPK6 (SIPK ortholog in Arabidopsis) gene-silencing lines than that in wild-type controls (Menke et al., 2004). One possible explanation for these results could be that MPK6 and MPK3 have partially redundant functions. The down-regulation of MPK6 could be compensated functionally by the up-regulation of MPK3.

Spatiotemporal Regulation

Signaling specificity also may be spatiotemporally regulated. In mammalian cells, it has been well documented that, upon activation, MAPK can translocate to the nuclei or to other subcellular organelles, where they activate a specific set of substrates (Widmann et al., 1999). Plant cells may have adopted a similar mechanism. Upon peptide elicitor Pep-13 treatment, parsley MAPKs (PcMPK3a/b, and PcMPK6) rapidly translocate and accumulate in nuclei, but the putative upstream MKK (PcMKK5) show constitutive cytosolic localization (Ligterink et al., 1997; Kroj et al., 2003; Lee et al., 2004). In Arabidopsis, immunolocalization in planta also has demonstrated that, upon ozone treatment, MPK3 and MPK6 rapidly translocate to nuclei (Ahlfors et al., 2004). Future research should address the mechanism by which MAPK translocation is regulated in plants.

ADDITIONAL PROTEIN KINASES IN ARABIDOPSIS

CDPK-SnRK Super-Family in Arabidopsis

The CDPK-SnRK (calcium-dependent protein kinase-SNF1 related protein kinase) super-family of protein kinases contains a predicted 84 members in the Arabidopsis genome (Hrabak et al., 2003). All of the members contain a Ser/Thr kinase domain but also have various other domains in the remainder of the protein, including a highly variable N-terminal domain and a C-terminal region that often includes regulatory domains. This super-family is sub-divided into smaller families including the CDPKs, the SnRKS, and a few families containing limited members in the Arabidopsis genome (Table 2).

CDPKs

The predicted 34 CDPKs in Arabidopsis contain four characteristic domains: a variable N-terminal domain, the kinase domain, an autoinhibitory domain, and a calmodulin-like domain (Harmon et al., 2000; Cheng et al., 2002; Hrabak et al., 2003). The autoinhibitory domain has a pseudosubstrate site that may act to inhibit the activity of the protein kinase (Harmon et al., 1994). This domain is located immediately C-terminal to the kinase domain and is sometimes called the junction domain. At the C-terminal end of the protein is a calmodulin-like domain. This domain contains at least one Ca^{2+}-binding elongation factor (EF) hand, and most members contain four EF hands (Cheng et al., 2002; Hrabak et al., 2003).

Although they do not contain transmembrane domains, many CDPKs are predicted to be membrane associated because the N-terminal region often contains putative myristoylation and palmitoylation sites (Hrabak et al., 2003). Indeed, one CDPK has been found to be associated with the endoplasmic reticulum (ER) membrane, and the first 10 amino acids containing these acylation sites were sufficient for this targeting (Lu and Hrabak, 2002). A more extensive study found several family members that are targeted to the plasma membrane and a few that are both cytoplasmic- and nuclear-localized or associated with the peroxisome (Dammann et al., 2003).

Regulation of the CDPKs is not completely clear. It is predicted that Ca^{2+} binding in the calmodulin-like domain induces a conformational change that results in the release of the autoinhibitory domain, which allows the kinase to be active (Harmon et al., 2000; Cheng et al., 2002). Additional work has shown different Ca^{2+}-binding affinities at different sites within the calmodulin-like domain (Christodoulou et al., 2004). The higher affinity sites may bind Ca^{2+}, but the kinase remains inhibited. Only when the lower affinity sites are filled is the inhibition released. In addition to Ca^{2+}, there is some evidence that phospholipids and 14-3-3 proteins also may play a regulatory role (Binder et al., 1994; Camoni et al., 1998). Autophosphorylation also may play a regulatory role as well (Cheng et al., 2002). Future work is needed to determine how the numerous CDPKs

| Table 2. Characteristics of the CDPK-SnRK families in Arabidopsis. |
|---|---|---|---|---|
| Family | Kinase domain | Ca^{2+}-binding domain | Autoinhibitory domain | Acylation sites (in multiple family members) |
| CDPK | Yes | Yes | Yes | Yes |
| SnRK | Yes | No | Variable | No |
| CRK | Yes | No | No | Yes |
| PPCK/PEPRK | Yes | No | No | No |

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are regulated to allow the proper signals to be transmitted in their respective pathways.

The in planta functions for the CDPKs are quite varied and not yet entirely understood. Studies in Arabidopsis and in other plants have shown that CDPKs are involved in hormone signaling, in various aspects of growth and development, in water relations, in metabolic functions, and in responses to abiotic and biotic stresses (Cheng et al., 2002; Lee and Rudd, 2002; Ludwig et al., 2004). Since Ca\(^{2+}\) fluxes are involved in many signaling events, future research into how specificity is maintained and into the role of CDPKs as signaling nodes will be of particular interest. As would be expected from the diverse signaling pathways in which CDPKs are active, the substrates identified in Arabidopsis and other species are also quite diverse (Cheng et al., 2002). For example, in Arabidopsis, CDPKs have been found to phosphorylate phenylalanine ammonia-lyase, an enzyme involved in pathogen defense, and the Ca\(^{2+}\) pump (ACA2) (Hwang et al., 2000; Cheng et al., 2001).

SnRKs

With a predicted 38 members, the SnRK (SNF1-related protein kinases) family is slightly larger than the CDPK family in Arabidopsis (Hrabak et al., 2003). This family is most closely related to SNF1 from yeast and AMP-activated protein kinases from animals. As in yeast and in animals, many of these family members are predicted to play important roles in metabolic regulation in Arabidopsis (Haldrow and Hardie, 1998; Halford et al., 2003; Halford et al., 2004). Unlike the CDPKs, the SnRKs do not have C-terminal Ca\(^{2+}\)-binding domains; however, Ca\(^{2+}\) does play a role in the regulation of some members. The SnRK family, for example, includes the calcineurin B-like (CBL)-interacting protein kinases (CIPKs). CBLs are a family of 10 Ca\(^{2+}\)-binding proteins in Arabidopsis that physically interact with CIPKs (Shi et al., 1999; Kim et al., 2000; Luan et al., 2002; Kolukisaoglu et al., 2004). Details for one CIPK (SOS2) and one CBL (SOS3) are discussed later in this chapter.

In yeast and in animals, the SnRK protein kinase forms a heterotrimeric complex. Homologs to these interacting partners have been isolated in Arabidopsis and have been shown to physically interact with SnRKs (Halford et al., 2003) and with members of the ubiquitin-mediated protein degradation pathway (Farras et al., 2001). Several metabolic substrates have been identified for the SnRKs (Halford et al., 2003). In addition, a transcription factor that mediates ABA-responsive gene expression also was identified recently as a substrate (Choil et al., 2005). Several stress-related functions have been reported for SnRK family members, including responses to hyperosmotic or salt stress and ABA-mediated responses to water stress (Yoshida et al., 2002; Boudsocq et al., 2004).

To date, the best-described SnRK is salt overly sensitive2 (sos2). sos2 is a member of the SnRK family and was identified as one of the sos mutants that hyperaccumulate Na\(^{+}\) during salt stress (Liu et al., 2000). Unlike most CDPK-

SnRK family members, SOS2 contains a Thr in the activation loop (Hrabak et al., 2003). A Thr to Asp mutation, as well as a Ser to Asp mutation or a Tyr to Asp mutation, in the activation loop results in a constitutively active SOS2, as does the removal of an interaction domain to which SOS3 binds (Guo et al., 2001; Gong et al., 2002). SOS3 is a Ca\(^{2+}\)-binding protein that contains an EF hand. SOS3 physically interacts with SOS2 and and binding is required for kinase activity (Halfter et al., 2000). The SOS3-binding site is an autoinhibitory domain that blocks substrate access to the SOS2 catalytic site in the absence of active SOS3 (Guo et al., 2001).

SOS2 and SOS3 are regulators of SOS1, a plasma membrane Na\(^{+}/H^{+}\) exchanger (Ooi et al., 2002). SOS3 recruits SOS2 to the plasma membrane where SOS1 is localized (Quintero et al., 2002). This suggests a model where the SOS3 Ca\(^{2+}\)-sensor recruits and activates the SOS2 protein kinase to the plasma membrane where the SOS1 Na\(^{+}\) transporter is phosphorylated and activated during salt stress (Quintero et al., 2002; Guo et al., 2004). In addition to SOS1, SOS2 also regulates the vacuolar H\(^{+}\)/Ca\(^{2+}\) antipporter CAX1 independently of SOS3 (Cheng et al., 2004). Future work into this dual regulation should prove interesting.

Other CDPK-SnRKs

The remaining CDPK-SnRK families contain fewer predicted members in the Arabidopsis genome. There are eight predicted CRK (CDPK-related protein kinase) family members (Hrabak et al., 2003). These are similar to the CDPKs, except that the Ca\(^{2+}\)-binding EF hands are degenerated and appear to be nonfunctional. Two PPCKs (phosphoenolpyruvate carboxylase protein kinases), which are calcium-independent protein kinases that phosphorylate PEP carboxylase, also are found in the Arabidopsis genome, as well as two PEPRKs (PEP carboxylase protein kinase-related protein kinases) (Hrabak et al., 2003). Most recently, a calmodulin-binding protein kinase, named CRCK1 (Calmodulin-Binding Receptor-Like Cytoplasmic Kinase1), that is involved in stress signaling pathways was isolated in Arabidopsis (Yang et al., 2004).

GSK-3/Shaggy-Like Protein Kinases

The glycogen synthase protein kinase 3 (GSK-3)/SHAGGY protein kinases are Ser/Thr kinases that carry out multiple functions in metazoans (Doble and Woodgett, 2003). GSK-3 was first characterized for its ability to phosphorylate and inactivate the enzyme glycogen synthase (Embi et al., 1980; Woodgett and Cohen, 1984). The GSK-3/Shaggy-like gene family in Arabidopsis contains at least 10 members, which are divided into three classes (Dornelas et al., 1998; Dornelas et al., 1999).

In Arabidopsis, the GSK-3/Shaggy-like family members are expressed in the embryo (Dornelas et al., 1999), the pollen (Tichbinsky et al., 1998), and in floral organs.
(Dornelas et al., 2000). Analyses of real-time reverse transcriptase PCR expression profiling indicated that many members of this gene family are expressed across multiple tissues while others are tissue-specific and/or responsive to abiotic stresses (Charrier et al., 2002).

Functional analyses revealed that the GSK-3/SHAGGY-like family members play both developmental and stress-responsive roles in Arabidopsis. AtSK11 (ASKα) and AtSK12 (ASKβ) antisense lines show morphological phenotypes in flowers, including increased perianth organs and altered gynoecium development (Dornelas et al., 2000). AtGSK1 can rescue a salt-sensitive yeast strain (Piao et al., 1999), and its overexpression results in a constitutive expression of salt-stress genes and an enhanced tolerance to salt stress (Piao et al., 2001).

The best-characterized member in Arabidopsis is BIN2/UCU1/DWF12. Two alleles of BR-INSENSITIVE 2 (BIN2) were uncovered in a screen of dwarf BR-insensitive plants (Li et al., 2001). Both alleles are dominant and have a phenotype similar to bri1. Subsequent cloning of BIN2 revealed it as a GSK-3/SHAGGY-like family member (Li and Nam, 2002). The two BIN2 alleles act as gain-of-function mutations and plants with either allele have BR-deficient phenotypes. Reduced BIN2 expression can partially rescue weak bri1 mutants, suggesting BIN2 is a negative regulator of BR signaling (Li and Nam, 2002).

Additional alleles of BIN2 have been isolated. Three alleles of ULTRACURVATA1 (UCU1) have been cloned and are named for the phenotype of strongly downward rolled leaves (Perez-Perez et al., 2002). In these plants, cell expansion is reduced, resulting in dwarfed plants that are also BR-insensitive. Studies in a different ecotype uncovered dwarf12 (dwl12-1D and dwf12-2D), which has identical mutations as ucu1-1 and bin2-1, respectively (Choe et al., 2002). Interestingly, six of the seven point mutations described thus far occur in a domain highly conserved with animal GSK-3s called the TREE domain, a possible phosphorylation site (Choe et al., 2002). However, in vitro phosphorylation target site studies do not suggest that the TREE domain is a direct BR1 target (Oh et al., 2000), and BR1 was not found to interact physically with BIN2 (Li and Nam, 2002). Future studies should identify what role, if any, this domain may play in the regulatory control of BIN2/UCU1/DWF12.

Two genes have been isolated that are candidate BIN2/UCU1/DWF12 substrates. The brassinazole-resistant 1-1D (bzz1-1D) mutant suppresses a BR-deficient phenotype and is a positive regulator of the BR-signaling pathway (Wang et al., 2002). BZZ1 is a member of a plant-specific gene family that comprises five members in Arabidopsis. bzz1-EMS-suppressor1 (Bes1), a mutant with a similar phenotype as bzz1-1D also has been identified (Yin et al., 2002). These two genes are closely related to each other. Furthermore, yeast two-hybrid and pull-down assays indicate that BES1 and BIN2 interact specifically and that BIN2 is able to phosphorylate BES1 (Yin et al., 2002). This suggests that BIN2 phosphorylation of BES1 acts as a regulatory mechanism to reduce the activity of BES1 at low BR concentrations. BIN2 also directly interacts with BZR1 in vitro and negatively regulates BZR1 accumulation in vivo, likely by phosphorylation (He et al., 2002). However, BIN2 phosphorylation of BZR1 and BES1 does not appear to be dependent on a priming phosphorylation or a scaffold protein, as has been reported in animal GSK-3s (Zhao et al., 2002b). This suggests that a novel activation mechanism may exist in the plant GSK-3/SHAGGY-like protein kinases.

**PINOID**

Mutations of the PINOID (PID) gene encode a Ser/Thr kinase that displays a phenotype similar to plants treated with an auxin-transport inhibitor (Christensen et al., 2000) (Figure 4). PID encodes a functional protein kinase that contains all the typical subdomains of protein kinases. Constitutive overexpression of PID demonstrated a role in auxin signaling (Christensen et al., 2000; Benjamins et al., 2001). Further work demonstrated a role for PID in mediating localization of Pin-Formed1 (PIN1, a transporter-like membrane protein) to create auxin gradients during patterning processes (Friml et al., 2004). PID also has been shown to play a role in the formation of cotyledons during embryogenesis (Furutani et al., 2004; Treml et al., 2005).

Interestingly, a yeast two-hybrid screen found two proteins that bind Ca2+ and interact with PID in a Ca2+-dependent manner (Benjamins et al., 2003). While this interaction stimulates autophosphorylation activity of PID, the Ca2+-binding proteins are not a substrate. Recently, two protein kinases similar to PID (WAG1 and WAG2) were found to be root-tip expressed protein kinases that negatively regulate a root-waving response (Santner and Watson, 2006) (Fig. 4).

**TOUSLED**

TOUSLED (TSL) encodes a Ser/Thr protein kinase that is important in development of both floral and vegetative tissue (Roe et al., 1993). The N-terminal region of TSL contains an essential nuclear localization signal and domains important for oligomerization, which are essential for kinase activity (Roe et al., 1997). TSL kinase activity is higher during G2/M-phase and during G1-phase cells compared to S-phase cells, suggesting a role for TSL during the cell cycle (Ehsan et al., 2004).

**Histidine Protein Kinases**

In plants, there are eight canonical histidine protein kinases in the genome. Histidine protein kinases are members of two-component signaling relays. Two-component systems have been shown to be important in both cytokinin and ethylene signal transduction in plants. Histidine protein kinases and two-component systems are covered in
Light-Responsive Protein Kinases

Two groups of protein kinases are important in light-signaling pathways in plants. Phytochromes are responsible for monitoring the red and far-red regions of the spectrum. PHYA is a potential Ser/Thr protein kinase in higher plants. Phytochromes are covered in more detail in another chapter of The Arabidopsis Book (Wang and Deng, 2004).

Phototropins (phot1 and phot2) are involved in the responses to unidirectional blue-light sources. It is likely that both phot1 and phot2 are capable of autophosphorylation by Ser/Thr kinase domains in response to blue-light illumination. Phototropins are covered in more detail in another chapter of The Arabidopsis Book (Liscum, 2002).

PROTEIN PHOSPHATASES IN ARABIDOPSIS

Protein phosphorylation and dephosphorylation are involved in almost all cell-signaling events. Reversible protein phosphorylation depends on coordinated actions of protein kinases and protein phosphatases. While both protein kinases and protein phosphatases exist as large protein families in Arabidopsis, protein kinases greatly outnumber protein phosphatases. This numerical imbalance gives rise to the question of how a limited number of protein phosphatases are fulfilling the job of maintaining the phosphorylation status of a cell. Protein phosphatases regulate a diverse array of processes in Arabidopsis, including auxin transport, ABA signaling, and RLK signaling. Based on phospho-amino-acid-substrate specificity, protein phosphatases are divided into two major groups: protein Ser/Thr phosphatases and protein tyrosine phosphatases.

Protein Ser/Thr Phosphatases

Further classification of the protein Ser/Thr phosphatases is based on substrate specificity, divalent cation requirements, and inhibitor sensitivity, as summarized in Table 3 (Smith and Walker, 1996). Based on sequence and structural analysis, the type one (PP1), type 2A (PP2A), and type 2b (PP2B) protein phosphatases are related enzymes and, hence, are defined as the PPP family. The type 2C protein phosphatases (PP2C) and other Mg²⁺-dependent Ser/Thr phosphatases, are closely related and share no sequence homology with PPP and, thus, are defined as the PPM family (Barford, 1996; Barford et al., 1998).

Types 1 and 2 Protein Phosphatases

The PPP family enzymes are multimeric holoenzymes. The core catalytic region (around 280 aa) is very conserved
In Arabidopsis, there is specificity, and activity of heterotrimeric PP2A (Li et al., 2002). While both A subunits and C subunits are highly conserved and ubiquitous phosphatases across all eukaryotes. In animals, the PP1C core catalytic subunit, which assembles with different regulatory subunits, is implicated in cell-cycle regulation, apoptosis, glycogen metabolism, and neuronal activities (Bollen, 2001; Gallego and Virshup, 2005).

In plants, there is only limited knowledge about PP1 so far (Smith and Walker, 1996; Lin et al., 1999; Luan, 2003). An initial survey identified eight PP1-type protein phosphatase catalytic subunit genes in Arabidopsis (Smith and Walker, 1993; Lin et al., 1998), which later were confirmed by whole-genome analysis (Kerk et al., 2002). Expression pattern analyses demonstrated that PP1 catalytic subunits are ubiquitously expressed in Arabidopsis (Lin et al., 1998). Most of the functional inferences of PP1 catalytic subunits are based on inhibitor approaches. As shown in Table 3, PP1s are sensitive to okadaic acid and insensitive to cantharidin, which is used to distinguish PP1 activity from other phosphatases in the cell. PP1 activity was found to be involved in the regulation of membrane channels, cell cycle control, and developmental regulation in plants (Smith and Walker, 1996; Luan, 2003). However, no loss-of-function phenotypes have been described yet for the genes encoding the PP1 catalytic subunits.

<table>
<thead>
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<th>Table 3. Classification of the protein Ser/Thr phosphatases.</th>
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<td><strong>Substrate specificity</strong></td>
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<tr>
<td><strong>Type I</strong></td>
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<tr>
<td>PP1</td>
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<tr>
<td><strong>Type II</strong></td>
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<tr>
<td>PP2A</td>
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<td>PP2B</td>
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<td>PP2C</td>
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PP2As are heterotrimeric enzymes formed by a catalytic core subunit (C subunit) that associates with a scaffold subunit and a regulatory B subunit. The Arabidopsis genome encodes 5 C subunits, 3 A subunits, and 17 B subunits. With different combinations of A, B, and C subunits, there could be up to 255 PP2A holoenzymes (Kerk et al., 2002). While both A subunits and C subunits are highly conserved across all eukaryotes, B subunits are quite varied, which is consistent with their regulatory functions. B subunits are further classified into B, B', and B", based on molecular weight (Luan, 2003). In animals, regulatory B subunits regulate the subcellular localization, substrate specificity, and activity of heterotrimeric PP2As (Li et al., 2002; Gallego and Virshup, 2005). In Arabidopsis, there is still only limited knowledge about the regulatory functions of the B subunits.

In Arabidopsis, PP2As play essential roles in plant development and auxin signal transduction. *rcn1* (root curl in Naphthylphthalamic acid) was identified in a screen for potential auxin-transport mutants by a root curling assay. *rcn1* roots tightly curl in the presence of the auxin-transport inhibitor Naphthylphthalamic acid (NPA), while the wild-type roots grow in straight lines. Auxin efflux in *rcn1* mutants was shown to be more sensitive to NPA than in wild-type seedlings (Garbers et al., 1996). *RCN1* encodes an A subunit of PP2A. Initial functional analysis showed that RCN1 was able to complement the yeast *tpd3*, a mutation in a PP2A A subunit (Garbers et al., 1996). Plants treated with the PP2A inhibitors okadaic acid and cantharidin can partially phenocopy the *rcn1* mutant, suggesting that decreased PP2A activity is responsible for the *rcn1* phenotype. In vitro enzyme assays demonstrated that RCN1 is a positive regulator of PP2A activity, as PP2A activity in an *rcn1* protein extract is much lower than that in wild-type. Inhibitor assays also showed that *rcn1* seedlings are more sensitive to okadaic acid and cantharidin, which is consistent with the reduced PP2A activity in the *rcn1* mutant (Deruere et al., 1999).

All three A subunits are functional, with RCN1 playing a major function and PP2AA2 and PP2AA3 playing relatively minor functions (Zhou et al., 2004b). The *pp2aa2/pp2aa3* double mutants display only minor defects, such as slightly reduced root elongation. However, the *rcn1/ppaa2* and *rcn1/pp2aa3* double mutants have an extremely severe developmental and growth defect phenotype. At the seedling stage, the mutants have greatly reduced hypocotyls and root elongation. The hypocotyls and roots show radially expanded, resulting from irregular cell expansion in the epidermis and cortical cells. In the adult mutant plants, small rosette leaves are clustered together, and the inflorescence blots are much shorter than in the wild-type. Moreover, the *rcn1/pp2aa2* double mutants are infertile (Zhou et al., 2004b). These systemic analyses suggest that all three PP2A A subunits are functionally overlapping but not equivalent.
The irregular cell expansion in both the rcn1/pp2a2a2 and rcn1/pp2a3a2 double mutants is reminiscent of the ton2/fass mutant phenotype. The ton2/fass is the only PP2A B regulatory subunit that has been characterized in Arabidopsis so far (Camilleri et al., 2002). In the ton2 mutants, defects in cortical microtubule organization cause irregular cell-size and cell-shape phenotypes. A yeast two-hybrid assay demonstrated that TON2 interacts with the A subunits of PP2A, indicating that protein phosphatase 2A may be involved in the control of cortical cytoskeleton organization (Camilleri et al., 2002).

**Type 2C Protein Phosphatases**

The PP2Cs belong to the PPM family of Ser/Thr phosphatases. Their catalytic activity requires the divalent cations Mn$^{2+}$ or Mg$^{2+}$. PP2Cs are monomeric enzymes that exist in all eukaryotes. PPM phosphatases do not share any sequence homology with PPP phosphatases. However, the protein structures of these two families of phosphatases are fairly similar, suggesting similar catalytic mechanisms and a convergent evolution (Das et al., 1996).

In the Arabidopsis genome, there are about 69 PP2Cs, which is much more complex and abundant than in other organisms (Kerk et al., 2002). Except for six genes, the 69 PP2Cs can be subdivided into 10 groups (Schweighofer et al., 2004). Catalytic domains of individual PP2Cs have various N- or C-terminal extensions (e.g., putative MAPK docking site, membrane localization signaling anchor, phospho-protein interacting FHA domain) that may contribute to their regulation, substrate specificity, and subcellular localization (Schweighofer et al., 2004).

Although relatively few have been functionally characterized, PP2Cs are implicated in diverse developmental and stress responsive signaling pathways in Arabidopsis.

**Abscisic Acid Insensitive 1 and 2.** There are three Ser/Thr protein phosphatases known to be involved in regulation of the ABA-signaling pathway: Abscisic Acid Insensitive 1 (ABI1) and ABI2, Homology to ABI1/ABI2 (HAB1), and AtPP2CA. The abi1-1 and abi2-1 mutants were identified in an EMS mutagen screen for ABA insensitive seed germination. Both mutants have reduced seed dormancy, and seed germination is less sensitive to inhibitory concentrations of ABA. At the vegetative stage, both mutants display seedling growth that is insensitive to ABA, reduced drought tolerance, and abnormal stomata regulation (Leung et al., 1994; Leung et al., 1997). ABI1 and ABI2 are highly homologous at the protein sequence level. A biochemical analysis showed that both ABI1 and ABI2 encode functional PP2Cs. abi1-1 and abi2-1 both carry the same metal-binding site mutation (G-D) that abolishes most of the PP2C protein phosphatase activity of the enzyme (Bertauche et al., 1996; Sheen, 1998). Based on the dominant nature of the original abi1-1 and abi2-1 mutations, the pleiotropic phenotype is believed to be due to dominant negative effects (Bertauche et al., 1996; Leung et al., 1997; Rodriguez, 1998).

Isolation and analysis of intragenic recessive revertant alleles of abi1-1 and abi2-1 showed that ABI1 and ABI2 are negative regulators of ABA signaling (Gosti et al., 1999; Merlot et al., 2001). All the revertant alleles carry missense mutations in the conserved catalytic domain. In vitro phosphatase assays demonstrated that these revertants either have greatly reduced or no PP2C activity, indicating that these revertants are loss-of-function alleles. In contrast to abi1-1, all the revertants of abi1-1 (abi1-1R) are hypersensitive to ABA, as evidenced by increased inhibition of seed germination, enhanced seed dormancy, and drought tolerance with exogenous ABA application. However, why the abi1-1 dominant negative allele shows an opposite ABA sensitivity than the recessive abi1-1R remains unclear. One possibility is that a dominant negative mutation may trap target protein kinases and, thus, inhibit their function in the ABA signaling. The recessive revertants, on the other hand, may lose their ability to trap and dephosphorylate the target protein kinases, thereby allowing the hyperphosphorylated protein kinases to function in the ABA signaling pathway. T-DNA insertion null mutants of ABI1 and ABI2 have been reported (Kuhn et al., 2005; Yoshida et al., 2006). Future, detailed analyses of such mutants are needed to help solve this puzzle.

**Homology to ABI1/ABI2.** Based on sequence homology, HAB1 is closely related to ABI1 and ABI2. Although sequence homology does not always indicate the same function, HAB1 does share a similar function as ABI1 and ABI2 (Rodriguez et al., 1998). Expression of HAB1 is ABA inducible. Transgenic plants constitutively expressing HAB1 have reduced ABA sensitivity, while hab1 T-DNA insertion mutants show ABA hypersensitivity. These results suggest that HAB1 also is a negative regulator of ABA signaling. However, how HAB1 differentially regulates ABA signaling remains unclear (Rodriguez et al., 1998; Saez et al., 2004).

**AtPP2CA.** AtPP2CA can rescue the cAMP phosphodiesterase defective sterile mutant pde1 in Schizosaccharomyces pombe (Kurumori and Yamamoto, 1994). Like ABI1/2 and HAB, AtPP2CA belongs to the A-type PP2C phosphatases (Schweighofer et al., 2004). A maize protoplast transient transfection experiment demonstrated that, like ABI1, AtPP2CA can block ABA-inducible gene expression (Sheen, 1998). AtPP2CA can be induced, in an ABA-dependent manner, by different abiotic stress conditions, such as drought, high salt, and low temperature.

To elucidate the function of AtPP2CA in abiotic-stress responses, antisense AtPP2CA transformant plants were generated (Tahtiharju and Palva, 2001). Cold acclimatization in the antisense lines was much faster than in the control plants, but no differences were observed in drought tolerance (Tahtiharju and Palva, 2001). Cold-induced genes were highly up-regulated in the antisense lines, which further confirms the role of AtPP2CA in cold tolerance. In addition, the antisense lines were hypersensitive to ABA, which indicates that AtPP2CA plays a negative regulatory role in ABA-mediated cold acclimatization (Tahtiharju and Palva, 2001).
Recently, ABA-hypersensitive germination 3 (ahg3) was cloned and found to be due to point mutation in AtPP2CA. Expression analysis demonstrated that ahg3 is highly expressed in seeds (Yoshida et al., 2005). Independently, AtPP2CA was identified by screening a library of 35S::cDNA transgenic lines for changes of ABA sensitivity. While 35::AtPP2CA plants were insensitive to applied ABA during seed germination, AtPP2CA T-DNA insertion alleles were hypersensitive to ABA application during seed germination (Kuhn et al., 2005). These results support the proposal that AtPP2CA is a strong negative regulator of ABA signaling.

How AtPP2CA function is regulated in Arabidopsis is still largely unknown. A yeast two-hybrid screen showed that AtPP2CA is an interaction partner of AKT2, a potassium channel inward rectifier (Vranova et al., 2000); this physical interaction was confirmed by in vitro pull-down assays. AtPP2CA modulates the AKT2 potassium-channel activity when coexpressed in COS cells and oocytes of Xenopus, which provides the evidence for a functional interaction between these two proteins (Cherel et al., 2002). However, more data are needed to generalize the regulatory mechanism of AtPP2CA’s function in Arabidopsis.

**KINASE ASSOCIATED PROTEIN PHOSPHATASE.** KAPP (kinase associated protein phosphatase) was the first downstream regulator of an RLK to be characterized. It was identified by screening an Arabidopsis cDNA expression library for interactions with an RLK protein kinase domain (Stone et al., 1994).

KAPP is a unique multi-domain protein. It has a type I membrane anchor at the N-terminus, a kinase interaction domain (KID)-forkhead-associated (FHA) domain in the central part, and a PP2C domain at the C-terminus (Stone et al., 1994). Transient expression experiments showed that the N-terminal signal anchor is functional in targeting KAPP to the cowpea mesophyll protoplast membrane (Shah et al., 2002). However, this localization pattern needs to be confirmed with a stable transgenic line. The functional relevance of KAPP’s membrane association in Arabidopsis remains unclear.

The interactions between KAPP and RLKs are mediated by KID. The KAPP KID domain, which contains 239 residues (aa 98-336), interacts with the RLK catalytic domain in a phosphorylation-dependent manner (Stone et al., 1994; Braun et al., 1997). Subsequent serial deletion and in vitro binding assays demonstrated that the minimal functional phosphoprotein binding unit of KAPP consists of 119 residues that span amino acids 180 to 298 (Li et al., 1999). In the KID domain, there is a 52-residue region (aa 208-259) that shares homology with the FHA domain (Hofmann and Bucher, 1995). Site-directed mutagenesis of four highly conserved residues (G211, S226, H229, N250) within the KAPP FHA-homology region and in vitro binding assays demonstrated that the 52 amino acid core region (208-259) is essential, but not sufficient for, its interaction with phosphorylated RLKs (Li et al., 1999).

Originally, the FHA domain was identified in a group of forkhead transcription factors. Subsequent research, however, showed that this domain exists in a wide variety of proteins from prokaryotes to eukaryotes (Hofmann and Bucher, 1995; Li et al., 2000). Supporting evidence that the FHA domain is a phosphoprotein-binding module comes from studies of the Rad53p in yeast (Sun et al., 1998). Rad53p is a protein kinase that is involved in DNA-damage responses and cell-cycle arrest in Saccharomyces cerevisiae. Rad53p has two FHA domains (a N-terminal FHA1 and a C-terminal FHA2 domain) that flank a central Ser/Thr kinase domain. Upon sensing DNA damage or inhibition of DNA replication, a kinase cascade is activated, and Rad9p is phosphorylated by Mec1p. The cell cycle is arrested upon recognition of phosphorylated Rad9p by Rad53p through the FHA2 domain. A mutation of the FHA2 domain can bypass the DNA damage that is induced by G2/M cell-cycle arrest, suggesting the biological function significance of this domain.

The C-terminus of KAPP is a PP2C domain. In vitro protein phosphatase assays showed that the activity of KAPP is consistent with its classification as a PP2C; Mg2+/Mn2+ is required for its activity and it is insensitive to high concentrations of okadaic acid, which is a specific inhibitor of PP1 and PP2A phosphatases (Stone et al., 1994).

Protein phosphorylation and dephosphorylation play essential roles in regulating signal perception and transduction. In RLK-mediated signaling pathways, the regulatory mechanism of phosphorylation and dephosphorylation are not completely understood. KAPP was the first and the only phosphatase to be shown to physically interact with multiple RLKs (Stone et al., 1994; Braun et al., 1997; Stone et al., 1998; Trotochaud et al., 1999; Gomez-Gomez et al., 2001; Shah et al., 2002; Rienties et al., 2005). It has been proposed that KAPP serves as a positive regulator of RLK signaling pathways, just like the Drosophila SRC homology 2 (SH2) domain protein tyrosine phosphatase (PTPase), corkscrew. Corkscrew has been shown to interact with multiple membrane-associated receptor tyrosine kinases (RTKs) and to transduce positively RTKs' signals to the intracellular components (Perkins et al., 1996; Roberts, 1996). Alternatively, KAPP may serve as a negative regulator of RLKs’ signaling pathway, to dephosphorylate and desensitize the ligand-activated RLKs (Roberts, 1996).

However, recent genetic and biochemical studies suggest that KAPP is a functional protein phosphatase and serves as a negative regulator in RLK signaling pathways. Two lines of evidence supporting this proposal come from the studies of CLV1 and FLS2. CLV1 is a LRR-RLK (Clark et al., 1997). Its primary function is to promote the shoot apical meristem to differentiate and to inhibit its proliferation by feedback down-regulation of WUS expression (Schoof et al., 2000; Clark, 2001). The clv1 mutants have abnormally enlarged meristems because of an excessive proliferation of meristem tissue. To address the possible regulatory role of KAPP in a CLV1-signaling pathway, KAPP was shown to interact with CLV1 and to dephosphorylate CLV1 in vitro. Transgenic analysis showed that KAPP overexpression lines have a similar phenotype to clv1, and KAPP co-suppression lines can suppress the clv1 weak-allele phenotype (Williams et al., 1997; Stone et
al., 1998). Gel filtration experiments suggested that KAPP and Rho GTPase coexist in an active 450 kDa CLV1 complex; in the kinase inactive mutant (clv1-10), this complex can not be assembled (Trotchaud et al., 1999). Additional evidence that supports the proposal that KAPP is a negative regulator of RLK signaling comes from studies of FLS2. As noted earlier, FLS2 is a LRR-RLK involved in the binding and recognition of the bacterial elicitor flagellin (fig22) (Gomez-Gomez and Boller, 2000). Yeast two-hybrid experiments showed that KAPP physically interacts with FLS2. In Arabidopsis, when KAPP is overexpressed, the plants show reduced binding to fig22 and become insensitive to flagellin treatment (Gomez-Gomez et al., 2001).

KAPP is a single-copy gene in Arabidopsis (Stone et al., 1994). No homologs of KAPP have been identified in the Arabidopsis genome (Kerk et al., 2002). However, KAPP orthologs have been identified in maize and rice; they also show binding to several RLKs (Braun et al., 1997; van der Knaap et al., 1999). Future research on the in vivo function of KAPP will be required to understand the role of this protein phosphatase.

**POLTERGEIST.** poltergeist (pol) was characterized as a suppressor of clv1 because it can suppress the massive accumulation of meristematic stem cells in CLV1 mutant backgrounds. POL and its homologs (PLLs, POL-like) encode a novel subtype of PP2C, which is distinguished by the insertion of 200 unique amino acids between subdomain III and IV in the PP2C catalytic domain (Yu et al., 2003). In vitro biochemical analysis demonstrated that POL is a functional PP2C protein phosphatase and that its N-terminus may function as a negative regulatory domain for the C-terminus catalytic domain (Yu et al., 2003). Its predicted nuclear localization signal, indicates that POL may function downstream of the CLV1 signaling cascade.

**Protein Tyrosine Phosphatases**

Protein tyrosine phosphatases (PTPs) super-family can be classified into tyrosine-specific PTPs that act on phosphotyrosine and dual-specificity protein tyrosine phosphatase (DsPTP), which can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine. The lack of sequence homology between PTPs and protein Ser/Thr phosphatases and the unique 3-D structure of PTPs' catalytic domains indicate that PTPs evolved independently (Fauman and Saper, 1996). However, the highly conserved structure of the catalytic domain within the PTP super-family suggests a common phosphate hydrolysis mechanism (Fauman and Saper, 1996). All of the members of the PTP super-family carry the signature motif of CX2R in their active site and the cysteine is required for PTP catalytic activity (Fauman and Saper, 1996).

There are 18 PTPs in the Arabidopsis genome (Kerk et al., 2002). The function of PTPs in plants was not known until AtPTP1 was characterized in 1998 (Xu et al., 1998). AtPTP1 expression is strongly induced under high-salt stress conditions. The catalytic domain of AtPTP1 shares high homology with mammalian tyrosine phosphatases. In vitro enzyme assays showed that AtPTP1 has the CX2R signature motif essential for the tyrosine-specific phosphatases activity (Xu et al., 1998). The first DsPTP (AtDsPTP1) was identified the same year (Gupta et al., 1998). In animals, substrates of tyrosine phosphatases come from phosphorylation product of tyrosine kinase and MAPKK. In Arabidopsis, there are no tyrosine protein kinases that can be identified based on the genome sequence, which suggests that the major function of plant PTPs is to counteract MAPK activity (Luan, 2003). Consistent with this, AtDsPTP1 was demonstrated to be able to dephosphorylate and inactivate AtMPK4 (Gupta et al., 1998).

The first genetic inference of the function of PTP in Arabidopsis came from the genetic characterization of AtMKP1. mkp1 is hypersensitive to genotoxic stress, UV-C and methyl methanesulphonate, (Ulm et al., 2001). Further research demonstrated that mkp1 is salt tolerant. A yeast two-hybrid screen found that MKP1 interacts with MPK3, MPK4, and MPK6, which are key signaling components in a diverse set of stress and environmental signaling responses. These data indicate MKP1 may regulate plant response to salinity and genotoxic stresses through the regulation of MAPK activities (Ulm et al., 2002).

Recently, another MKP-like gene Propyzamide-hypersensitive1 (PHS1) was cloned (Naoi and Hashimoto, 2004). The original phs1-1 mutant, which carries a point mutation of Arg to Cys in the non-catalytic N-terminal, is semidominant. The phs1-1 mutant has cortical microtubule organization defects, whereas the phs1-2 null allele is recessive embryo lethal. In animals, the corresponding Arg is critical for the interaction between MKP and MAPK substrates, and mutation of the highly conserved Arg disrupts this interaction (Tanoue et al., 2002). By analogy, the Arg-Cys mutation in phs1-1 may inhibit the kinase-phosphatase

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A similar regulation mechanism has been observed in reversibly (Meng et al., 2002; Xu et al., 2002; Tonks, 2005). This is a well-designed mechanism to regulate PTP activity, as cytokines and growth factors can stimulate transient and rapid production of ROS, which is correlated with the activation of MAPK6 by H$_2$O$_2$, suggesting that AtPTP could be a major target for oxidative stress in plants (Gupta and Luan, 2003).

As a second messenger, Ca$^{2+}$ functions in a diverse array of signaling transduction pathways in plants. As a Ca$^{2+}$ sensor, calmodulin may regulate a wide range of downstream proteins that help to translate differential Ca$^{2+}$ inputs into specific physiological responses (Ng and McAinsh, 2003). It has been demonstrated that AtPTP1 interacts with CaM; depending on the substrates of AtPTP1, CaM binding can either increase or decrease the dephosphorylation activity of AtPTP1 (Yoo et al., 2004). NtMKP1 (the AtMKP1 analog in tobacco) also is regulated by CaM binding, indicating that CaM regulation of PTP activity could be a common regulatory mechanism of plant protein phosphatase activity (Yamakawa et al., 2004).

**CONCLUSIONS**

The Arabidopsis genome is rich with genes that encode in protein kinases and protein phosphatases. There has been an impressive advance in our understanding of the functional roles of these enzymes over the past few years. These studies reinforce the idea that protein kinases and phosphatases are major regulators of cellular and developmental processes. However, much of the work has focused on trying to understand the role of a single gene. We are just beginning to elucidate the complex networks involving protein kinase and phosphatases in cellular function. Many more questions remain to be addressed. While convergence and divergence of functions on the different levels of protein kinase cascades has been proposed, how exactly is signaling specificity maintained in each pathway? What are the spatiotemporal dynamics of the active components of in a protein phosphorylation cascade? What are the substrates and interacting regulators?

A combination of approaches will required to address these questions. Future research should go beyond the loss-of-function approaches such as T-DNA insertion mutant screening (Alonso et al., 2003), EMS mutagenized TILLING mutant screening (McCallum et al., 2000a, 2000b), and RNAi gene silencing (Wesley et al., 2001). The approaches of systems biology (e.g., whole-genome expression profiling by microarray, yeast two-hybrid screening, protein microarray proteomics, and phosphoproteomic techniques), combined with the genetics, should aid in the identification of interacting factors and substrates. For example, about 48 potential substrates for MPK3 and MPK6 have been recently identified by a protein microarray-based proteomics approach (Feilner et al., 2005). Phosphoproteomic approaches have also yielded tremendous information about cellular phosphorylation events (Nuhse et al., 2003, 2004; Peck, 2006). Newly developed approaches that allow comparison of multiple parallel samples will contribute to our ability to quantitatively and comparatively track cellular phosphorylation events (Shadforth et al., 2005). Future work will need to
focus how these proteins are integrated into regulatory networks and how the dynamics of these networks influence plant development and responses to the environment.

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