ABP1 and ROP Mediated Auxin Signaling in Arabidopsis

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ABSTRACT OF THE DISSERTATION

ABP1 and ROP GTPases Mediated Auxin Signaling in Arabidopsis

by

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Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
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Dr. Zhenbiao Yang, Chairperson

Auxin is a multi-functional hormone essential for plant development and pattern formation. A nuclear auxin perception and signaling system controlling auxin-induced gene expression has been established. However this nuclear-based auxin signaling cannot explain all the actions of auxin in plant cells. In this dissertation, we have demonstrated a cytoplasmic auxin signaling mechanism that can regulate cell morphogenesis through ROP GTPase signaling.

In the first chapter, we use leaf pavement cells (PCs) as a model system to investigate how auxin modulate the interdigitation growth between adjacent cells. PCs develop interdigitated lobes and indentations to form a puzzle-piece shape and provide a useful system to investigate the spatial coordination of cell expansion among cells in a two-dimensional plane. PC interdigitation is compromised in leaves deficient in either auxin biosynthesis or its export by the auxin transport facilitator
PINFORMED 1 (PIN1) which is localized at the lobe tip. Auxin coordinately activates two Rho GTPases, ROP2 and ROP6, which promotes the formation of complementary lobes and indentations across cells, respectively. Activation of both ROP2 and ROP6 by auxin occurs within 30 seconds and depends on AUXIN-BINDING PROTEIN 1 (ABP1). These findings reveal ROP-based novel auxin signaling mechanisms, which modulate the spatial coordination of cell expansion across a field of cells.

However, the mechanism about how auxin activates ROPs is still unknown since ABP1 localized to the outer surface of plasma membrane and cannot directly bind with ROPs. In the second chapter, we reported a family of receptor like kinases (RLK) called TMK that could bind with ABP1 on plasma membrane and activate ROP GTPase in pavement cells. tmk mutant showed similar phenotype and auxin insensitivity as in abp1 mutant. And both ROP2 signaling and ROP6 signaling are not activated in tmk mutant. ABP1 and TMK1 are confirmed in a same complex on PM by co-immunoprecipitation. In the meanwhile, TMK kinase domain can directly bind with N terminal of SPK1, which is a putative ROPGEF that can activate ROP2.

Therefore, we concluded that TMK and ABP1 are forming an auxin receptor complex on PM that mediate auxin activation of ROP signaling pathway.
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Introduction
1.1 Abstract

Auxin is considered as a key phytohormone that participates in nearly every process of plant development. Recently a TIR1-based nuclear auxin perception and signaling system controlling auxin-induced gene expression has been well established. PIN mediated auxin polar transport maintains auxin gradients during plant development. This gradient provides certain threshold concentrations of auxin at specific localizations that induces specific gene expression pattern through the TIR1 pathway, which further determines the cell fate (Mockaitis and Estelle, 2008). ABP1, considered as another auxin receptor candidate, participates in many auxin actions, but the role of ABP1 as auxin receptor has been in debate because its downstream pathway is unknown to date (Badescu and Napier, 2006). Rho GTPase is conserved as a molecular switch of signal transduction in both animal and plant kingdoms. Plant has a single subfamily of Rho GTPases, named ROP (Rho-like GTPases from plants), which regulates many aspects of cellular processes including cytoskeleton organization, vesicular trafficking, and polarity establishment (Yang, 2008). Two classes of downstream effectors of ROP have been indentified in plants including RICs and ICRs/RIPs, some of which function in the regulation of cytoskeleton and vesicular trafficking (Yang and Fu, 2007). It is not surprising that some conserved regulators of ROPs are also found in plants, such as DHR2-type GEFs, RhoGAPs, and RhoGDIs. However plant contains a specific group of PRONE-type RhoGEFs, which might indicate the different regulation mechanism in plants. RhoGAPs and RhoGDIs function as negative regulators that provide spatial regulation of ROP activity in plant cells (Yang and Fu, 2007).
1.2 Auxin signaling in Arabidopsis.

1.2.1 Auxin acts as a key hormone that regulates many developmental aspects in plants.

The influence of auxin in plant is first demonstrated by Charles and Francis Darwin in early 1887, when they found that after perceiving light at one side of plant, an “influence” could be transported to another side that causes the plant bend towards the light, which is termed phototropism later on. Then in 1926, the chemicals were identified from the agar block, to which the “influence” activity from the grafted plant tissue was transported. These chemicals were further termed to be auxin, which has been implicated in regulating most of the quantitative growth changes in plants. The molecular mechanisms for auxin regulation in plant growth is becoming clear after more than a century of research.

Different from animals that can move freely, plants use self-regulation strategies to survive in the complicated ever-changing environment. Auxin participates in most of these self-regulation processes including phototropism and gravitropism. Light regulates asymmetric auxin distribution by controlling auxin transport and further promotes the orientation of shoot growth to the light source that optimize plant’s survival (Blancaflor and Masson, 2003). Similarly in gravitropism, roots bend in response to gravity due to a regulated movement of auxin by polar transport (Wisniewska et al., 2006). Both gravitropism and phototropism are corresponding to auxin-regulated cell expansion that is depending on auxin concentrations. Alterations in auxin distribution lead to the differential growth patterns among cells, which further determine the organ shape or growth orientation that affect the whole plant.
Auxin is also required for embryogenesis, meristem maintenance and organ initiation. Different from auxin-regulated cell expansion in differentiated cells, auxin is essential for the determination of cell fate and cell division pattern during these processes as early as the first zygotic division (Weijers and Jurgens, 2005; Weijers et al., 2005b). During embryogenesis, auxin distribution pattern that is regulated by polar auxin transport, determines the fate of the initial cell for both shoot or root meristems (Weijers and Jurgens, 2005; Weijers et al., 2005b)(Friml et al., 2003; Weijers et al., 2006). In root development, an auxin transport loop generates a stable auxin distribution pattern that is necessary and sufficient for maintaining the continuous root growth by regulating different cellular responses in the different zones that contain different concentrations of auxin (Benjamins and Scheres, 2008; Blilou et al., 2005; Grieneisen et al., 2007). Auxin is also found to promote lateral organ formation such as lateral root initiation, leaf primodium formation, and floral primodium formation (Heisler et al., 2005)(Benjamins and Scheres, 2008; Benkova et al., 2003; Casimiro et al., 2001; Friml et al., 2002). All these functions illustrate the key role of auxin in regulating cell differentiation, and cell proliferation during organ initiation and development.

The complicated but precisely regulated auxin activity in plants indicates an elegant molecular basis for these processes. Recent research progress provides the foundation of our understanding in auxin actions, and begins to address how auxin regulates diverse developmental processes. The most significant advances that have been made
over the past few years are from the identification of TIR1-based auxin receptor system in the nucleus (Mockaitis and Estelle, 2008), and PIN-based auxin polar transport in plant tissues (Kleine-Vehn and Friml, 2008).

1.2.2 TIR1/AFB mediated auxin-signaling pathway

After a few decades of research, valuable information is accumulated to explain the nature of auxin actions in plants. The first important finding is the rapid effects of auxin on altering gene expression that normally happens within minutes (Goda et al., 2004; Mockaitis and Estelle, 2008; Nemhauser et al., 2006; Overvoorde et al., 2005). Aux/IAA family proteins were first found to be encoded by mRNAs, which increased many folds in response to exogenously applied auxin (Abel and Theologis, 1996; Mockaitis and Estelle, 2008). And ARFs (auxin response factors) family proteins are identified as transcriptional factors that can bind with auxin-responsive promoters (Abel and Theologis, 1996; Casimiro et al., 2001; Guilfoyle and Hagen, 2007; Ulmasov et al., 1999a). The Arabidopsis genome contains 29 members of Aux/IAA genes, and 23 members of ARFs (Guilfoyle and Hagen, 2007). These two groups of well-characterized genes mediate the complicated framework of auxin-regulated responses. The genes that are up-regulated or down-regulated are finally responsible for the physiological effects on plant growth (Guilfoyle and Hagen, 2007; Rouse et al., 1998; Ulmasov et al., 1999a).

Loss-of-function analysis of Aux/IAA proteins demonstrated the compensatory functions among the family members although they have high variation in amino acid identity (Dreher et al., 2006)(Abel and Theologis, 1996; Nemhauser et al., 2006; Reed, 2001; Worley et al., 2000). The functions of each individual Aux/IAA that
ARFs contain a conserved N-terminal DNA binding domain demonstrates its role as transcriptional factors (Ulmasov et al., 1999a). More interestingly, both Aux/IAA and ARF proteins contain conserved domain III and IV at C-terminus. These domains mediate the formation of homo- or heterodimer between Aux/IAA and ARF proteins (Kim et al., 1997; Ulmasov et al., 1999b; Weijers et al., 2005a). Further study showed that the interaction between Aux/IAA and ARF inhibits the transcriptional regulation activity of ARFs (Ulmasov et al., 1999b; Zenser et al., 2001). Loss of function of ARF5 in *mp* (*monopteros*) mutant showed the complete inhibition of root formation (Schlereth et al., 2010). A similar phenotype was found in IAA12 gain-of-function *bdl* (*bodenlos*) mutant, which is due to a point mutation in the GWPPV motif of the conserved domain II that causes the stabilization of IAA proteins (Hamann et al., 2002; Hamann et al., 1999). These provide a possible mechanism about how Aux/IAA and ARF together regulate gene expression (Weijers et al., 2005a). Aux/IAA proteins functioned as negative regulators of ARFs by the direct binding with ARFs, while ARFs are transcription factors that directly control gene expression. The point mutation in domain II of IAA proteins that enhanced their stabilization indicated this domain might regulate the degradation of IAA proteins (Ramos et al., 2001). A series of auxin-resistant mutants were also indentified and characterized that indicated the ubiquitin-mediated protein degradation pathway is involved in auxin responses (Leyser et al., 1993)(Nemhauser et al., 2006; Woodward et al., 2007). Indeed, the core motif of domain II, termed as “degron”, was sufficient for the degradation of IAA proteins. The proteins fused with “degron” sequence become the
direct target of E3 ubiquitin ligase complex (Yang et al., 2004; Zenser et al., 2001) that lead to the ubiquitination of the proteins, which were further degraded by the 26S proteasome. TIR1 gene, indentified from auxin resistant mutant, encoded a F-box protein that is a part of ubiquitin ligase complex SCF^{TIR1} (Dharmasiri et al., 2005a). Aux/IAA proteins were further proved to bind with and act as the substrates of the SCF^{TIR1} complex since several Aux/IAA proteins are more stabilized in tir mutant (Dharmasiri et al., 2005b). It was significant breakthrough in plant biology that TIR1 was finally confirmed as an auxin receptor that mediates Aux/IAA protein degradation and further influences gene expression by releasing the ARF transcriptional factors (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). The structure of TIR1-auxin-Aux/IAA complex addressed the mechanism about how this receptor complex works (Tan et al., 2007). Auxin bound within a hydrophobic cavity of the TIR1 that were further closed up by the degron motif of IAAs. This binding does lead to any major conformational changes of the receptor complex, but provides more binding surface and enhances the affinity between TIR1 and Aux/IAA. Therefore, auxin was considered as molecular glue between TIR1 and Aux/IAAs until Aux/IAAs are ubiquitinated for degradation (Tan et al., 2007).

1.2.3 ABP1 mediated auxin-signaling pathway

This decent auxin perception mechanism in nucleus reveals the partial mystery of auxin function in plants, but not all. A serious of rapid cellular responses mediated by auxin, including the regulation of cytoskeleton organization and the ion transport, cannot be explained by TIR1-based nucleus auxin signaling pathway since the processes were too fast to be due to the transcriptional changes (Badescu and Napier,
2006). Auxin itself can also promote its own transport by regulating PIN localization, which did not require TIR1 receptor either (Paciorek et al., 2005). All these suggested that other auxin signaling pathways exist in plant. The function of the membrane and ER associated Auxin Binding Protein 1 (ABP1) provided a possible mechanism in regulating auxin cellular responses. ABP1 binds auxin with high affinity and specificity in different plant species. Loss-of-function of ABP1 caused the embryo lethal phenotype that was due to the severe defects in both cell division and cell expansion (Chen et al., 2001). On the other hand, over-expression of ABP1 promoted an auxin dependent cell expansion (Jones et al., 1998). Recent work further confirmed the role of ABP1 in auxin mediated cell division and cell expansion by using conditional repression of ABP1 (Braun et al., 2008). Unfortunately, no downstream components of ABP1 that mediated auxin responses were identified, which made the role of ABP1 as an auxin receptor remained a matter of debate (Badescu and Napier, 2006).

1.2.4 Auxin polar transport in plant

Auxin distribution pattern is generated by auxin transport in response to stimulus. Auxin efflux from inside to outside of cells by two groups of exporter proteins (PINs and PGPs); while auxin influx is majorly via diffusion but also mediated by importer proteins (AUX). PIN mediated polar transport is a subject of intense investigation for recent years.

The PIN family proteins, which contain 8 family members, mediate auxin efflux in plant. The asymmetric localization of PIN on plasma membrane in one cell is always
coincident with the auxin accumulation in the adjacent cell, which indicates PIN proteins might regulate the direction of auxin flow (Galweiler et al., 1998; Kleine-Vehn and Friml, 2008; Liu et al., 1993). The first characterized loss-of-function mutant was *pin formed 1* mutant, which was the protein family named after. The mutant formed a pin-like plant architecture, which totally lacked flowers, and was similar to the phenotype generated by auxin transport inhibitor (Galweiler et al., 1998; Liu et al., 1993; Okada et al., 1991). The reduced auxin transport activity was detected in this mutant, which further confirmed its role in auxin export (Galweiler et al., 1998). PIN1 was highly expressed in stele cells in different tissues, such as leaf vein or root stele. PIN1 preferentially localized to one side of these cells, such as the basal side in the root stele cells, which is consistent with the direction of auxin flow (Blilou et al., 2005).

Gravitropism response was defect in *pin2* mutant, and PIN2 localized to the upper side of epidermal cell, which indicates PIN2 mediate auxin transport from root tip to the elongation zone. Auxin promotes cell elongation in the elongation zone; therefore asymmetric auxin distribution causes asymmetric growth of cells, and further regulates the root curling after response to gravity (Bennett et al., 1996; Luschnig et al., 1998; Muller et al., 1998). More interestingly, PIN2 also localized to the lower side of cortex cells, and the inner side of the elongated cells in the elongation zone, which provide the hints that auxin can transport back again to the root tip via cortex (Benkova et al., 2003; Blilou et al., 2005; Friml et al., 2003).

The localization of PIN3, PIN4, and PIN7 proteins further confirmed the existence of auxin transport loop in root. PIN3 was detected in columella cells, and also at the basal side of the cortex cells and the inner side of pericycle cells, which is similar to
PIN2. PIN4 was essential for maintaining the auxin maximum in the quiescent centre, therefore it localized to the cells around the meristem. PIN7 also localized to the basal side of the contex cells, and in columella cells as PIN3. Together with PIN1 and PIN2, all these PINs generated an auxin transport loop in root cells (Blilou et al., 2005). The auxin was transported out from the meristem to the different zones along the root, and transported back to the meristem through the vascular cells. This auxin transport loop was sufficient for generating auxin distribution patterns along the root cells that is essential for root growth (Grieneisen et al., 2007).

PIN5, PIN6 and PIN8 belong to a unique subfamily of PINs in plant. They were surprisingly detected in ER rather than plasma-membrane where other PINs localized, and further functional analysis showed that PIN5 mediated auxin transport from the cytoplasm to the lumen of ER, which might regulate the auxin homeostasis by compartmentalization (Mravec et al., 2009). These findings support a possibility that PIN5-mediated auxin transport from cytoplasm to ER can reduce the auxin availability for PM based auxin efflux, and might also induce auxin signaling in ER since ABP1 also localized to ER (Mravec et al., 2009).

1.3 Rho GTPase signaling pathway in Arabidopsis.

1.3.1 Rho GTPase: molecular switch of signaling transduction in plant.

Rho GTPases are considered as molecular switches in signaling networks that control many cellular processes by regulating cytoskeletal organization and vesicular trafficking in eukaryotes. There are three subfamilies of Rho GTPase in animals named as Rho, CDC42 and Rac in animals and only CDC42, Rho two subfamilies in yeasts. Plants genome contains a specific family of Rho GTPase proteins named ROP
(Rho-like GTPases from plants) (Cheung et al., 2003; Kawasaki et al., 1999; Winge et al., 1997). There are 11 ROPs in *Arabidopsis*. Phylogenetic analysis of all Rho GTPases indicated that ROPs are evolved from the common ancestor of Rho, CDC42 and Rac proteins (Yang et al., 2004).

In plants, ROPs also participate in many signaling pathways that control cell polarization, polar growth, and cell morphogenesis by regulating cytoskeletal organization or vesicular trafficking (Fu et al., 2005; Fu et al., 2002; Fu et al., 2001; Fu et al., 2009; Gu et al., 2005; Hazak et al., 2010; Kawasaki et al., 2006; Lavy et al., 2007; Lee et al., 2008). Tip growth in pollen tubes and cell morphogenesis in leaf pavement cells provided two model systems for the functional analysis of RhoGTPase in plants (Lee and Yang, 2008; Yang, 2008) (Figure 1.1). Although the functions of plant Rho GTPases are high conserved as in animals or yeast, a group of plant-specific downstream effectors are found to modulate these fundamental cellular processes. Plants also have some specific up-stream regulators such as RopGEFs and AtRENs in spite of the conserved DHR2-type guanine nucleotide exchange factors (GEFs), RhoGAPs, and RhoGDIs.

1.3.2 ROP downstream effecters and signaling pathways

**RICs**

The first important family of ROP downstream effector proteins is identified by yeast-two-hybrid screening, named as *ROP INTERACTIVE CRIB MOTIF-CONTAINING PROTEINS (RICs)* (Wu et al., 2001). There are 11 RICs in Arabidopsis genome that contain a highly conserved CRIB (Cdc42/Rac-interactive binding) motif for the bind with ROPs. is commonly found in Cdc42/Rac effector proteins such as WASPs and
p21PAK, and is responsible for their interactions with the GTP-bound form of Cdc42 and Rac GTPases. The sequences of these 11 RICs showed high diversity except the CRIB motif, which might indicate the variety of their downstream signaling pathways (Gu et al., 2005; Hsu et al., 2010).

The tip-growing pollen tube provides an excellent system for investigating the mechanism of ROP regulated polar growth (Figure 1.1). ROP1, which localized to the apical plasma membrane (PM), was proved to regulate F-actin dynamics that are required for pollen tube growth (Fu et al., 2001; Lee et al., 2008). How a single tip localized ROP1 regulate F-actin dynamics became a focus of interest since F-actin dynamics that involved both the actin assembly and disassembly were supposed to be activated by two independent signaling pathways. The functional analysis of RICs in pollen tube provides the hints to explain this question. Over-expression (OX) of RIC3 and RIC4 both caused the depolarized pollen tube, however RIC3-OX caused the F-actin to disappear, whereas RIC4-OX made it stable (Gu et al., 2005; Wu et al., 2001). Co-overexpression of RIC3 and RIC4 generated the normal polarized pollen tube and regular F-actin dynamics (Gu et al., 2005). Further investigation showed that RIC4 promoted the assembly of F-actin when activated by ROP1, which was similar to its function in leaf pavement cells where RIC4 was activated by ROP2 to promote the formation of cortical F-actin in the lobe region (Fu et al., 2005). RIC3 in pollen tubes is proved to activate the disassembly of F-actin by increasing cytosolic Ca$^{2+}$ levels when activated by ROP1. Therefore, the RIC4 and RIC3 pathways, both activated by ROP1, coordinately control actin dynamics by activating assembly and disassembly of F-actin respectively at the tip of pollen tubes (Gu et al., 2005).
RIC1, another well-characterized RICs, has been shown to regulate microtubule organization in leaf pavement cells (Fu et al., 2005; Fu et al., 2009) (Figure 1.1). Leaf pavement cells (PCs) showed a specific jigsaw-puzzle shape that were formed by interdigitating growth of the lobes and indentations. The interdigitation growth of PCs was also proved to be regulated by two coordinated counteractive pathways. The ROP2-RIC4 pathway are activated at the lobe tips that promote the accumulation of F-actin while the ROP6-RIC1 pathway are activated at the indentation region to promote the formation of well-organized Microtubules there (Fu et al., 2005; Fu et al., 2009). RIC4’s function has been described above to promote the assembly of F-actin at lobe site that is essential for outgrowth. Over-expression of RIC1 led to the formation of the dense and paralleled cortical MTs that can inhibit outgrowth, whereas loss of function of RIC1 caused the randomized MTs (Fu et al., 2005). RIC1 was further proved to be associated with MTs that was dependent on the activation by ROP6 (Fu et al., 2009). Interestingly, the ROP2-RIC4 pathway and the ROP6-RIC1 pathway mutual inhibited each other (Fu et al., 2005). Therefore, the RIC4 and RIC1 pathway, which are activated by ROP2 and ROP6 respectively, can counteract with each other to promote the accumulation of F-actin for lobe formation and the well-organized MTs for indentation.

ICR/RIPs

Another family of ROP effector proteins, named as ICR1 (Interactor of Constitutively Active ROP1) or RIP1 (ROP-interactive Partner 1), was identified by yeast-two-hybrid screens for interacting proteins of active ROPs (Lavy et al., 2007; Li et al.,
Arabidopsis genome encodes five ICR/RIP family proteins. Analysis of truncated proteins indicated the C-terminus of ICR/RIPs contain the motif for ROP binding, which was different from CRIB-containing RICs (Lavy et al., 2007). Recent findings demonstrated that ICR1/RIP1 acts as a scaffold protein linking ROP GTPase to exocyst, which was similar to CDC42 in yeast. CDC42 directly interacts with SEC3 that contained a Rho GTPase binding motif to recruit the exocyst complex to the site where CDC42 was activated (Zhang et al., 2001). However, in plants and animals, SEC3 homologs did not contain any Rho GTPase binding motif. In mammalian cells, CDC42/RhoA took advantage of its downstream effector protein IQGAP1 to interact with Sec3/Sec8 (Sakurai-Yageta et al., 2008). Plants used similar strategy to link Rho GTPase to exocyst that ICR1/RIP1 bind with AtSEC3A, a subunit of the exocyst complex (Lavy et al., 2007; Zajac et al., 2005). Loss-of-function mutant of ICR1 (icr1) shows defects in plant morphogenesis, while leaf pavement cells became cubical caused by the defect of cell expansion. It was consistent with the essential role for the exocyst in cell expansion in plants (Hala et al., 2008; Synek et al., 2006). icr1 mutant also showed the disruption of its root meristem, a phenotype similar to polar auxin transport defective mutant (Lavy et al., 2007). Recent work showed that ICR1/RIP1 could regulate the polar localization of PIN to generate auxin maxima in roots and embryos (Hazak et al., 2010). Regulation of PIN polar localization was facilitated by both localized inhibition of PIN endocytosis and localized recycling (Dhonukshe et al., 2008). ICR1 was required for endocytic PIN recycling to the site where ICR1 was accumulated (Hazak et al., 2010). This is consistent with the function of ICR1 in tethering of endocytic recycling vesicles to the ICR1 site. Previous work also suggested that auxin inhibited PIN
endocytosis to promote its own efflux (Paciorek et al., 2005). Interestingly my dissertation research suggests that extracellular auxin activates lobe-localized ROP2, which in turn is required for PIN1 polarization to the lobes in pavement cells, suggesting that auxin activation of ROP2 and PIN1 polarization form a positive feedback loop. Therefore it is reasonable to speculate that auxin activation of ROP2 promotes PIN1 polarization by coordinating two downstream pathways: an unknown pathway inhibits PIN1 endocytosis and the ICR1/RIP1 pathway promotes its recycling.

1.3.3 ROP Regulators: Providing Spatial Signals for ROPs

A key hallmark of Rho GTPase signaling is its spatial control of cellular processes. In this capacity, Rho GTPases are typically activated in local domains of the PM, such that they are capable of regulating the polarization of the cytoskeleton and of vesicular trafficking as discussed above for ROP effectors. Given the conservation of this spatial control in all eukaryotic cells, it is not surprising that some Rho regulators such as RhoGAPs (Rho GTPase activating proteins) and RhoGDIs (Rho guanine nucleotide dissociation inhibitors) are conserved for the spatial regulation of ROPs in plants. Investigation of these regulators in plant cellular systems may reveal paradigms for the spatial control of Rho GTPases (e.g., see REN1 below). Since the types of cues that determine spatial activation of ROPs are likely plant cell-specific, one might expect that unique ROP activators could have evolved for the perception and transmission of these cues. Indeed as discussed below a plant-specific class of RhoGEFs.
Animal and fungal RhoGEFs fall into two types: one containing a DBL homology (DH) domain and plestrin homology (PH) domain, and the other containing Dock homology region (DHR) (Meller et al., 2005). In mammals, each type of RhoGEFs is encoded by a large gene family. However plants lack DH-type RhoGEFs, which are often activated by receptor tyrosine kinase (RTK) or G protein coupled receptor (GPCR) signaling in mammalian systems (Fukuhara et al., 1999; Sah et al., 2000; Schiller, 2006; Schlessinger, 2000). This is consistent with the fact that RTK is missing in plants and that a single Ga in Arabidopsis has a minimal effect on plant growth and development (Yang, 2002). Furthermore, only a single DHR-type RhoGEF, named SPIKE1 (SPK1) is found in Arabidopsis (Qiu et al., 2002). Knocking out SPKI in Arabidopsis caused adult lethality and defects in the morphogenesis of leaf epidermal cells (Qiu et al., 2002). Some of these changes such as the loss of jigsaw-puzzle-piece shape in pavement cells are similar to those induced by down regulation of ROPs (Fu et al., 2005; Fu et al., 2002; Qiu et al., 2002). Recent work showed that SPIKE1 could bind to ROPs in a DHR2-dependent manner and act as a RhoGEF (Basu et al., 2008). SPIKE1 may also have the function not observed in animals, as it seems to act in the WAVE and ARP2/3 complexes to control the actin cytoskeleton (Basu et al., 2008). SPK1 does not affect other ROP-dependent processes such as tip growth in pollen tubes and root hairs, suggesting the existence of other types of RhoGEFs in plants.

Indeed a novel and plant-specific RhoGEF family, termed RopGEF, was identified (Berken et al., 2005; Gu et al., 2006). The RopGEF family contains 14 members in
Arabidopsis, which share a conserved PRONE (plant-specific Rop nucleotide exchanger) domain with all RopGEFs from other plant species (Berken et al., 2005; Gu et al., 2006). This PRONE domain functions as the GEF catalytic domain (Berken et al., 2005; Gu et al., 2006). Although they do not share amino acid sequence similarity with animal and fungal RhoGEFs, the crystal structure analysis shows that their 3-D structure resembles that of other RhoGEFs, and they function as a dimer like other RhoGEFs (Thomas et al., 2007). In vitro experiments suggest that the N- and C-termini of RhoGEF1 contain structural domains that interact with and inhibit the central PRONE domain, a regulatory feature that appears to be common among various RhoGEFs (Gu et al., 2006).

The cellular function of the RhoGEF family is largely uncharacterized. Overexpression of RopGEF1 or RopGEF12 induced depolarization of pollen tube growth, similar to that induced by ROP1 overexpression, suggesting that these RopGEFs may act as an activator of ROP1 in controlling polar growth in pollen tubes (Gu et al., 2006; Zhang and McCormick, 2007). Growth depolarization is similarly induced by the overexpression of the full-length RopGEF1 and the PRONE domain, from which the autoinhibitory domains were removed, implying the existence of cellular mechanism for releasing the autoinhibition (Gu et al., 2006). A possible mechanism for removing C-terminal autoinhibiton was also suggested for RhoGEF12 (Zhang and McCormick, 2007). Interestingly, a tomato RopGEF homolog was independently identified from a yeast two-hybrid screen for proteins that interact with the kinase domain of pollen-expressed receptor-like protein kinases, LePRK1 and LePRK2 (Kaothien et al., 2005; Zhang et al., 2008; Zhang and McCormick, 2007).
This led to testing a physical and functional interaction between the pollen-specific Arabidopsis RopGEF12 and PRK2a, a homolog of LePRK2 (Zhang and McCormick, 2007). Indeed the C-terminal region of RhoGEF12 interacts with the kinase domain of the Arabidopsis PRK2a. A series of overexpression experiments suggest that this interaction recruits RopGEF12 to the PM and releases the inhibition of the C-terminal region on the GEF activity of the PRONE domain (Zhang and McCormick, 2007). A subclass of RopGEFs, all of which are expressed in pollen, contains conserved phosphorylation sites at the C-terminal region, and S510D mutation within this region has an impact on the function of RopGEF12 in pollen tubes (Zhang and McCormick, 2007). Thus PRK2a may regulate RopGEF12 via PRK2a-mediated phosphorylation of the C-terminal region.

These observations suggest that plant-specific RopGEFs might have evolved to transmit plant-specific signals that are perceived by the RLK superfamily (>400 members in Arabidopsis), which is the predominant cell-surface receptor ser/thr kinases in plants, given lack of RTKs and scarcity of GPCRs (if any) in plants. Thus it is anticipated that RLKs could be a primary mechanism by which extracellular signals activate ROPs in plants. Another RLK candidate for ROP activation PAN1 has been reported in maize (Cartwright et al., 2009). PAN1 is required for the accumulation of a membrane-associated phosphoprotein, and also the accumulation of actin patches that is essential to polarize subsidiary mother cell (SMC) for asymmetric cell division (Cartwright et al., 2009). A recent study suggests that extracellular auxin activates both ROP2 and ROP6, respectively localized to the complementary lobing and indenting regions in leaf pavement cells (see Figure 1.2) (Xu et al., 2010). Auxin
activation of these two ROP signaling pathways requires auxin-binding protein 1 (ABP1), which apparently acts in the cell surface and does not contain a transmembrane domain (Figure 2). Presumably a cell surface protein with transmembrane domains such as an RLK could act with ABP1 as auxin co-receptor to transmit extracellular auxin signals to ROPs. ROP GTPase signaling in this system has been suggested to coordinate cell polarization and cell morphogenesis between adjacent cells (Xu et al., 2010). Having an RLK-type cell surface receptor to perceive a ROP activating signal from adjacent cells could be a common mechanism for cell-cell coordination for the spatial control of cellular processes in multicellular plant tissues consisting of walled cells. Therefore we predict that identification of RLKs that regulate RopGEFs will be a fertile field for future studies of ROP signaling in connection to developmental mechanisms in plants.

RhoGAPs and RhoGDIs

Two types of RhoGAPs, both of which contain conserved RhoGAP domain, are known in plants, and both of them have been implicated in the spatial control of ROP activity (Hwang et al., 2008; Klahre and Kost, 2006). One family of Arabidopsis RhoGAPs with six members, termed RopGAPs, was initially identified in a yeast two-hybrid system for proteins interacting with constitutive active ROP1 (G15V) (Wu et al., 2000). These RopGAPs contain a conserved GAP-like domain and a N-terminal CRIB motif that enhances their affinity for the GTP-bound form of ROP and the GAP activity (Wu et al., 2000). In tobacco pollen tubes, NtRhoGAP1 overexpression inhibited pollen tube elongation and caused narrower tubes, and GFP-tagged NtRhoGAP1 was localized preferentially in the shoulder of the tube apex (Klahre and
Kost, 2006). These observations led to the proposal that RopGAPs restrict ROP signaling to the apex of pollen tubes by lateral inhibition (Klahre and Kost, 2006), as shown for several RhoGAPs in animal systems (Jenkins et al., 2006; Knaus et al., 2007; Wells et al., 2006). However, loss of RopGAP function in Arabidopsis did not induce tip swelling, as it would be expected if RopGAPs were important in regulating ROP polarization (Hwang et al., 2010).

Another type of RhoGAPs with three members in Arabidopsis was identified from genetic screen for ren (ROP enhancer) mutations that enhance pollen tube tip swelling induced by ROP1 overexpression in Arabidopsis. REN1 contains an N-terminal PH domain and a RhoGAP domain, and two coiled-coil (CC) domains at the C-terminus (Figure 7). This novel RhoGAP doesn’t contain CRIB domain and is distinct from RhoGAPs in its cellular function (Hwang et al., 2008). Knocking out REN1 causes swollen pollen tubes that are reminiscent of those induced by the constitutively active form of ROP1. The ren1 phenotype together with biochemical data indicates that REN1 acts as RhoGAP to negatively regulate tip-localized ROP1. REN1 is localized to exocytic vesicles in the pollen-tube apex as well as the apical PM region where ROP1 is activated. These results suggest that REN1 act as a global inhibitor to spatially restrict ROP1 activity to the apical PM (Hwang et al., 2008). Analysis of spatiotemporal dynamics of ROP1 activity in tobacco pollen tube tips also supports the existence of a global inhibitor in the restriction of ROP1 activity to the pollen tube apex (Hwang et al., 2010). These findings reveal a new paradigm for the spatial regulation of Rho GTPase activity by RhoGAPs, which is distinct from the lateral inhibition known for several RhoGAPs found in animal systems (Jenkins et al.,
It was proposed that the global mode of action for the REN RhoGAP facilitates the self-regulation of Rho GTPases such as that found in pollen tube tips (Hwang et al., 2005; Hwang et al., 2010). Indeed, it was shown that the apical PM localization of REN1 oscillates behind ROP1 activation and ahead of ROP1 deactivation (Hwang et al., 2008). Furthermore, REN1 function is dependent upon its targeting to the apical PM through its association with the exocytic vesicles, implying the presence of a negative feedback mechanism, in which ROP1-mediated polar exocytosis is important for REN1-mediated down-regulation of ROP1 (Hwang et al., 2008) (Figure 2). Thus the REN1 RhoGAP action appears to couple the temporal regulation with the spatial regulation of ROP activity, generating a self-organizing system to control oscillating polar growth. Such a self-organization design principle might also work in other highly efficient Rho GTPase-mediated cellular processes such as fungal hyphal growth, neuronal growth, and cell movement.

RhoGDIs are also conserved and play an important role in the spatial regulation of ROP GTPase in plants (Hwang et al., 2010; Klahre and Kost, 2006). The Arabidopsis genome encodes three RhoGDI highly similar to mammalian RhoGDIs (Bischoff et al., 2000). Loss-of-function of SCN1/AtRhoGDI1 (scn1) causes a phenotype in root hairs similar to that induced by ROP2 overexpression, since it leads to the mislocalization of ROP2 during root hair initiation and growth (Carol et al., 2005; Jones et al., 2002; Molendijk et al., 2001). In wild type, ROP2::YFP localization is restricted to the PM at root hair growth sites or the apical PM of growing hairs;
however in $scn1$ mutant, it accumulates at the cell surface in ectopic locations where numerous root hairs are initiated and remains at the cell surface in growing root hairs (Carol et al., 2005). RhoGDI2a RNAi ($gdi2a$-RNAi) expression caused the depolarization of pollen tubes that is also observed in GFP-ROP1 overexpression lines. The localization of ROPs in pollen tubes is depolarized in $gdi2a$-RNAi mutant, which is similar to mislocalization of ROP2 in $scn1$ mutant root hairs (Hwang et al., 2010). In tobacco pollen tubes, transient expression of NtRhoGDI2 inhibited pollen tube growth while NtRac5 caused depolarization in pollen tube (Klahre et al., 2006). However, co-expression of both proteins produced normal pollen tube, which indicates that NtRhoGDI2 and NtRac5 act together to control the polar growth of pollen tubes. NtRhoGDI2 was localized to the cytoplasm, and co-expression of NtRhoGDI2 and YFP:NtRac5 inhibits the accumulation of NtRac5 at PM and translocates NtRac5 to the cytoplasm. A point mutation in NtRac5 (R69A) that abolished the specific binding between NtRhoGDI2 and NtRac5 caused the mislocalization of NtRac5 to the flanks of pollen tube apex and enhanced the ability of NtRac5 to depolarize pollen tube when over-expressed (Klahre et al., 2006). These findings demonstrate a role of RhoGDI in control of polar localization of Rho GTPases by sequestering them in the cytosol.
1.4 Objectives:

Rho GTPases and their downstream pathways are illustrated to regulate the cell morphogenesis in both pollen tube and leaf pavement cells. However the original developmental signal that can initiate these processes is still unknown. My dissertation research takes advantage of leaf pavement cells system to figure out the upstream signaling mechanism that can activate ROP signaling.

1. The discovery of tip-to-margin-to-center growth gradient of cotyledon pavement cells indicate the auxin might be the signal that initiate the pavement cell morphogenesis since the distribution pattern of auxin also shows a tip-to-margin-to-center gradient. The first part of my work is to illustrate the role of auxin in regulating pavement morphogenesis, and whether auxin is the signal that can activate ROP2-RIC4 and ROP6-RIC1 pathways that promote lobe protrusion and indentation respectively. We also want to indentify the auxin receptor that mediates this process.

2. ABP1 is considered as an auxin receptor candidate that mediates auxin activation of ROP signaling on plasma membrane. However, ABP1 is not a trans-membrane protein and does not contain any functional domain that can transmit auxin signal to downstream pathway. The second part of my research is to indentify the co-partner proteins of ABP1 on plasma membrane and find out the molecular mechanism about how auxin signal is transmitted to ROP pathways.
1.5 References


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1.6 Figures and Legends

Figure 1.1 ROP signaling pathways in pollen tube and pavement cells.
**Figure 1.1. ROP signaling pathways in pollen tube and pavement cells.**

**A.** ROP1 is locally activated at the apex of pollen tube PM. ROP1 activates the downstream effector RIC4 that promotes F-actin assembly. ROP1 also activates another effector RIC3 that promotes actin disassembly through regulating calcium concentration. These two pathways coordinately regulate actin dynamics that is essential for exocytosis in the apical region of growing pollen tubes. REN1, a RhoGAP in plants, is translocated to the apical region by exocytosis, suggesting REN1 involvement in the negative feedback regulation of the ROP1 activity.

**B.** In leaf pavement cells that contain interdigitated lobes and indentations, ROP2 is locally activated at the apex of lobes by auxin through ABP1, which leads to the activation of RIC4 at lobe sites that promotes accumulation of F-actin. Local active ROP2 also promotes PIN1 polar localization to apical PM of lobes. PIN1-exported auxin in turn activates ROP2 at the cell surface to initiate a positive feedback loop, which is essential for continuous outgrowth of lobe. PIN1-exported auxin at the lobe sites can diffuse across the cell wall to activate the ROP6 pathway at adjacent cell. ROP6 activates RIC1 that binds MTs and regulates MT organization to restrict growth in this region.
Chapter 1.

ABP1 and ROP GTPase-dependent auxin signaling modulates cellular interdigitation in Arabidopsis
2.1 Abstract

Auxin is a multi-functional hormone essential for plant development and pattern formation. A nuclear auxin perception and signaling system controlling auxin-induced gene expression has been established (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Mockaitis and Estelle, 2008). We have demonstrated a cytoplasmic auxin signaling mechanism that modulates the interdigitating growth of Arabidopsis leaf epidermal pavement cells (PCs). PCs develop interdigitated lobes and indentations to form a puzzle-piece shape and provide a useful system to investigate the spatial coordination of cell expansion among cells in a two-dimensional plane. PC interdigitation is compromised in leaves deficient in either auxin biosynthesis or its export by the auxin transport facilitator PINFORMED 1 (PIN1) which is localized at the lobe tip. Auxin coordinate ally activates two Rho GTPases, ROP2 and ROP6, which promotes the formation of complementary lobes and indentations across cells, respectively. Activation of both ROP2 and ROP6 by auxin occurs within 30 seconds and depends on AUXIN-BINDING PROTEIN 1 (ABP1). These findings reveal ROP-based novel auxin signaling mechanisms, which modulate the spatial coordination of cell expansion across a field of cells.
2.2 Introduction

Auxin is a multifunctional plant hormone regulating various aspects of plant growth and development. Recent elegant studies have established a simple auxin perception and signaling mechanism controlling auxin induced gene expression (Leyser, 2006; Parry and Estelle, 2006), which involves auxin stabilizing the interaction between the TIR1-family F-box proteins and the IAA/AUX proteins, transcriptional repressors (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Mockaitis and Estelle, 2008; Tan et al., 2007). This activates the IAA/AUX degradation pathway, releasing auxin-induced gene expression (Leyser, 2006; Parry and Estelle, 2006). However, this pathway cannot account for auxin-induced rapid cellular responses such as cell expansion, cytosolic Ca$^{2+}$ increase, and proton secretion, which occur within minutes (Badescu and Napier, 2006; Senn and Goldsmith, 1988; Shishova and Lindberg, 2004; Vanneste and Friml, 2009). AUXIN BINDING PROTEIN1 (ABP1) has been proposed to be an auxin receptor that rapidly activates cell expansion (Badescu and Napier, 2006; Chen et al., 2001a; Chen et al., 2001b; Jones, 1994). ABP1 knockout cause lethality of early embryos due to their failure to polarize (Chen et al., 2001b). Auxin has also been implicated in the regulation of cell polarization including polar distribution of the auxin efflux carrier PIN proteins to the plasma membrane (PM) and determination of root hair initiation sites in the root epidermal cells (Dhonukshe et al., 2008; Fischer et al., 2006; Ikeda et al., 2009; Jones et al., 2009; Paciorek et al., 2005). However, signaling events downstream of ABP1 and those underlying auxin to
control cell polarization are unknown.

Coordinate spatial control of cell expansion or asymmetry across an entire field of cells in a tissue or organ is important for pattern formation and morphogenesis. In animals, this type of spatial coordination is required for cellular intercalation that drives convergent extensions fundamental to many developmental processes, including early embryogenesis (Green and Davidson, 2007; Heasman, 2006; Wallingford et al., 2002). In plants, PINFORMED (PIN) auxin export facilitators are located in the PM either basally, apically, or laterally of a specific cell type to generate directional flow of auxin through a tissue (Petrasek et al., 2006; Vieten et al., 2007; Wisniewska et al., 2006). This directional auxin transport is essential for tissue pattern formation such as in roots (Blilou et al., 2005; Grieneisen et al., 2007). Spatial coordination among epidermal cells is important for patterning of the epidermal tissues such as the positioning of root hairs in the basal end of the root trichobast and the jigsaw puzzle appearance of pavement cells (PCs) in the leaf (Fischer et al., 2006; Fu et al., 2005; Fu et al., 2002; Ikeda et al., 2009; Jones et al., 2009). The molecular mechanisms underlying the spatial coordination in these systems are poorly understood.

We used Arabidopsis leaf epidermal PCs as a model system to investigate the mechanisms for the cell-cell coordination of interdigitated cell expansion (Fu et al., 2005; Fu et al., 2002; Settleman, 2005; Yang, 2008). PCs have a “jigsaw-puzzle” appearance resulting from intercalary growth that produces interdigitated lobes and
indentations (Figure 1A). This cellular interdigitation resembles cell intercalation required for convergent extension during early embryogenesis in animal cells. Interestingly, embryonic cell intercalation and PC interdigitation share common mechanisms, including Rho GTPase signaling and its effect on the cytoskeleton (Fu et al., 2005; Settleman, 2005; Yang, 2008). ROP2 and ROP4, two functionally-overlapping members of the Rho GTPase family in Arabidopsis, promote lobe development (Fu et al., 2005; Fu et al., 2002). ROP2 is activated locally at the lobe-forming site and consequently activates its effector RIC4, which in turn results in the formation of cortical diffuse F-actin required for lobe outgrowth (Fu et al., 2005). In the nascent outgrowing lobe tips, ROP2 suppresses well-ordered cortical microtubule (MT) arrays by inactivating another effector, RIC1 (Fu et al., 2005; Fu et al., 2002), thus relieving MT-mediated outgrowth inhibition. In the opposing indenting zone, ROP6 activates RIC1 leading to formation of well-ordered MT arrays and the inhibition of ROP2 activation (Fu et al., 2005; Fu et al., 2009). What activates the ROP2 and ROP6 pathways and how these two pathways coordinate across cells to produce the cellular interdigitation remains unknown.

In this chapter, we demonstrate that auxin promotes interdigitated PC expansion by coordinately activating the antagonistic ROP2 and ROP6 pathways in an ABP1-dependent manner and that ROP2 is required for the targeting of PIN1 to the lobing regions of the PM, which is crucial for the interdigitated PC expansion. These findings establish a new auxin signaling mechanism that is required for
cytoplasmic events including cytoskeletal organization, PIN protein targeting, and spatially coordinated cell expansion. Our data support a molecular framework underpinning cellular interdigitation that requires cell-cell coordination of lobe protrusion and indentation formation, which involves ABP1-dependent auxin perception, its activation of two opposing ROP signaling pathways, and the function of PIN1 auxin efflux facilitator.

2.3 Results

**Auxin is a developmental signal that activates PC interdigitation**

To investigate how the jigsaw-puzzle piece appearance of the intercalary PCs is developmentally controlled, we used expanding embryonic leaves (cotyledons), because their PCs change shape from near-square into jigsaw puzzle-piece shape in a predictable manner within 2-3 days after germination (DAG). Essentially all PCs of the cotyledon at 1-DAG lack interdigitating lobes and indentations (Figure 2.1A, left). Interdigitation first started at the tip of the cotyledon at 2 DAG, progressively spread to their margin (Figure 2.1A, middle), and finally reached the center and the base of the cotyledon at 3 DAG. Quantitative analysis indicated that the interdigitation initiation, which is measured by the lobe number per area (the lobe number of each cell divided by the cell area), displayed dynamic tip- and margin-high gradients: first tip-to-margin/base and then margin-to-center gradients (Figure 2.1A). The interdigitation gradient was also found at the single cell level for cells located near the
cotyledon base at 4 DAG. The interdigitation was first seen at the tip-margin sides of the cell and then the base-center sides (Figure 2.1B). These dynamic gradients of interdigitation imply the existence of some development signal(s), which spreads from the tip first to the margin and then to the center/base of the cotyledons to activate the formation of the jigsaw puzzle appearance.

Auxin is one of the major long- and short-range signals in plants and displays properties of a morphogen by forming concentration gradients in a tissue/organ to modulate developmental pattern formation. Auxin gradients are commonly monitored using a transcriptional fusion of the synthetic auxin responsive promoter, DR5, with a reporter gene such as the β-glucuronidase (GUS) gene. At 1 DAG, the DR5::GUS signal formed a tip-high gradient at the tip of cotyledons (Figure 2.1C), which was correlated with the developmental gradient of PC interdigitation observed at 2 DAG (Figure 2.1A, middle). At 2 DAG, the GUS signal formed a gradient along the margin of cotyledons (Figure 2.1D), again corresponding to the progression of interdigitation that occurred at 3 DAG (Figure 2.1A, right). Similar dynamic gradients of the DR5 promoter activity was found in a DR5rev::GFP line (data not shown). This is consistent with direct auxin measurements in tobacco leaves, which shows an auxin maximum at the tip of youngest leaves, moving basally as the leaf expands. Therefore, the dynamic formation of tip- and margin-high auxin gradients closely correlates with the progression of the activation of PC interdigitation from the cotyledon tip to the margin and then towards the center and the base. We
hypothesize that auxin is a development signal that initiates PC interdigitation.

**Auxin promotes and is required for PC interdigitation**

Given the widespread role of auxin in the regulation of pattern formation in plants, we sought to evaluate its involvement in the control of the interdigitated growth of PCs in Arabidopsis. We first examined the effect of exogenous auxin on the degree of PC interdigitation, which was measured by the number of lobes per cell area in a two-dimensional plane of the leaf surface (Figure 2.3A). A lobe is defined as an outgrowth of at least 1 µm in depth from the periphery of a PC (Figure 2.3A). Treatments of wild-type seedlings with the synthetic auxin naphthalene-1-acetic acid (NAA) significantly increased PC interdigitation in a dose-dependent manner with an effective NAA concentration as low as 5 nM and optimal concentration around 20 nM (Figure 2.2B and 2.2C; Figure 2.3C). We next determined the requirement of endogenous auxin for PC interdigitation by using mutants defective in YUCCA gene family-dependent auxin biosynthesis (Cheng et al., 2006; Zhao et al., 2001). The cotyledon PCs of the *yuc1 yuc2 yuc4 yuc6* quadruple mutant, which accumulates a lower amount of auxin than the wild type (Cheng et al., 2006), exhibited reduced interdigitation (Figure 2.2B and 2.2C). This *yuc1 yuc2 yuc4 yuc6* PC phenotype resembled that of the *ROP2RNAi rop4-1* line (Figure 2.2B and 2.2C), in which *ROP2* and *ROP4* expression is reduced (Fu et al., 2005). Interestingly, NAA treatment rescued the interdigitation defect of the *yuc1 yuc2 yuc4 yuc6* mutant but not that of
the ROP2 RNAi/rop4-1 line (Figure 2.2B and 2.2C; Figure 2.3C and 2.3D). These results suggest that auxin is a signal that induces lobe formation possibly by activating ROP2 and ROP4.

**Auxin activates the ROP2-RIC4 pathway at the plasma membrane**

To test whether auxin activates ROP2, we first determined the effect of auxin on ROP2 activity using an effector binding-based assay to measure active GTP-bound ROP2 (Baxter-Burrell et al., 2002). In this assay, the ROP2 effector-RIC1 fusion protein conjugated to agarose beads is used to specifically capture GTP-bound GFP-ROP2 isolated from transgenic plants expressing GFP-ROP2. RIC1-bound active GFP-ROP2 is then detected by western blotting analysis using anti-GFP antibody. Using this assay to monitor ROP2 activation in protoplast extracted from Arabidopsis leaves, we found that ROP2 activity doubled by addition of as low as 1 nM NAA and reached saturation at 20 to 100 nM NAA (Figure 2.4A and 2.4B), which is consistent with the concentrations of NAA for the induction of PC interdigitation (Figure 2.3C and 2.3F). Time course analysis showed that ROP2 activity doubled within 30 sec after NAA treatment (Figure 2.4C and 2.4D). This ROP2 activation is one of the most rapid auxin responses characterized to date. The rapid activation of ROP2 by auxin suggests that auxin perception directly leads to ROP2 activation at the PM.

Localization of GFP-RIC4 to the PM is a display of *in vivo* activation of ROP2,
because RIC4 specifically binds the active form of PM-delimited ROPs (Fu et al., 2005; Hwang et al., 2005). In wild-type PCs, GFP-RIC4 was preferentially localized to the PM domains associated with initiating or growing lobes where ROP2 is activated. In the yuc quadruple mutant, GFP-RIC4 localization to these PM domains was reduced, with a corresponding increase of its level in the cytoplasm (Figure 2.5A and 2.5B). Treatment with 20-nM auxin recovered GFP-RIC4 localization to the PM in the yuc quadruple mutant (Figure 2.5A and 2.5B), but not in the rop2-1rop4-1 double mutant (data not shown). Fine cortical F-actin, a RIC4 signaling target, was also markedly reduced in the yuc quadruple-mutant PCs as in the ROP2RNAi rop4-1 PCs (Fu et al., 2005) (Figure 2.5C). Taken together, our results indicate that auxin is required for localized ROP2 activation in the cortex of the lobing region in PCs.

**ABP1 is required for auxin promotion of PC interdigitation**

There are two well-characterized auxin-binding protein families in Arabidopsis, AUXIN-BINDING PROTEIN 1 (ABP1) and the TRANSPORT INHIBITOR RESISTANT 1 (TIR1) proteins. The TIR1-family of F-box proteins (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Tan et al., 2007) controls auxin-induced gene expression by activating the degradation of the IAA/AUX transcriptional repressors (Leyser, 2006; Mockaitis and Estelle, 2008). Therefore, this nuclear TIR1 pathway is unlikely to mediate ROP2 activation and other PC responses that are rapidly induced by auxin within 30 sec (Badescu and Napier, 2006); the most rapid
auxin-induced changes in mRNA expression occur within 2-5 minutes after auxin treatments (Abel and Theologis, 1996). ABP1 is partially localized to the cell surface, and the cell surface localized ABP1 is associated with a GPI-anchored PM protein and rapidly activates cell expansion (Badescu and Napier, 2006; Braun et al., 2008; Chen et al., 2001a; Chen et al., 2001b; Jones, 1994; Shimomura, 2006; Steffens et al., 2001). Because null alleles of abp1 are embryo lethal (Chen et al., 2001b), we isolated a weak allele, abp1-5 containing a point mutation (His94>Tyr) in the auxin-binding pocket (Woo et al., 2002) (Figure 2.6A). PCs of abp1-5 mutants showed an interdigitation defect similar to that observed in the yuc quadruple mutant (Figure 2.6B and 2.6C; Figure 2.2B and 2.2C). This phenotype was rescued to wild type by transgenic expression of ABP1 (Figure 2.7A and 2.7B), indicating that the abp1-5 PC phenotype was due to the reduced ABP1 function in the mutant. The role of ABP1 in PC interdigitation was further confirmed by similar phenotypes induced by inducible expression of an ABP1 antisense RNA and a RNA encoding single-chain fragment variable 12 derived from anti-ABP1 mAb12 antibody (Braun et al., 2008) (Figure 2.6D and 2.6E; Figure 2.7C and 2.7D).

We hypothesized that auxin would not rescue the defects in PC interdigitation in the abp1-5 mutant and the ABP1 antisense line if ABP1 perceives the auxin that activates PC interdigitation. Indeed we found that, in contrast to the wild-type and yuc quadruple mutant PCs, exogenous auxin did not induce lobe formation in PCs containing the abp1-5 mutation or expressing ABP1 antisense RNA (Figure 2.6B to
ABP1 is required for auxin activation of the ROP2-RIC4 pathway

We next tested whether ABP1 is required for the auxin activation of the ROP2 pathway. The abp1-5 mutation greatly reduced GFP-RIC4 localization to the lobe tip and PM (Figure 2.9A and 2.9B), as well as localized accumulation of diffuse cortical F-actin (Figure 2.9C). Thus, ROP2 signaling is greatly compromised by the abp1-5 mutation. Furthermore, in contrast to the yuc quadruple mutant, in the abp1-5 mutant, the defect in RIC4 localization could not be rescued by auxin treatment (Figure 2.9A). Finally, both the analysis of GFP-RIC4 localization to the PM and measurement of GTP-bound ROP2 showed that the rapid auxin activation of ROP2 in protoplasts was abolished by the abp1-5 mutation and ABP1 antisense expression (Figure 2.8A and 2.8B; Figure 2.9D to 2.9F). Therefore, our results show that ABP1 function is required for auxin-dependent ROP2 activation. This suggests that ABP1 acts upstream of the PM-localized ROP2 in the perception of auxin.

ROP2-dependent lobe-localized PIN1 is required for interdigitation.

The presence of ABP1 in the cell surface (Diekmann et al., 1995; Henderson et al., 1997; Jones and Herman, 1993; Leblanc et al., 1999) and the localization of its downstream component ROP2 to the lobe PM region imply that the perception of extracellular auxin leads to localized ROP2 activation. Thus, the presence of a
mechanism to induce local accumulation of auxin at the extracellular regions of PCs is expected. Consistent with this notion, we found PIN1 preferentially localized to the apical PM of PC lobes, as demonstrated by PIN1-GFP localization in a pPIN1::PIN1-GFP line, as well as by PIN1 immunostaining with anti-PIN1 antibody (Figure 2.10A). PCs of a PIN1 loss-of-function mutant, pin1-1, showed a defect in interdigitation, and were long and narrow (Figure 2.10B; Figure 2.11A and 2.11B), a phenotype highly similar to the ROP2 RNAi/rop4-1 line (Fu et al., 2005). Other loss-of-function pin1 alleles such as pin1-5 showed a similar phenotype (Figure 2.11E and 2.11F). Furthermore, GFP-RIC4 localization to the PM was eliminated in the pin1-1 mutant, with the fluorescence diffusely distributed in the cytosol (Figure 2.10D and 2.10E). Application of NAA failed to rescue the lobing defect in the pin1-1 mutant (Figure 2.10B and 2.10C; Figure 2.11A and 2.11B), indicating a critical role for PIN1-mediated localized auxin export in lobe formation and a role for auxin in the localized ROP2 activation. The inability of auxin to rescue pin1-1 phenotype also implies a role for PIN1 in a positive feedback, i.e., PIN1 localization to the lobe tip may require ROP2 signaling. Consistent with this implication, PIN1 localization to the PM was compromised in the ROP2RNAi rop4-1 line, the abp1-5 mutant, and the ABP1 antisense line, which all showed greatly enhanced PIN1 internalization in PCs and reduced localization to the lobe PM (Figure 2.10A, right panel; Figure 2.11G and 2.11H). Transient expression of a dominant negative ROP2 mutant protein also increased PIN1-GFP internalization, suggesting that PIN1 localization to the PM is
directly affected by ROP2 signaling, not indirectly through ROP2/4-mediated cell shape changes (Figure 2.11C and 2.11D). Taken together, these results support the hypothesis that a PIN1-dependent positive feedback loop is required for localized ROP2 signaling and lobe outgrowth. The requirement of localized PIN1 for interdigitation implies a role for localized extracellular auxin in the regulation of this process.

**Auxin also activates the ROP6-RIC1 pathway in an ABP1-dependent manner.**

PIN1-exported auxin in the lobing side is expected to diffuse across the cell wall to the complementary indenting side of the neighboring cell, where the ROP6-RIC1 pathway operates (Fu et al., 2009). Thus we speculated that PIN1-exported auxin could serve as a cross-cell signal to activate the ROP6-RIC1 pathway, hence providing a mechanism for the cell-cell coordination of lobe outgrowth with indentation formation. The initial clue to this notion came from our observation that both of the quadruple *yuc1 yuc2 yuc4 yuc6* and single *abp1-5* mutants exhibited an additional cell shape phenotype that was observed in *rop6-1* and *ric1-1* mutants (Fu et al., 2005; Fu et al., 2009), specifically, wider neck regions (Figure 2.12A and 2.12B). This PC phenotype could not be accounted for by the sole defect in the ROP2-RIC4 pathway, because the *ROP2RNAi rop4-1* and *ric4-1* knockout mutants lacked this phenotype (Fu et al., 2005; Fu et al., 2002). The wide neck phenotype suggests that auxin and ABP1 may also activate the ROP6-RIC1 pathway, which promotes
indenting. Therefore, we sought to test whether ABP1 perception of auxin activates the ROP6-RIC1 pathway.

ROP6 is required for RIC1 decoration of cortical MTs like beads on a string and for its function in promoting the ordering of cortical MTs (Fu et al., 2009). If auxin is required for the activation of the ROP6-RIC1 pathway, one would expect that RIC1’s association with cortical MTs is disrupted in the abp1-5 and yuc1 yuc2 yuc4 yuc6 mutants, as in the rop6-1 null mutant (Fu et al., 2009). Indeed, RIC1 association with cortical MTs was greatly abolished in both yuc1 yuc2 yuc4 yuc6 quadruple and in abp1-5 single mutant PCs (Figure 2.12C; Figure 2.13A). Consistent with the defect of RIC1 distribution, the arrangement of cortical MTs in these mutants became mostly random, similar to that seen in rop6-1 and ric1-1 mutants (Figure 2.13B). This indicates that auxin and ABP1 are required for the activation of the ROP6-RIC1 pathway.

If auxin activates the ROP6-RIC1 pathway, auxin should promote RIC1 association with cortical MTs. We previously showed that ROP2, which is also activated by auxin, inhibits RIC1 function by sequestering RIC1 from cortical MTs in PCs. To circumvent the possible complication of the ROP2 effect on RIC1 localization (Fu et al., 2005), we analyzed YFP-RIC1 localization in the rop2-1 rop4-1 double mutant, in which ROP2 function is severely compromised. YFP-RIC1 appeared as beads lining cortical MTs as shown previously (Figure 2.12C and 2.12D) (Fu et al., 2005). Ten min after the application of 10 nM auxin, the number of YFP-RIC1 associated MTs
dramatically increased, and MTs became more ordered, especially in the indented region of the PC (Figure. 2.12D). Furthermore, both the number of YFP-RIC1 beads and their intensity greatly increased as rapidly as 4 min after auxin application (Figure. 2.12E and 2.12F). In the abp1-5 mutant, auxin failed to change the localization pattern of RIC1 (Figure. 2.12D to 2.12G), suggesting that ABP1 acts upstream of the ROP6-RIC1 pathway. These results support the hypothesis that auxin activates the ROP6-RIC1 pathway in an ABP1-dependent manner.

**Auxin also activates ROP6 rapidly**

To further confirm auxin activation of the ROP6-RIC1 pathway, we determined the effect of auxin on ROP6 activity. Indeed, auxin treatments increased the amount of active ROP6 in a dosage-dependent manner (Figure 2.4A and 2.4B). At low auxin concentrations (<10 nM NAA), ROP6 activation was similar to that of ROP2. ROP6 activation was much greater compared to ROP2 when NAA concentrations were 10 nM or higher. The activation of ROP2 by auxin was saturated at 100-nM NAA treatment, while ROP6 activation was not saturated until 1 µM of NAA. The significance of this difference in the kinetics of ROP2 and ROP6 activation remains to be determined, but it appears to be an important parameter that is required for the localized activation of ROP2 and ROP6 in the complementary lobing and indenting sides by uniformly applied auxin (see Discussion). Like ROP2, ROP6 was rapidly activated within 30 seconds after 20 nM NAA treatment (Figure 2.4C and 2.4D),
consistent with a role for ABP1 in the perception of auxin that activates ROP6. ABP1-dependent ROP6 activation by auxin was further demonstrated by our finding that the auxin-dependent increase in ROP6 activity was abolished by the \textit{abp1-5} mutation (Figure 2.12H).

### 2.4 Discussion

The findings here have several important implications. First, these results establish a cytoplasmic auxin signaling mechanism that is distinct from the TIR1-based nuclear auxin-signaling pathway and provides a new perspective of auxin action at the cellular level. Second, our findings give new insights into hormonal signaling leading to changes in the cytoskeleton and vesicular trafficking, which is crucial for hormone action in plants yet scarcely studied. Third, we show that ABP1 acts upstream of ROP GTPase signaling, which gives an unprecedented understanding of signaling events downstream of the auxin perception by ABP1, an essential auxin-binding protein that has long been implicated in auxin function but whose mode of action has been enigmatic. Finally, our results suggest that the ABP1- and ROP-dependent auxin signaling plays a pivotal role in the spatial coordination of cell expansion within and between cells during interdigitated growth of PCs. Since auxin is a multi-functional hormone polarly transported out of cells, this auxin signaling mechanism could serve as a common mode of intracellular and intercellular coordination of cell growth, morphogenesis and polarity in plants.
A new auxin signaling mechanism regulates cytoplasmic pathways

Genetic studies have revealed an essential role for the TIR1/AFB-dependent nuclear auxin signaling system in the regulation of auxin-mediated growth, development, and patterning that rely changes in gene expression (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005; Mockaitis and Estelle, 2008). Previous work hints the existence of other auxin signaling mechanisms (Badescu and Napier, 2006), and our findings here clearly establish a distinct auxin signaling mechanism that exists in the cell boundary/cytoplasm and is capable of responding to auxin in seconds. The function of this ABP1/ROP-dependent auxin signaling system is complementary to the TIR1/AFB1-dependent pathway, which directly regulates auxin-mediated gene expression. Different from TIR1/AFB1 pathway, the ABP1/ROP-dependent pathways directly regulate cytoplasmic events such as actin and microtubule organization and PIN protein trafficking. Our findings provide new insights into the mechanism by which auxin modulates cytoskeletal reorganization and cell morphogenesis in multicellular tissues of plants. Although our work here focuses on the roles of this new auxin signaling mechanism in pavement cell morphogenesis, it is likely that similar ABP1-ROP signaling pathways may operate in other plant cells and tissues because of widespread expression and functions of ABP1 and ROPs in plants (Braun et al., 2008; Chen et al., 2001a; Chen et al., 2001b; Fu et al., 2005; Fu et al., 2002; Fu et al., 2009; Jones, 1994; Jones and Herman, 1993; Jones et al., 1998; Tromas et al.,
Our findings here do not exclude the involvement of ROPs in the regulation of TIR1/AFB-dependent auxin responses. In fact, it was shown in tobacco and Arabidopsis protoplasts, expression of dominant negative or constitutively active forms of the tobacco NtRac1 ROP affected auxin-induced gene expression (Tao et al., 2002), and thus ROP may also regulate the nuclear pathway in addition to the cytoplasmic pathways.

**ABP1 may be a cell surface auxin receptor that activates ROP2 and ROP6 signaling**

We have shown that ABP1 is required for the rapid activation of the PM-localized ROP2 and ROP6 pathways by auxin. ABP1 is associated with the outer surface of the PM through its binding to a GPI-anchored protein (Shimomura, 2006), though ABP1 is partially localized to endoplasmic reticulum. Furthermore, it is the cell surface-associated ABP1 that mediates auxin activation of cell expansion (Chen et al., 2001a; Jones et al., 1998). Hence we propose that ABP1 is a cell surface receptor of auxin that activates the ROP signaling pathways involved in PC interdigitation. Consistent with the cell surface site for the ABP1-mediated auxin perception, we found that the PIN1 mediated auxin export is required for ROP2 activation. ABP1 is not a transmembrane protein and likely works with a trans-membrane partner or co-receptor. Identification of such an assumed trans-membrane protein will be crucial.
for understanding how auxin is perceived at the cell surface and how it leads to ROP activation in the cytoplasmic side of the PM.

**A working model for the coordination of interdigitated cell growth and beyond**

Based on our current and previous results (Fu et al., 2005; Fu et al., 2009; Yang, 2008), we propose a working model for the auxin signaling pathways required for interdigitated growth (i.e., development of complementary lobes and indentations) in PCs (Figure 2.14). In this paper, we demonstrate that auxin activates both of the ROP2 and ROP6 pathways via ABP1. Our previous work suggests that ROP2 and ROP6 are locally activated at opposing sides of the cell wall and that the ROP2 and ROP6 pathways are mutually exclusive along the PM of a PC (Fu et al., 2005; Fu et al., 2009; Yang, 2008). At the steady state, therefore, extracellular auxin is not expected to simultaneously activate ROP2 and ROP6 at the same side, and thus simultaneous activation of ROP2 and ROP6 by extracellular auxin must occur at the opposing sites (lobe and indentation bordered by the cell wall). A key aspect of this working model is the existence of an auxin-ROP2-PIN1-auxin positive feedback loop, which is required for local accumulation of extracellular auxin. Our mathematical modeling suggests that localized auxin accumulation in the cell wall region is theoretically feasible (Grieneisen et al., 2010), but experimental proof of local auxin accumulation in the cell wall requires an auxin probe that could report localized extracellular auxin level, which has yet to be developed. Importantly, this working
model can explain how extracellular auxin coordinates lobe and indentation development at the steady state, once the interdigitation pattern has been initiated (i.e., the cell region for lobe formation or indentation has already been established).

Can this working model also explain how uniformly applied auxin leads to the initial establishment of cell cortical regions that define lobe- or indentation-forming sites to initiate the interdigitation pattern (Figure 2.2)? In other cell types, a uniform field of signal activates cell polarization through a stochastic local activation of the positive feedback loop at the leading edge and its interaction with an antagonistic pathway in the trailing edge (Hazak et al., 2010; Paciorek et al., 2005; Van Keymeulen et al., 2006; Xu et al., 2003). Similarly, we speculate that a uniform auxin field could transform into a localized signal through a stochastic local activation of the ROP2-PIN1 positive feedback loop and its interaction with the antagonistic ROP6 pathway. Given stochastic activation of an intracellular ROP2 signaling component, the positive feedback loop would be transiently initiated only at one side of but not simultaneously at both of the opposing sides of a cell wall region. Given the ROP2-PIN1 loop activated at a random site in cell 1, ROP6 would be suppressed at this site, due to the mutual inhibition of the ROP2 and ROP6 pathways in this cell (Fu et al., 2005; Fu et al., 2009; Yang, 2008). The feedback loop would rapidly increase the level of extracellular auxin, which would preferentially activate the ROP6 pathway at the complementary site in cell 2, because at these higher levels, auxin causes greater ROP6 activation compared to ROP2 (Figure 2.4A). Once ROP6 were
preferentially activated at the complementary side in cell 2, ROP2 would be inhibited at this site, and the ROP2-PIN1 feedback loop would be stabilized in cell 1. Therefore the differential ROP2 and ROP6 activation and the mutual inhibition between the ROP2 and ROP6 pathways within one PC could assure that the ROP2 and ROP6 pathways are respectively activated stably at the two opposing sides of the extracellular auxin. Interestingly a mathematical model constructed based on the structure depicted in Figure 2.14 simulates such a stable generation of the interdigitated ROP2 and ROP6 activation and the localized accumulation of extracellular auxin only if the kinetics for activation of ROP2 and ROP6 by auxin exhibits a difference shown in Figure 2.4A and 2.4B (Fu et al., 2009), the mutual inhibition of the ROP2 and ROP6 pathways also coordinates lobing and indenting within a PC (Fu et al., 2005; Fu et al., 2009; Grieneisen et al., 2010). One shortcoming of this model seems to be that due to fast auxin diffusion, it is difficult to envision how the ROP2-PIN1 feedback loop occurring at an asymmetric side of the cell wall and extracellular auxin accumulating locally could be possible. Remarkably, mathematical simulation based on this model shows that symmetric activation of ROP2 at both sides of the cell wall was unstable and was dynamically and rapidly converted to the stable interdigitated ROP2 and ROP6 activation (Grieneisen et al., 2010). Therefore the stable ROP2 and ROP6 interdigitation along with the localized extracellular auxin can be produced via a dynamic mutual interaction between ROP6 and the ROP2-PIN1 positive feedback under the condition
that auxin preferentially activates ROP6 compared to ROP2.

Positive feedback loop initiated by a stochastic local change in Rho GTPase signaling has been proposed to be a general mechanism for the establishment of self-organizing cell polarity in yeast and animal cells (Altschuler et al., 2008; Hazak et al., 2010; Paciorek et al., 2005; Van Keymeulen et al., 2006; Xu et al., 2003). In neutrophil and other animal cells, the perception of uniform concentrations of chemoattractants by a single receptor leads to establishment of the frontness and backness polarity by activating two antagonistic cytoskeleton-regulating Rho GTPase pathways (Hazak et al., 2010; Paciorek et al., 2005; Van Keymeulen et al., 2006; Xu et al., 2003). This would be remarkably similar to the activation of the antagonistic ROP2 and ROP6 pathways by the ABP1 perception of uniform concentrations of auxin. Therefore the self-organization design principles for the spatial coordination of cell growth and movement might be conserved in both single and multi-cellular tissue across eukaryotic kingdoms.

Our working model shown in Figure 2.14 may serve as a unifying mechanism for the coordination of cell morphogenesis and polarity within various plant tissues. Indeed, studies suggest a role for auxin in orchestrating PIN localization in files of cells directing auxin flow (Paciorek et al., 2005; Sauer et al., 2006) and in coordinating hair positioning toward the basal side of root-hair-forming cells (Fischer et al., 2006; Jones et al., 2009). The position of root hair formation can be predicted by the polar localization of ROP2 in the hair forming epidermal cells (Jones et al., 2002), and
ROP2 polar localization is affected by auxin (Fischer et al., 2006; Jones et al., 2009; Payne and Grierson, 2009; Yang, 2008), raising the possibility that the auxin-mediated ROP signaling may also underlie the coordination of polar cell growth among root epidermal cells.

Our working model here could also be used to explain how auxin may coordinate the polarization of PIN proteins to the same cell end among a file of cells that direct auxin flow, i.e., auxin could activate a ROP2-like pathway that forms a positive feedback loop at the end of PIN localization as well as a ROP6-like pathway that antagonizes with the ROP2-like pathway at the side lacking PIN localization. Auxin was shown to inhibit PIN internalization in root cells (Dhonukshe et al., 2008; Paciorek et al., 2005), which is also in agreement with our finding in this report that PIN1 internalization is increased when ROP2 function is compromised in PCs. In further support of a role for ROP signaling in the modulation of PIN polarization, Interactor of Constitutively active ROP 1 (ICR1), a likely ROP effector protein, was recently found to be required for PIN polarization both in Arabidopsis embryonic and root cells (Hazak et al., 2010). Therefore we anticipate that the elucidation of the ROP-based cytoplasmic auxin signaling pathways in various auxin-mediated processes will likely be an exciting and fertile area of research in cell and developmental biology in the coming years.
2.5 Materials And Methods

Plant materials and growth condition

Arabidopsis plants were grown at 22°C on MS agar plates or in soil with 16-hr light/8-hr dark cycles unless indicated otherwise. The DR5::GUS line and the yuc1 yuc2 yuc4 yuc6 quadruple mutant were kindly provided by Tom Guilfoyle and Yunde Zhao, respectively (Cheng et al., 2006; Hagen and Guilfoyle, 2002). The double-mutant ROP2RNAi rop4-1 line was described previously (Fu et al., 2005; Fu et al., 2002). The pin1-1 and pin1-5 mutants are T-DNA insertional lines obtained from ABRC (SALK, CS8065 and 097144, respectively) and their genotypes were confirmed by PCR analysis.

The tilling allele abp1-5 was obtained by the following method: ABP1 gene-specific primers (5’- TGATTCTAGGCTTGCCAATC GTGAGG -3’ and 5’-AGCCTTGCAGCAGTGTGTGAGTATA -3’) were designed to amplify the region between 1st intron/2nd exon and 5th exon of ABP1 gene. This region covers 996 bp of genomic DNA sequence that contains most of the open reading frame of ABP1 gene (excluding the 1st exon) (Till et al., 2003). This pair of primers was submitted for TILLING (Henikoff et al., 2004). The abp1-5 allele contains a missense mutation of C→G in the 94 codon of the coding sequence. The mutation causes an H→Y conversion at position H59 of the ABP1. H59 lies within the binding pocket of ABP1 and coordinates with the carboxylic acid of auxin and with a zinc ion (Woo et al., 2002). The mutation was confirmed by sequencing at the University of North
Carolina-Chapel Hill. Tilling mutant *abp1*-5 was backcrossed 6 times with Col-0 and genotyped by restriction digestion of PCR fragments. The primers (Atabpe1x2FW 5’-TGACCTTCCTCAGGATAACTATGG-3’ and Atabp1ex4RV 5’-CCAACACCTGCAGGTCCTCATGAC-3’) were designed to amplify the exon 3 region where the point mutation occurs. This 802-bp PCR product was digested with Rsa I to distinguish the wild type (523-, 207- and 73-bp fragments) from *abp1*-5 (523-, 165-, 73- and 45-bp fragments). For genetic complementation, *abp1*-5 was transformed with the Arabidopsis wild type ABP1 cDNA driven by the 35S cauliflower mosaic virus promoter.

Conditional plants for ABP1 expression were obtained by expressing either a full-length antisense construct or the recombinant single-chain fragment variable 12 derived from the monoclonal anti-ABP1 antibody mAb12 under the control of the ethanol inducible system as described (Braun et al., 2008; David et al., 2007). Ethanol induction was obtained by exposure of siblings to ethanol vapor generated from 500 µL of 5% ethanol in a microtube placed at the bottom of sealed square plate.

**Confocal and imprinting analysis of leaf Arabidopsis PC shape**

PC shape from Arabidopsis cotyledons was imaged directly on confocal microscopy (Leica SP2 confocal microscope) or indirectly by an imprinting method (Mathur and Koncz, 1997). Since PCs are auto-fluorescent, their cell outlines can be imaged on confocal microscopy with the following settings: excitation 351 nm or 364 nm, 50%
laser power and emission 400-600 nm. For some treatments, the cotyledons were curved, so analyzing cell shapes by confocal microscopy was difficult. In this case, an agarose imprinting method was used (Mathur and Koncz, 1997). Briefly, 3% agarose gel with low melting points (30-35°C) was boiled and dropped onto a slide. The cotyledon was quickly laid out on the unsolidified agarose. After the agarose was solidified at 4°C for 5 min, the cotyledon was peeled off, and cell outlines imprinted on the agarose were imaged on bright field microscopy (Nikon). Additional image analyses involved use of Metamorph 4.5. The images are edited by photoshop 7.0 by adjusting figure sizes and resolution and adding labels.

**Ballistics-mediated transient expression in leaf epidermal cells**

Subcellular localization of GFP-RIC4, YFP-RIC1 and F-actin was analyzed by use of transiently-expressed pBI221:GFP-RIC4, pUC:YFP-RIC1 and pBI221:GFP-mTalin constructs as described previously (Fu et al., 2005; Fu et al., 2002). We used 0.8 µg pBI221:GFP-mTalin, 1 µg pBI221:GFP-RIC4 and 1 µg pUC:YFP-RIC1 for particle bombardment. GFP and YFP signal was detected 5 hrs after bombardment by use of a Leica SP2 microscope (GFP: 488 nm excitation, 25% power; excitation 520 nm-600 nm, gain at 600; YFP: 514 nm excitation, 25% power; excitation 530 nm-600 nm, gain at 600). Cells at stage II showing similar medium levels of GFP (Fu et al., 2005; Fu et al., 2002) were chosen for GFP marker analysis. For 3-D reconstruction, optical sections in 1.0-µm increments were imaged for each cell by use of the Leica
Naphthalene-1-acetic acid (NAA) treatments

NAA (Sigma, St. Louis, MO) was dissolved in DMSO as 0.5 M stock solutions, which were diluted to the indicated concentrations in liquid MS (for seedling treatments) or W5 media (for protoplast treatments). Seeds were germinated in the liquid MS media containing NAA or NPA. Each treatment was repeated at least three times with the corresponding controls.

Protoplast preparation and PEG-mediated transient expression

Protoplast preparation and PEG-mediated transient expression were described previously (Sheen, 2001). The 2nd or 3rd pair of rosette leaves from 2- or 3-weeks-old seedlings was used to prepare protoplasts. Protoplasts were counted by use of a hemacytometer (Hausser scientific, Cat # 1483). An amount of $10^5$-$10^6$ protoplasts were used for ROP2 activity assay, and $10^4$-$10^5$ protoplasts were used for transient expression.

ROP2 and ROP6 activity assays in protoplasts

Two different methods were used to analyze auxin activation of ROP2 in protoplasts. The first method involves a biochemical assay, in which GFP-tagged active ROP2 or ROP6 was pulled down by use of MBP-RIC1. Protoplasts were isolated from leaves.
of 2- or 3-week old 35S::GFP-ROP2 or –ROP6 transgenic seedlings as described previously (Jones et al., 2002; Sheen, 2001). Isolated protoplasts were treated with different concentrations of NAA, or with 100 nM for various times and frozen by liquid nitrogen. Total protein was extracted from $10^5$-$10^6$ treated protoplasts using extraction buffer (25 mM HEPEs pH 7.4; 10 mM MgCl$_2$; 10 mM KCl; 5 mM DTT; 5 mM Na$_3$VO$_4$; 5 mM NaF; 1 mM PMSF; 1% protease inhibitor from Sigma, St. Louis; 1% TritonX-100) at 4 °C. Twenty micrograms of MBP-RIC1-conjugated agarose beads were added to the protoplast extracts, and incubated at 4 °C for 3 hrs. The beads were washed using a washing buffer (25 mM HEPEs pH 7.4; 1 mM EDTA; 5 mM MgCl$_2$; 1 mM DTT; 0.5% TritonX-100) three times at 4 °C (5 mins each). GTP-bound GFP-ROP2 or –ROP6 that was associated with the MBP-RIC1 beads was used for analysis by western blotting with an anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Prior to the pull-down assay, a fraction of total proteins was analyzed by immunoblot assay to determine total GFP-ROP2 or –ROP6 (GDP-bound and GTP-bound GFP-ROP2 or -ROP6). The amount of GTP-bound GFP-ROP2 or -ROP6 was normalized to that of total GFP-ROP2 or –ROP6. The level of GTP-bound ROP2 or ROP6 relative to the control (0 nM NAA at 0 mins) was calculated by dividing the amount of normalized GTP-bound ROP2 or ROP6 from each treatment by the normalized amount from the control, which is defined as “1”.

To evaluate the significance of the differences between ROP2 and ROP6 activities at different auxin concentrations, we performed a repeated-measures analysis of
variance with ROP activity level as the dependent variable, and auxin concentration, ROP type and interaction between auxin concentration and ROP type as the independent variables (Proc Mixed with first-order autoregressive covariance estimation; SAS Institute, INC., Cary, NC). We found there is significant interaction between the ROP type and concentration (p-value<0.001), which suggests the difference in ROP activity level between ROP2 and ROP6 depends on the concentration. We therefore tested the significance of difference in ROP activity level between ROP2 and ROP6 at concentration level using F-test and all the p-values are less than 0.05 except at concentration 0 and 1 nm and the difference increases with concentration level. We also compared mean values of activity level at auxin concentration 0, 1 nM, 10 nM, 20 nM, 100 nM, 1 µM and 10 µM for ROP2 and ROP6 separately using Tukey pairwise mean comparison. We found that for ROP2 the activity levels significantly increase at lower concentration and stabilize at median concentration and significantly decrease at high concentration, while for ROP6 the activity level significantly increase at low and median concentration level and then stabilize at high concentration level.

We also performed a repeated-measures analysis of variance with ROP activity level as the dependent variable, and time (in minutes), ROP type and interaction between time and ROP type as the independent variables. We found there is significant interaction between the ROP type and time (p-value<0.001), which suggests the difference in ROP activity level between ROP2 and ROP6 depends on
the time. We therefore tested the significance of difference in ROP activity level between ROP2 and ROP6 at each time points using F-test and all the p-values are less than 0.05 except at time 0. We also compared mean values of activity level at times 0, 0.5, 1, 2, 5 and 8 minutes for ROP2 and ROP6 separately using Tukey pairwise mean comparison and found that the activity levels significantly increase from the beginning until 2 minutes and then stabilize afterwards for ROP6, and slowly but significantly increase again after 5 minutes for ROP2.

In the second method, changes in GFP-RIC4 localization to the PM were monitored in isolated protoplasts. Protoplasts were isolated from leaves of wild-type plants (Col 0) or mutants as described above. Two micrograms of a 35S::GFP-RIC4 construct was introduced into $10^4$-$10^5$ protoplasts by PEG-mediated transformation. Typically, 70%-80% of the protoplasts were transformed. Protoplasts were incubated at 23 °C for 5 hrs to overnight, treated with NAA (1 µM final concentration), and imaged immediately by use of a Leica SP2 confocal microscope. The earliest possible time for imaging was 2 min after NAA application. Time-lapse images were taken every 2-3 min.

**Quantitative analysis of GFP-RIC4 and YFP-RIC1 localization**

The images of GFP-RIC4 localization in both pavement cells and protoplasts were taken by Leica SP2, and image analysis were conducted by Metamorph 4.5 using region function. First we created a region along cell cortex. The average intensity of
GFP for this was calculated by Metamorph. Then we created a region just inside of the cell cortex, which included all cytoplasm signals, and the average cytoplasmic signal was calculated. The average signals were then used to calculate the ratio of PM/Cyto.

YFP-RIC1 was transiently expressed in pavement cells using the ballistics-mediated method as described above. Four to five hours after bombardments, leaves were treated with 10 nM auxin, and time-series YFP-RIC1 images are taken using a Leica SP2 confocal microscope 2 minutes after auxin treatment. Image analysis was conducted using Metamorph 4.5. The average signal intensity of YFP-RIC1 dots along MT and the length of MT bundle were directly measured by the Metamorph software, and the number of YFP-RIC1 dots was counted by eyeballing. YFP-RIC1 dots No./µm indicates the number of YFP-RIC1 dots divided by MT length.

**Immunolocalization of PIN1, RIC1, and MT in pavement cells**

Whole mount immunostaining of Arabidopsis leaves was previously described (Fu et al., 2005; Wasteneys et al., 1997). Briefly, the fixed, shattered and permeabilized leaves were incubated with primary antibody (anti-PIN1 1:200, anti-RIC1 1:100, anti-αTubulin 1:200) overnight at 4°C (Paciorek et al., 2005), and then incubated with the second antibody (FITC conjugated anti-rabbit IgG 1:200, TRITC conjugated anti-mouse IgG 1:200) for 2 hours at 37°C. Stained cells were observed by use of a Leica SP2 confocal microscope. Cells at stage II (Fu et al., 2005; Fu et al., 2002)
were chosen for comparison between wild type and mutant cells.

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2.7 Figures and Legends

Figure 2.1 Tip-to-margin-to-center/base progression of pavement cell (PC) interdigitation follows the tip- and margin-high auxin gradients in young Arabidopsis cotyledons.
Figure 2.2 Auxin activation of PC interdigitation requires ROP2/4
Figure 2.3 Dose-dependent auxin induction of interdigitation in pavement cells (PCs) occurs in wild type but not in mutants that are compromised in ROP2 or ABP1 function.
Figure 2.4 Auxin rapidly activates ROP2 and ROP6 in a dosage dependent manner
Figure 2.5 Auxin is required for the activation of ROP2 downstream signaling events.
Figure 2.6 ABP1 is required for auxin perception that promotes PC interdigitation
Figure 2.7 Reduced ABP1 function in the *abp1-5* tilling line and ethanol inducible antisense or antibody line is responsible for the PC interdigitation defect.
Figure 2.8 Auxin can activate ROP2-RIC4 pathway through ABP1
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Figure 2.10 PIN1 is localized to the lobe tip and is essential for auxin promotion of PC interdigitation.
Figure 2.11 PIN1 is essential for the activation of ROP2-dependent lobe growth, and transiently expressed dominant mutant of ROP2 promotes PIN1 internalization.
Figure 2.12 Auxin induces ROP6 activation and the association of RIC1 with cortical MTs in an ABP1-dependent manner
Figure 2.13 ABP1-perceived auxin is essential for activation of ROP6-RIC1 pathway
Figure 2.14 A model for the ABP1- and ROP GTPase-based auxin signaling mechanisms underlying interdigitating growth in PCs.
Figure 2.1 Tip-to-margin-to-center/base progression of pavement cell (PC) interdigitation follows the tip- and margin-high auxin gradients in young Arabidopsis cotyledons.

(A): PC interdigitation first occurs in the tip and progressively spreads to the margin and the center/base of germinating cotyledons. Various days after germination (DAG), wild type Col-0 cotyledon PCs were imaged using confocal microscopy, and the degree of interdigitation was quantified based on the number of lobes per cell and was color-coded as shown in the color bar.

(B): A gradient of interdigitation within a single PC. PCs near the base of the cotyledon at 4 DAG. Cell outlines were imaged from a transgenic line expressing a tubulin-GFP using confocal microscopy. Arrow indicates the waviest region of the cell outline, which is proximal to the tip/margin; arrowhead indicates the straightest region of the cell outline, which is distal to the tip/margin. Scale bar = 25 mm.

(C-D): GUS staining in a DR5::GUS line suggests a tip-high auxin gradient in the cotyledon at 1-DAG and a margin-high gradient in the cotyledon at 2-DAG. Scale bar = 50 mm.
Figure 2.2 Auxin activation of PC interdigitation requires ROP2/4.

(A): A schematic showing three stages of PC morphogenesis. At stage 1, near-rectangular-shaped PCs lack obvious lobes and indentations but exhibit localized ROP2 activation (i.e., RIC4 localization) and accumulation of diffuse F-actin in certain cortical regions where lobes will emerge. At stage 2, lobes protrude at the site of ROP2 activation and F-actin accumulation, and ROP6 is activated, leading to well-ordered cortical microtubule arrays form in the indenting regions. At stage 3, the jigsaw-puzzle appearance is fully developed.

(B): Auxin increased interdigitation of wild-type PCs and suppresses the PC interdigitation defect in the yuc1 yuc2 yuc4 yuc6 (yuc 1/2/4/6) quadruple mutant but not in the ROP2RNAi rop4-1, which exhibited a similar interdigitation defect. Seedlings were cultured in liquid MS with or without 20 nM NAA, and cotyledon PCs were imaged 4 days after stratification.

(C): Quantitative analysis of PC interdigitation. The degree of interdigitation in PCs shown in (B) was quantified by determining the density of lobes for each PC. Data are mean lobe number per mm2 ± SD (n>400 cells from three individual plants). The yuc 1/2/4/6 mutant had a significantly lower density of lobes than Col-0 wild type, and NAA significantly increased the mean density of lobes in Col-0 wild type and the yuc 1/2/4/6 mutant (t-test, p<0.001) but not in the ROP2RNAi rop4-1 line (t-test, p>0.1). Non-biased double blind analysis confirms all of the phenotypic differences between mutants and treatments described in this Figure (Figure. 2.3B).
Figure 2.3 Dose-dependent auxin induction of interdigitation in pavement cells (PCs) occurs in wild type but not in mutants that are compromised in ROP2 or ABP1 function.

(A): We considered the lobe number in the round initial cells as 0. The initial cells will develop lobes in two ways. The first kind of cells will expand radically and in the meanwhile protrude the lobes around the cell outline. The number of the sides where is outgrowing from initial cell is considered as lobe number (upper panel). The second kind of cells will elongate first, and protruding lobe at the side of elongated cell. The number of the outgrowing sites along the elongation side is considered as lobe number (lower panel).

(B): To confirm the pavement cell phenotypes described in the paper, we conducted double blind analysis, in which wild type control and the mutants or untreated control and treated samples were blindly labeled and were analyzed without the knowledge of the nature of the analyzed samples. Here is double blind analysis for the quantification of PC phenotype in wild type, yuc 1/2/4/6, and rop2RNAI rop4-1 mutants treated with auxin. The data set was identical to that described in Figure 1, and the double blind analysis produces nearly identical quantitative results as those shown in Figure 1.

(C): Auxin (NAA) dosage responses in wild-type PCs. To assess whether auxin induction of PC interdigitation is similar to other physiological responses to different concentrations of auxin, we applied various concentrations of NAA to Arabidopsis seedlings grown in liquid media. NAA promotion of PC interdigitation exhibits a
typical auxin dose-response curve. NAA induced increased interdigitation at a concentration as low as 5 nM, with an optimal concentration of 20 nM. The induction decreased with NAA concentration of 100 nM.

(D): The rop2RNAi rop4-1 mutant is insensitive to auxin induction of PC interdigitation: NAA did not induce any cell shape changes at various concentrations, which indicates the rop2RNAi rop4-1 mutant loses auxin responsiveness.

(E): The abp1-5 mutant fails to respond to auxin. As in the rop2RNAi rop4-1 mutant, abp1-5 did not respond to various concentrations of NAA. These data support an important role for both ABP1 and ROP2/4 in the auxin induction of PC interdigitation.

(F): Quantitative analysis of PC phenotypes shown in (C-E). The mean number of lobes per area of PCs was measured as described in Figure 1. Data are mean lobe number per \( \mu \text{m}^2 \pm \text{SD} \) (n>400 cells from three individual plants).

(G-H): The cotyledon development is mostly normal in different mutants. We found that the morphology of cotyledons in the yuc1246, abp1-5, and ROP2RNAi rop4-1 mutants was not significantly different from wild type (G). Furthermore, the mean pavement cell size in these mutants (and thus the cell number) is not different from that in wild type (H). This indicates the PC phenotype is not the indirect effect caused by cotyledon or leaf morphology change and cell division pattern change.
Figure 2.4 Auxin rapidly activates ROP2 and ROP6 in a dosage dependent manner.

(A-B): Auxin dosage responses of ROP2 and ROP6 activation. Protoplasts were isolated from leaves of transgenic GFP-ROP2 (upper panels) or GFP-ROP6 (lower panel) seedlings and treated with the indicated concentrations (1 nM to 10 μM) of NAA for 2 min (A), or treated with 100 nM NAA for the indicated times (0.5 to 8 min) (B). GTP-bound active GFP-ROP2 or GFP-ROP6 was coimmunoprecipitated with MBP-tagged RIC1 and detected by immunoblot analysis with anti-GFP antibody. An aliquot of total GFP-ROP2 or GFP-ROP6 (GDP and GTP forms) was included as the loading control (Total ROP2 or ROP6). Results from one representative experiment out of five independent experiments with similar results are shown. ROP2 and ROP6 experiments were conducted in parallel under identical conditions.

(C-D): Quantitative analysis of data from A and B. The relative ROP2 or ROP6 activity level was determined as the amount of GTP-bound ROP2 divided by the amount of total GFP-ROP2. The relative ROP activity in different treatments was standardized to that from mock-treated control, which was arbitrarily defined as “1”. Data are mean activity levels from five independent experiments ± SD. We tested the significance of difference in ROP activity level between ROP2 and ROP6 at various auxin levels using F-test. All the p-values are less than 0.001 except at 0 and 1 nM of auxin and the difference increased with auxin levels. We also compared mean values of ROP activity level using Tukey pairwise mean comparisons and found that
ROP2 activity significantly increased at lower auxin levels, stabilized at median auxin levels, and significantly decreased at high auxin levels. In contrast, ROP6 activity significantly increased at low and median levels and stabilized at high auxin levels. Further details of the statistical analysis methods can be found in the Experimental Procedures. (Also See Figure 2.5)
Figure 2.5 Auxin is required for the activation of ROP2 downstream signaling events.

(A): GFP-RIC4 distribution pattern in PCs of wild type, yuc1 yuc2 yuc4 yuc6 (yuc1/2/4/6) mutant, and yuc1/2/4/6 mutant treated with 20 nM NAA. GFP-RIC4 was localized to the cell cortex preferentially in lobe tips or lobe emergent sites of wild type PCs but was mostly diffuse in the cytosol in yuc1/2/4/6 PCs. NAA treatment increased the cortical localization of GFP-RIC4 and decreased its cytosolic signal in the mutant.

(B): Quantitative analysis of the cortical GFP-RIC4 signal. Analysis was performed for the cortical signal (PM signal divided by signal in the cytosol). The cortical signal of GFP-RIC4 was greatly decreased in yuc1/2/4/6 mutant (n>40, t-test p<0.001), but NAA treatment rescued GFP-RIC4 distribution to the cortex in yuc1/2/4/6 mutant (n>40, t-test p<0.01).

(C): RIC4-dependent cortical fine F-actin, visualized with transiently expressed GFP-mTalin, was found to preferentially associated with lobes in wild-type PCs (left) but was nearly absent in yuc1 yuc2 yuc4 yuc6 PCs (right). At least 20 cells are examined and had a similar pattern.
Figure 2.6 ABP1 is required for auxin perception that promotes PC interdigitation.

(A): The *abp1*-5 mutant is a TILLING line containing a point mutation (His59->Tyr) within the auxin binding pocket (Till et al., 2003; Woo et al., 2002). (Left) The crystal structure of maize Auxin-binding Protein 1 with bound NAA (PDB 1lrh). Maize ABP1 is a glycosylated homodimer that binds two NAA molecules (shown in red). High-mannose type oligosaccharide (shown in stick format) adorns the top, presumably the outward face of the dome-like complex, whereas the flat bottom presumably interfaces with a plasma membrane-docking protein. ABP1 monomers have a jelly-roll barrel fold formed by two antiparallel β sheets. Maize and Arabidopsis share 68% identity overall and 100% conservation in the binding pocket (outlined by the red box). (Right) The auxin-binding pocket is highlighted to show how H59 (sphere format) interacts with the carboxic acid group of NAA shown in red and with a zinc ion not shown (for clarity). The residue number is the same for both the mature maize and Arabidopsis protein sequences. H59 is mutated to a Y in the *abp1*-5 mutant.

(B): Defect in PC interdigitation in the *abp1*-5 mutant was not rescued by auxin. A recessive *abp1*-5 mutant allele, which contains a point mutation (H59Y) in the auxin binding pocket (A), shows a PC phenotype similar to that of the *yuc* quadruple mutant. Treatment with 20 nM NAA did not rescue the *abp1*-5 defect in PC interdigitation.

(C): PC interdigitation shown in (A) was quantitated by determining the mean lobe
number per µm² ± SD (n>400 cells from three individual plants). Wild type had significantly higher lobe number per µm² than *abp1*-5 (t-test, p<0.001). No significant difference was found between treatment with or without NAA (t-test, p>0.1).

(D): The defect in PC interdigitation in an inducible *ABP1* antisense line was not rescued by auxin. An *ABP1* antisense construct was expressed upon ethanol treatment (Braun et al., 2008). Without ethanol treatment, the PCs in this line were similar to wild-type PCs. Upon ethanol induction, *ABP1* antisense PCs were similar to the *abp1*-5 cells. As in the *abp1*-5 mutant, in the induced antisense line, treatment with 20 nM NAA did not rescue the PC interdigitation defect.

(E): PC interdigitation in the antisense line shown in (C) was quantitated by determining the mean lobe density ± SD (n>400 cells from three individual plants). Wild type had a significantly higher lobe density than the *ABP1* antisense line (t-test, p<0.001). No significant difference was found between treatment with or without NAA (t-test, p>0.1).

A double-blind analysis was performed and the results confirmed all of the phenotypic differences between mutants and treatments described in B and D) of this figure (See Figure 2.7).
Figure 2.7 Reduced ABP1 function in the *abp1-5* tilling line and ethanol inducible antisense or antibody line is responsible for the PC interdigitation defect.

(A): After five rounds of backcrosses to the wild type to eliminate background mutations in this tilling line, the PC interdigitation defect was retained, which suggests that the H59Y mutation was responsible for the phenotype. To further confirm this, we determined whether wild-type *ABP1* cDNA complemented the *abp1-5* phenotype by introducing a 35S::*ABP1* into the mutant. Three independent lines examined show the wild-type PC phenotype, as indicated by the mean lobe number and lobe length. An example of the complemented lines is shown in (A).

(B): Quantitative data confirm the recovery of *abp1-5* phenotype by 35S::*ABP1* (A). This indicates that *abp1-5* is a reduction-of-function mutant. PC interdigitation (both lobe number and lobe length) was reduced in *abp1-5* mutant (t-test, p<0.001, n>200), and this reduction was rescued in the complemented lines (t-test, p<0.001, n>200)

(C): Inducible ABP1 inactivation was generated by expressing antisense construct or the recombinant single-chain Fv12 anti-ABP1 under the control of the ethanol system (Braun et al., 2008). The plants were grown in MS solid media without sugar. A tube containing 5% ethanol was placed into the plate after seed germination to induce the Fv12 antiABP1 gene (Braun et al., 2008; David et al., 2007) and the PCs from 4- or 5-day old cotyledons were imaged. C shows representative images of PC phenotype without (left) or with ethanol induction in wild type, and mutants described above.
(D): Quantitative analysis of PC phenotypes shown in (C). At least 400 cells from 6 individual plants were analyzed in each line. (t-test for wild type with or without ethanol induction, p>0.1; for ABP1 antisense line, p<0.001; for ABP1 antibody line, p<0.001)

(E): Double blind assay for the quantification of PC phenotype in wild type, abp1-5, abp1 antisense mutants treated with auxin. The data set was identical to that described in Figure 2.5, and the double blind analysis produces nearly identical quantitative results as those shown in Figure 2.6.
Figure 2.8 Auxin can activate ROP2-RIC4 pathway through ABP1

(A) Measurement of GTP-bound GFP-ROP2 in protoplasts isolated from a abp1-5 line stably expressing 35S::GFP-ROP2 by co-immunoprecipitation assay described in Figure 2. This line was generated by crossing the abp1-5 mutant to a 35S::GFP-ROP2 (Jones et al., 2002). F2 seedlings expressing GFP and homozygous for abp1-5 were pooled and used for protoplast isolation. Auxin can not activated ROP2 in abp1-5 mutants compared to in wild type where auxin activate ROP2 within 30secs (Figure 2.4C).

(B-C): Loss of auxin activation of ROP2 in the abp1-5 mutant and the induced ABP1 antisense line. GFP-RIC4 distribution to the PM in isolated protoplasts was used to report ROP2 activation by auxin. (B) Representative images of GFP-RIC4 distribution in protoplasts isolated from different lines before and 5 min after auxin application. The bright field images (left) show intact protoplasts corresponding to the GFP-RIC4 fluorescent images at time 0. See Figure. 2.9 for representative images from the complete time course analysis. (C) Quantitative analysis of GFP-RIC4 distribution to the PM (as indicated by relative GFP intensity in the PM standardized with the cytosolic GFP intensity). Data are mean values from 10 protoplasts analyzed ± SD.
Figure 2.9 Auxin activation of ROP2 and ROP2-dependent accumulation of cortical fine F-actin is abolished by the abp1-5 and antisense expression.

(A): GFP-RIC4 localization pattern in wild type and the abp1-5 mutant. Left, confocal images showing that cortical localization of GFP-RIC4 was lost in abp1-5 PCs and was not rescued by NAA treatment.

(B): Quantitative analysis of GFP-RIC4 signal on both PM and lobe was decreased in the abp1-5 mutant (n>=25, t-test p<0.01) and could not be rescued by NAA application (n>=25, t-test p>0.1). Scale bar = 25 μm.

(C): RIC4-dependent cortical fine F-actin, visualized with transiently expressed GFP-mTalin (Fu et al., 2005), was found to preferentially associate with lobes in wild-type PCs (left), but was nearly absent in abp1-5 PCs (right). At least 20 cells were examined and exhibited similar GFP-mTalin localization patterns.

(D-F): Auxin-induction of RIC4 distribution to the plasma membrane was abolished by the abp1-5 mutation and ABP1 antisense expression. Protoplasts were isolated from leaves of the yuc1 yuc2 yuc4 yuc6 mutant (yuc1/2/4/6) (D), the abp1-5 mutant (E), and the inducible ABP1 antisense line (F). The GFP-RIC4 construct was introduced into isolated protoplasts, which were then treated with DMSO or 1 μM NAA for indicated lengths of time before being imaged on confocal microscopy. GFP-RIC4 distribution to the PM was significantly increased by 1-μM NAA treatment in the yuc1 yuc2 yuc4 yuc6 (yuc1/2/4/6) protoplasts but not in the abp1-5 or ABP1 antisense protoplasts. Quantitative analysis of GFP-RIC4 localization is
shown in Figure 2.8.

**Figure 2.10 PIN1 is localized to the lobe tip and is essential for auxin promotion of PC interdigitation.**

(A): Left: PIN1-GFP was preferentially localized to the tip of lobes in PC. Middle: Immunostaining of PIN1 in PCs. Arrows indicates the accumulation of PIN1 at the lobe region. Right: Immunostaining of PIN1 in ROP2RNAi rop4-1 mutant. Arrows (yellow) indicates the accumulation of PIN1 at the lobe region was lost in ROP2RNAi rop4-1. Arrowheads indicate internalized PIN1, which was greatly increased in the cytoplasm of ROP2RNAi rop4-1 cells. The experiment was repeated three times; more than 75 cells were examined, showing a similar localization pattern.

(B): PC shapes in wild type (left) and pin1-1 mutant (middle). pin1-1 PCs were slender with few lobes, a phenotype similar to a rop2-1rop4-1 double knockout mutant (data not shown). 20 nM NAA was unable to rescue pin1-1 phenotype in PCs (right).

(C): Quantitative data for (B). Lobe numbers per cell area in pin1-1 mutant and pin1-1 mutant treated with 20 nM NAA were quantified using double blind analysis as described in Figure 2.6. pin1-1 cells showed significantly reduced lobe formation compared to wide type (n=400, T-test p<0.001), and 20 nM NAA did not rescue this phenotype (n=400, T-test p>0.1). Higher NAA concentrations had no effect on the pin1-1 phenotype either (Figure 2.11A and B).

(D): GFP-RIC4 distribution pattern in PCs of wild type and pin1-1 mutant.
GFP-RIC4 was localized to the cell cortex preferentially in lobe tips or lobe emergent sites of wild type PCs but was mostly diffuse in the cytosol in *pin1-1* PCs.

(E): Quantitative analysis of the cortical GFP-RIC4 signal was performed as described in Figure 2.5. Cortical signal of GFP-RIC4 dramatically decreased in *pin1-1* mutant (n>25, t-test p<0.001),
Figure 2.11 PIN1 is essential for the activation of ROP2-dependent lobe growth, and transiently expressed dominant mutant of ROP2 promotes PIN1 internalization.

(A): NAA treatments did not alter pavement cell phenotype in pin1-1. Left panel shows the PCs before NAA treatment. Right panel show the PCs after 3 days of auxin treatment. Neither 20-nM nor 100-nM increased interdigitation in pin1-1 mutant, while 20 nM is optimal concentration that can dramatically induce interdigitation in wild type plants (Figure 2.2).

(B): Quantitative analysis for the phenotype in (A). No significant difference was found before and after auxin treatment (n>200 cells was measured from 3 individual plants for each treatment. t-test for all treatments, p>0.1)

(C): We used transient expression of a dominant negative mutant form of ROP2 (DN-ROP2) (Fu et al., 2002) to confirm that reduced PM localization and increased internalization of PIN1 in the rop2RNAi rop4-1 line (Figure 2.10A) was the direct effect of ROP2 and ROP4 but not the indirect result of lobing defect. In other cell types, it has been shown that PIN1 polarization to the PM involves asymmetric endocytosis and recycling of PIN1 (Dhonukshe et al., 2008). Our data described below show that inhibition of ROP2 directly causes increased PIN1 localization to a cytoplasmic structure (presumably an endosomal structure), implying that ROP2 promotes PIN1 localization to the PM by inhibiting PIN1 endocytosis from the ROP2 active site and/or by promoting its recycling to this site. (C) Left: Transient
expression of PIN1-GFP in Arabidopsis PCs. 1 µg 35S:: PIN1-GFP was bombarded to PC and incubated 24 hrs before imaging by confocal microscopy. Most PIN1-GFP signals were found on the PM. Accumulation of PIN1-GFP was preferentially associated with the lobe tips and putative emerging lobes (Arrowhead). Right: Co-expression of PIN1-GFP and DN-ROP2 in Arabidopsis PCs. 1 µg 35S::PIN1-GFP and 0.5 µg DN-ROP2 were both bombarded to PCs. Bombarded cells were incubated 24 hrs before imaging. DN-rop2 expression decreased PIN1-GFP signal on the PM, but increased the number of endosome-like PIN1-GFP dots in the cytoplasm (Arrow).

(D): Left: Quantitative analysis of PIN1-GFP signal on the PM. Ten cells from each experiment were used for quantification. Analysis was performed for the relative cortical signal (PM signal divided by signal in the cytosol) from images taken at medial confocal sections as shown in C. When co-expressed with DN-ROP2, the cortical signal was decreased (t-test p<0.01). Right: Quantification of endosome-like PIN1-GFP dots. Ten cells from each experiment were used for quantification. The mean number of internal PIN1-GFP dots per area was determined from images taken at medial confocal sections as shown in B. DN-ROP2 expression greatly increased the number of PIN1-GFP dots (t-test p<0.0001).

(E-F): PC phenotype in pin1-5. PCs from pin1-5 leaves show similar interdigitation defects as in pin1-1 mutant (Figure 5B). (E) Representative images of pin1-5 cell shape. (F) Quantification of lobe numbers in pin1-5 PCs (n>400, t-test p<0.001).
(G): Immunostaining of PIN1 in abp1-5 and ABP1antisense lines. Arrow indicates the localization of PIN1 in lobes, and arrowhead indicates internalized PIN1, which was greatly increased in abp1-5 and ABP1-antisense mutants as in ROP2RNAi rop4-1 mutant (Figure 2.10A) while preferential localization to the lob PM in these mutants was severely compromised.

(H): Quantification of PIN1 localization shown in G and Figure 2.10A. Number of internalized PIN1 dots per cell area was determined to quantify PIN1 internalization level in different mutants (Left). In ROP2RNAi rop4-1, abp1-5 and ABP1antisense lines, more PIN1 internalized to the cytoplasm and form endosome-like dots (n>75, t-test p<0.001). The PIN1 intensity ratio at lobe region vs indentation region is used to indicate PIN1 preferential localization to lobes in wild type cells, which is lost in mutants (n>75, t-test p<0.001).
Figure 2.12  Auxin induces ROP6 activation and the association of RIC1 with cortical MTs in an ABP1-dependent manner.

(A): PCs phenotype in yuc1 yuc2 yuc4 yuc6 (yuc1/2/4/6) and abp1-5 mutants. PCs in both yuc1/2/4/6 and abp1-5 had wider neck regions than wild type, similar to both rop6-1 and ric1-1 mutants (Fu et al., 2009), but in contrast to ROP2RNAi rop4-1 which had narrower neck (Fu et al., 2005).

(B): Quantitative analysis of PCs phenotype shown in (A). Both yuc1/2/4/6 (t-test, p<0.01) and abp1-5 (t-test, p<0.001) shows wider neck regions compared to wild type, which is similar to in rop6-1 and ric1-1 mutants. Data are mean neck width ± SD. (n>400 cells from three individual plants)

(C): YFP-RIC1 localization in yuc1/2/4/6, abp1-5 and rop6-1 mutants. YFP-RIC1 formed dot-like structure along cortical MTs (left) as shown previously (Fu et al., 2005); This localization pattern is dependent on ROP6 (Fu et al., 2009). In yuc1/2/4/6 and abp1-5 mutants, YFP-RIC1 lost its association with MTs as in rop6-1, implying that ROP6 is inactivated in the yuc1/2/4/6 and abp1-5 mutants (n>25). Arrowhead indicated in rop6-1 mutants, YFP-RIC1 was mostly shifted to lobe regions where ROP2 was presumably activated. This YFP-RIC1 localization pattern is different from that in the yuc1/2/4/6 and abp1-5 mutants, where YFP-RIC1 became diffusely localized to the cytosol because ROP2 is inactivated in these mutants. In addition, some YFP-RIC1 signal remained associated with MTs in rop6-1 but not in yuc1/2/4/6 and abp1-5, likely due to the existence of another ROP functionally overlapping with
ROP6 or of a ROP6-independent pathway downstream of auxin/ABP1.

(D): Auxin enhanced the association of YFP-RIC1 with cortical MTs like beads on a string in a rop2-1 rop4-1 double mutant, but not in the abp1-5 mutant. YFP-RIC1 construct was introduced PCs using a projectile-mediated transformation and transformed leaves were treated with NAA (10 nM), and YFP-RIC1 localization pattern was visualized by confocal microscopy before and 10 min after treatment. In rop2-1 rop4-1 PCs, YFP-RIC1 was strongly associated with MTs and exhibited a beads-on-a-string pattern. NAA enhanced this localization pattern. Arrowheads indicate the region where YFP-RIC1-lining filaments were increased. In abp1-5 cells, YFP-RIC1 association with MTs was weak and did not show the dotted pattern. NAA treatment did not alter the YFP-RIC1 localization in these cells. At least 15 cells were tracked for each mutant and showed similar response to NAA. (Scale bar =10 μm)

(E): A time-course analysis of YFP-RIC1 localization on individual MTs. YFP-RIC1 was imaged by confocal microscopy at the indicated times after NAA treatment. YFP-RIC1 dots gradually increased in both intensity and number by auxin treatment in rop2-1 rop4-1 but not abp1-5 cells.

(F-G). Quantitative analysis of YFP-RIC1 dot number and intensity shown in (D and E). (F), The number of YFP-RIC1 increased along the MT bundle which was measured by the number of YFP-RIC1 dots divided by the length of this MT. Data are mean Dots Number per μm ± SD (n=50) (G), Average intensity of YFP-RIC1 dots
was measured from 0 mins to 8 mins. The intensity at time 0 was standardized as 1. Data are relative average intensity compared to time 0 ± SD (n=100).

(H) Measurement of GTP-bound GFP-ROP6 in protoplasts isolated from a \textit{abp1-5} line stably expressing \textit{35S::GFP-ROP6} by co-immunoprecipitation assay described in Figure 2. This line was generated by crossing the \textit{abp1-5} mutant to a \textit{35S::GFP-ROP6}. F2 seedlings expressing GFP and homozygous for \textit{abp1-5} were pooled and used for protoplast isolation. Auxin can not activate ROP6 in \textit{abp1-5} mutants compared to in wild type where auxin activate ROP6 within 30secs (Figure 2.4C).
Figure 2.13 ABP1-perceived auxin is essential for activation of ROP6-RIC1 pathway

(A): Immunostaining of RIC1 in yuc1/2/4/6 and abp1-5 mutants confirmed that RIC1 lost its binding with MT bundle shown in (Figure 2.12C). Dot-like localization pattern seen in wild type cells were absent in either mutant. At least 15 cells were examined which showed a similar localization pattern.

(B): Immunostaining of MT in yuc1/2/4/6 and abp1-5 mutants showed the disorganized MT arrays in these mutants, which is similar to rop6-1 and ric1-1 mutants (Fu et al., 2009). At least 15 cells were examined which showed similar localization pattern.
Figure 2.13 A model for the ABP1- and ROP GTPase-based auxin signaling mechanisms underlying interdigitating growth in PCs.

(A): Localized extracellular auxin, which results from a PIN1-mediated positive feedback loop, coordinately activates two ABP1-dependent pathways. The ROP2-RIC4 pathway promotes lobe formation through actin microfilaments and the accumulation of localized auxin through PIN1-dependent positive feedback loop. The ROP6 pathway promotes indentation through ordered cortical MTs.

(B): A model for auxin control of interdigitating growth in PCs through the intercellular and intracellular coordination of the ROP2 and ROP6 pathways that are both activated by PIN1-dependent localized extracellular auxin. We propose that at the lobing side of the cell wall bordering two neighboring cells, an auxin→ROP2→PIN1→auxin feedback loop is initiated by an unknown mechanism, leading to a localized accumulation of auxin in the same cell wall region. This rapidly establishes the site for lobe formation and subsequent lobe outgrowth. The PIN1-exported auxin rapidly diffuses to the complementary side of the neighboring cell and activates the ROP6-RIC1 pathway, which locally promotes the formation of well-ordered MT arrays and indentation in this cell. Within each of these cells, the ROP2-RIC4 and ROP6-RIC1 pathways are mutually exclusive, which allows for the formation of alternating lobes and indentations (Fu et al., 2005).
Chapter 2:

ABP1 and TMK form an auxin receptor complex on the plasma membrane to activate ROP signaling in Arabidopsis.
3.1 Abstract:

ROP small GTPase controls pavement cell morphogenesis through an elaborate signaling network. Auxin acts as a coordinator that activates both ROP2 pathway and ROP6 pathways that promote lobe protrusion and neck indentation in pavement cells, respectively (Xu et al., 2010). Auxin binding protein 1 (ABP1) has been suggested to be the auxin receptor that mediates perception of auxin that can activate ROP signaling (Xu et al., 2010), however the mechanism for auxin activation of ROPs is still unknown since ABP1 localized to the outer surface of the plasma membrane and can not directly bind with ROPs. Here we report a family of receptor like kinases (RLK) called TMK that can bind with ABP1 on the plasma membrane and activate ROP GTPase in pavement cells. tmk mutant showed similar phenotype and auxin insensitivity to abp1 mutant. Both ROP2 signaling and ROP6 signaling are not activated in tmk mutant. ABP1 and TMK1 are confirmed in a same complex on PM by co-immuno-precipitation. In the meanwhile, TMK kinase domain can directly bind with and phosphorylate N terminal of SPK1, which is a putative ROPGEF that can activate ROP2 (Basu et al., 2008). Therefore, we concluded that TMK1 and ABP1 may form an auxin receptor complex on the PM that mediates auxin activation of ROP signaling pathways.
3.2 Introduction

Auxin is a multifunctional plant hormone regulating various aspects of plant growth and development. Recent elegant studies have established a simple auxin perception and signaling mechanism controlling auxin induced gene expression, which involves auxin stabilizing the interaction between the TIR1-family F-box proteins and the IAA/AUX proteins, transcriptional repressors (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005; Tan et al., 2007). This activates the IAA/AUX degradation pathway, releasing auxin-induced gene expression (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). However, this pathway cannot account for all the actions of auxin in plant cells.

AUXIN BINDING PROTEIN1 (ABP1) has been proposed to be an auxin receptor that rapidly activates cell expansion. (Chen et al., 2001a; Chen et al., 2001b; Jones et al., 1998) Recent work has demonstrated the role of ABP1 in mediating activation of ROP GTPase by auxin (Xu et al., 2010). In leaf pavement cells, ABP1 mediated fast activation of both ROP2 and ROP6 by auxin, which will further regulate F-actin accumulation at the lobe region or MT organization at the indentation region, respectively (Fu et al., 2005; Fu et al., 2009; Xu et al., 2010). However, ABP1 is not a transmembrane protein and likely works with a trans-membrane partner or co-receptor. Identification of such an assumed trans-membrane protein will be crucial for understanding how auxin is perceived at the cell surface and how it leads to ROP activation at the cytoplasmic side of the PM.
The structural features of plant receptor-like kinases (RLKs), which contain an extracellular domain to perceive signals and a transmembrane domain and intracellular kinase domain to transmit signals, imply their function as cell surface receptor capable of transmitting extracellular signals to intracellular signaling pathways. BRI1 is identified as BR receptor on the cell-surface that mediates a signaling cascade after perceiving BR signal (Kinoshita et al., 2005; Li et al., 2002; Tang, 2008; Wang and Chory, 2006). CLV1 is essential for maintaining the meristem after perceiving signal peptide CLV3 that is secreted from adjacent cells (Brand et al., 2000; Fletcher et al., 1999; Ogawa et al., 2008; Schoof et al., 2000). These examples reveal the important roles of RLKs in plant development and also provide the possible working model for RLKs in plant. However, there are more than 400 RLKs with divergent extracellular motifs and cytoplasmic serine/threonine kinase domains (Shiu and Bleecker, 2001). Most members from this superfamily of RLKs are not assigned to specific function.

In this chapter, we describe a four-members family of RLK proteins called TMK that I showed participating in the auxin mediated activation of ROP signaling. We provide the evidence that ABP1 and TMK functionally work as an auxin receptor complex on the plasma membrane that mediates auxin activation of ROPs. We also proved that ABP1 and TMK are in the same protein complex in vivo. Furthermore, we demonstrated that TMK1 kinase domain could interact with a putative RhoGEF called SPIKE1 that may further activate ROP GTPase.
3.3 Results:

Phenotype characterization of \textit{tmk} mutant in plant polar growth

TMK encodes a receptor like kinase with leucine-rich repeats in the extracellular domain (Chang et al., 1992). The kinase domain of TMK can be autophosphorylated on serine and threonine residues (Schaller and Bleecker, 1993). Four members of the TMK subfamily were identified by the sequence similarity in kinase domain. All four TMKs contain 10 LRR repeats in the extracellular domain that are separated into two blocks by a unique island sequence region (Figure 3.1A) (Dai, N., unpublished). T-DNA insertion mutants of these four TMKs are identified, and multiple \textit{tmk} mutants are generated by Bleecker’s group (Dai, N., unpublished). The \textit{tmk1;tmk2;tmk3;tmk4} quadruple mutant (\textit{tmk1234}) showed severe defects in growth and development, including roots, hypocotyls, leaves and stamen filaments due to inhibition of cell growth and cell proliferation (Dai, N., unpublished). Here I show that TMKs also control polar growth and cell morphogenesis of different cells in Arabidopsis. In \textit{tmk1234}, cotyledon and leaf morphology is altered. 46\% of \textit{tmk1234} seedlings showed single cotyledon similar to \textit{pin1} mutant (Friml et al., 2003) (Figure 3.1C and 3.1D), suggesting a possible defect in polar auxin transport in this mutant. Fused leaf is also found in \textit{tmk1234} mutant (Figure 3.1C) that is similar to the fused cotyledon in \textit{pin1-1} mutant (Figure 3.1D). Cotyledon morphology defects indicate patterning defect during embryogenesis. Therefore, we check the phenotype of \textit{tmk1234} mutant during embryo development. Interestingly, we found the pattern of
Embryo development is totally disrupted because cell division pattern is disorganized (Figure 3.2C), which is similar to abp1-1 mutant (Figure 3.2B) (Chen et al., 2001b). The orientation of cell division is determined by cell polarity that is controlled by polar auxin distribution (Friml et al., 2003). Thus, TMK might regulate cell polarity establishment during embryo development. In pavement cells, tmk1234 also showed morphogenesis defects. Mutant pavement cells nearly lost all lobes compared with wild type (Figure 3.3A). Previous work showed that lobe outgrowth is considered as local polar growth that is controlled by PIN1 polar transport of auxin at the lobe region of pavement cells (Xu et al., 2010). Therefore, we considered that TMKs also regulate polar growth in pavement cells.

**Phenotype similarity between tmk mutant and abp1 mutant**

An ABP1 mediated auxin-signaling pathway regulates cytoskeleton organization through ROP GTPase in plant cells (Xu et al., 2010). However, the molecular mechanism about how ABP1 activates ROP GTPase after perceiving auxin is still unknown. ABP1 is partially located on the outer surface of the plasma membrane and does not contain any functional domain that may transmit auxin signal to the ROP GTPase signaling pathway. We propose that ABP1 has a functional partner protein on the plasma membrane that facilitates the transmission of the auxin signal to intracellular downstream pathways. Interestingly, the phenotype similarity between tmk1234 and abp1 mutants indicated TMK and ABP1 might be in the same signaling
pathway. In pavement cells, ABP1 is proved to mediate the auxin activation of interdigitating growth (Xu et al., 2010). ABP1 can activate both ROP2 pathway and ROP6 pathway that promote outgrowth at lobe site, and restrict growth at indentation site, respectively (Xu et al., 2010). Therefore, PCs in abp1 mutant showed less and weaker lobes, and wider necks compared to wild type PCs. Interestingly, tmk1234 mutant showed similar defects as in abp1 mutant (Figure 3.2A) (Xu et al., 2010). In embryo, similar disorganized cell division pattern in both tmk1234 and abp1 mutants also implies that TMK and ABP1 may function in the same pathway (Figure 3.2) (Chen et al., 2001b). To test this hypothesis, we further investigated whether tmk1234 mutant also lose auxin sensitivity as in abp1 mutant. In leaf pavement cells, auxin can promote formation of more lobes in wild type seedlings but not in abp1 mutant (Xu et al., 2010). Similarly, auxin did not induce lobe formation in tmk1234 mutant (Figure 3.3). These results demonstrated that ABP1 and TMK are both required for auxin promotion of interdigititation growth in PCs.

**Both ABP1 and TMKs mediated auxin activation of ROP GTPase in pavement cells**

ABP1 mediates fast activation of ROP GTPase by auxin in pavement cells (Xu et al., 2010). When auxin activates ROP2 in lobe region, active ROP2 recruits downstream effector called RIC4 to this region that promotes accumulation of F-actin. Since RIC4 can only bind with active ROP2, we use GFP-RIC4 as an in vivo marker for active
ROP2 in pavement cells (Fu et al., 2005; Xu et al., 2010). In the meanwhile, auxin also activates ROP6 in the indentation region, and active ROP6 activates downstream effector RIC1, causing RIC1 to bind with MT and further promote the formation of well-organized MT. Because RIC1 binds with MT only when it is activated by ROP6, we use YFP-RIC1 as an active ROP6 marker in pavement cells (Fu et al., 2009; Xu et al., 2010). To test whether ABP1 and TMK are in the same pathway that mediates auxin activation of ROP GTPase, we examined how ROP2 and ROP6 activities were affected in tmk1234 mutant. GFP-RIC4 is transient expressed in PCs of tmk1234 mutant. In wild type, GFP-RIC4 prefers to localize at the tip of the lobes where ROP2 is activated, however in tmk1234 mutant, GFP-RIC4 lose lobe tip localization and PM localization, as in abp1-5 mutant and rop2RNAirop4-1 mutant. This implies that ROP2 is not activated in the tmk1234 mutant (Figure 3.4A and 3.4B). We also transiently expressed YFP-RIC1 in pavement cells of tmk1234 mutant. YFP-RIC1 was associated with MT in wild type PCs and formed dots along the MT, however YFP-RIC1 nearly lose all MT binding activity in tmk1234 mutant, as in abp1-5 and rop6-1 mutants (Figure 3.4C and 3.4D) (Fu et al., 2009; Xu et al., 2010), which indicates that the ROP6 pathway is also abolished in tmk1234 mutant.

To further test whether TMK also mediates auxin activation of ROP GTPases, we performed a similar ROP activity assay as described in (Xu et al., 2010). We crossed GFP-ROP2 or GFP-ROP6 to tmk1234 mutant, and screened homozygous plants by genotyping. Protoplasts isolated from 2-week old leaves were treated with different
concentrations of auxin for 2 mins. RIC1 fusion protein conjugated to agarose beads was used to specifically capture GTP-bound GFP-ROP2 or GFP-ROP6 in auxin-treated samples. In wild type cells, activity of ROP2 and ROP6 increased dramatically to nearly four folds after 100 nM NAA treatment (Figure 3.5) (Xu et al., 2010). However in tmk1234 mutant auxin activation of ROP2 and ROP6 was abolished as in abp1-5 (Figure 3.5) (Xu et al., 2010). Together these data indicated that TMKs functionally act in the pathway leading to auxin activation of ROP GTPase signaling pathways as does ABP1.

**ABP1 and TMK1 are in the same complex in vivo**

We have confirmed that ABP1 and TMKs are both functionally required for auxin activation of Rho GTPase in pavement cells. It is likely that ABP1 and TMK formed an auxin receptor complex on the PM to mediate auxin activation of ROP GTPase. To test this, we used Co-IP method to detect the interaction between ABP1 and TMKs in vivo. We used TMK1-GFP transgenic plants to extract proteins, and used GFP antibody to immuno-precipitate the TMK1 complex, and finally used ABP1 antibody to detect whether ABP1 is in TMK1 complex. In the meanwhile, we also used ABP1 antibody to immuno-precipitate the ABP1 protein complex, and used GFP antibody to test whether TMK1 is in the ABP1 complex. Both methods confirmed that ABP1 and TMK1 are in the same protein complex in vivo (data not shown). Therefore we proposed that ABP1 and TMK1 together formed an auxin receptor
complex on the PM to mediate auxin activation of ROP signaling.

**TMK1 sub-cellular localization implies a role for TMKs in polar growth.**

Since ABP1 and TMKs appear to form an auxin receptor complex on the PM, the sub-cellular localization pattern of TMK may indicate the sites where auxin perform its function. We examined TMK localization in a transgenic line expressing TMK1-GFP, in which GFP was fused to the C-terminus of TMK1 and was expressed behind the native TMK1 promoter (Dai, N., unpublished). This GFP fusion construct complemented the tmk1;tmk4 mutant phenotype, which indicates it is a functional TMK in plant cells (Dai, N., unpublished). In pavement cells, TMK1-GFP localized to the lobe initiation sites at very early stages (Figure 3.6A); and in later stages, it localized to both lobe region and neck region (Figure 3.6B), which is consistent with the finding that TMKs can mediate activation of ROP2 at lobe region and ROP6 at neck region. In early stages, auxin can activate ROP2 that will initiate a positive feedback that can continuously activate ROP2 (Xu et al., 2010). In the meanwhile, this positive feedback will increase the auxin concentration in extracellular regions, and this auxin will activate ROP6 to form indentation coordinately in adjacent cells (Xu et al., 2010). And both activations are dependent on ABP1/TMK1 receptor. More interestingly, TMK1-GFP is also polarly localized to certain side to cells from other organs, including root cells, and embryo cells. In both root and embryo, TMK1-GFP polar localization pattern is similar to PIN1 localization,
which further supports the role of TMK1 in determining polarity of cells (Figure 3.7C and 3.7D) (Friml et al., 2004; Scheres and Xu, 2006).

**TMK1 kinase domain interacts with SPIKE1**

Guanine nucleotide exchange factors (RhoGEFs) are direct activators for RhoGTPase by switching the GDP-bound inactive form of RhoGTPase to the GTP-bound active form. SPIKE1 is identified as RhoGEF in plants, and spk1 mutant showed severe cell and organ shape defect which is similar to the phenotype of rop2 mutants (Fu et al., 2005; Fu et al., 2002; Qiu et al., 2002). Recent work showed SPIKE1 could bind to ROPs in a DHR2-dependent manner and act as a RhoGEF (Basu et al., 2008).

ABP1 and TMK1 may form the auxin receptor complex on the PM that mediate auxin activation of ROP GTPases, but the mechanism how TMK1 activates ROPs is still unknown. We propose that TMK activate ROPs through RhoGEFs. First, we investigated whether TMK1 interacts with SPIKE1. We used TMK1 kinase domain as bait, and truncated SPIKE1 proteins as prey in a yeast-2-hybrid assay. Interestingly, TMK1 kinase domain interacted with the N terminal (1-135aa) of SPIKE1 (Figure 3.7). The N-terminus of SPIKE1 interacts with the C-terminus of SPIKE1 to self-inhibit the GEF activity of the C-terminaus (Zhen, X., unpublished). We propose that TMK1 will phosphorylate SPIKE1 N-terminal and release the self-inhibition of SPIKE and release its activity. Further work need to analysis whether TMK1 change the phosphorylation of N terminal and further change the GEF activity of SPIKE1.
3.4 Discussion:

These findings here have several important implications. First, we show that ABP1 and TMK act as an auxin receptor complex on PM that transmits auxin signal to ROP GTPase signaling. This gives an unprecedented understanding of molecular mechanism and signaling events downstream of the auxin perception by ABP1, which illustrate the role of ABP1 as an auxin receptor. Second, our findings give new insights into auxin signaling that happens on plasma membrane to regulate cell morphogenesis, which is distinct from TIR1-based nuclear auxin signaling pathway. Finally, our results provide a possible mechanism about how RhoGEFs are activated by the developmental signal through receptor like kinase in plants.

**ABP1 and TMK together form a cell surface auxin receptor that activates ROP signaling**

Previously, we have shown that ABP1 is required for the rapid activation of the PM-localized ROP2 and ROP6 pathways by auxin (Xu et al., 2010). Cell surface-associated ABP1 also mediates auxin activation of cell expansion although ABP1 also partially localize to ER (Chen et al., 2001a; Chen et al., 2001b). Consistent with the cell surface site for the ABP1-mediated auxin perception, we found that the PIN1-exported extracellular auxin is required for ROP2 activation on plasma membrane (Xu et al., 2010). However, ABP1 is not a transmembrane protein and may work with a trans-membrane partner to transmit auxin signal to downstream pathways. Identification of TMK, as a co-receptor for ABP1, will be crucial for
understanding how auxin is perceived at the cell surface and how it leads to ROP activation in the cytoplasmic side of the PM.

**A working model for ABP1/TMK mediated auxin signaling on cell surface.**

Based on our previous and current results (Fu et al., 2005; Fu et al., 2009; Xu et al., 2010), we proposed a working model for ABP1/TMK auxin receptor on cell surface. Once auxin is perceived by ABP1, it enhances the interaction between ABP1 and TMK that is similar to the role of auxin as “molecular glue” in TIR1-mediated pathway (Tan et al., 2007). This interaction promotes the dimerization of TMKs on PM (Figure 3.8), and further activates their self-phosphorylation and releases their kinase activity. Function analysis of RhoGEFs in plant and the structure biology of RhoGEF-ROPs complex indicate the auto-inhibition mechanism of RhoGEFs that the C-terminal inhibits GEF activity of PRONE domain (Gu et al., 2006; Thomas et al., 2007; Zhang and McCormick, 2007). SPIKE1, another RhoGEF candidate, also has self-inhibition through the interaction between N-terminus and C-terminus that can inhibit GEF activity of C-terminus (Zhen, X., unpublished) (Basu et al., 2008). Our results showed that TMK kinase domain can interact with N-terminal of SPIKE1, therefore we propose TMK can phosphorylate SPIKE1 N-terminal and release its GEF activity and finally activate downstream ROPs.
3.5 Methods and Materials

Plant materials and growth condition

Arabidopsis plants were grown at 22°C on MS agar plates or in soil with 16-hr light/8-hr dark cycles unless indicated otherwise. The tmk1234 quadruple and the pTMK1::TMK1-GFP transgenic line were kindly provided by Bleecker’s group {Hagen, 2002 #57;Cheng, 2006 #35}. abp1-5 mutant is described in (Xu et al., 2010). The following gene-specific primers were used for genotyping (Dai, N., unpublished):

TMK1, 5’ CGATCCTTGTACTAACTGGATTGGGATA 3’
5’ CCGAAACTGTAATCTTAACACTCTCTATT 3’;
TMK2, 5’CCGAAATCTAGTGGTTCTTCA-TGGTTA3’
5’ GTGTCTCTTCCGTTGACCTCCATTGCTT 3’;
TMK3, 5’ CTGTAGTATTTCGTTGCGTTCCTACTGAA3’
5’ GAAGAGTGACCGATTCTGCTGATT 3’;
TMK4, 5’ GACCTAGGAATGTCAGGAACGATCGAA 3’
5’ TTCACCCTAGCCGGAACCTTAGGTAT 3’.

Confocal and imprinting analysis of leaf Arabidopsis PC shape

PC shape from Arabidopsis cotyledons was imaged directly on confocal microscopy (Leica SP2 confocal microscope) or indirectly by an imprinting method {Mathur, 1997 #63}. Since PCs are auto-fluorescent, their cell outlines can be imaged on confocal microscopy with the following settings: excitation 351 nm or 364 nm, 50%
laser power and emission 400-600 nm. Additional image analyses involved use of Metamorph 4.5. The images are edited by photoshop 7.0 by adjusting figure sizes and resolution and adding labels.

**Ballistics-mediated transient expression in leaf epidermal cells**

Subcellular localization of GFP-RIC4, YFP-RIC1 and F-actin was analyzed by use of transiently-expressed *pBI221:GFP-RIC4, pUC:YFP-RIC1* constructs as described previously (Fu et al., 2005; Fu et al., 2002). We used 1 mg *pBI221:GFP-RIC4* and 1 mg *pUC:YFP-RIC1* for particle bombardment. GFP and YFP signal was detected 5 hrs after bombardment by use of a Leica SP2 microscope (GFP: 488 nm excitation, 25% power; excitation 520 nm-600 nm, gain at 600; YFP: 514 nm excitation, 25% power; excitation 530 nm-600 nm, gain at 600). Cells at stage II showing similar medium levels of GFP (Fu et al., 2005; Fu et al., 2002) were chosen for GFP marker analysis. For 3-D reconstruction, optical sections in 1.0-mm increments were imaged for each cell by use of the Leica software.

**Naphthalene-1-acetic acid (NAA) treatments**

NAA (Sigma, St. Louis, MO) was dissolved in DMSO as 0.5 M stock solutions, which were diluted to the indicated concentrations in liquid MS (for seedling treatments) or W5 media (for protoplast treatments). Seeds were germinated in the liquid MS media containing NAA or NPA. Each treatment was repeated at least three
times with the corresponding controls.

**Imaging analysis of embryo development**

Young silique is opened partially and fixed in fixation buffer (50% Methonal, and 10% Acetic Acid) overnight at 4°C. The fixed siliques are transferred to cleaning buffer (1%SDS, 0.2N NaOH) and incubate at room temperature overnight. The siliques are rinsed in water and transfer to 25% bleach solution (2.5% active Cl) for 1 min to 5 mins and then are rinsed in water again. The embryo outline is imaged on bright field microscope (Nikon).

**ROP2 and ROP6 activity assays in protoplasts**

ROP activity biochemical assay is described in (Xu et al., 2010), in which GFP-tagged active ROP2 or ROP6 was pulled down by use of MBP-RIC1. Protoplasts were isolated from leaves of 2- or 3-week old 35S::GFP-ROP2 or 35S::GFP-ROP6 transgenic seedlings in both wild type background or tmk1234 mutant background as described previously (Jones et al., 2002; Sheen, 2001). Isolated protoplasts were treated with different concentrations of NAA and frozen by liquid nitrogen. Total protein was extracted from $10^5$-$10^6$ treated protoplasts using extraction buffer (25 mM HEPEs pH 7.4; 10 mM MgCl$_2$; 10 mM KCl; 5 mM DTT; 5 mM Na$_3$VO$_4$; 5 mM NaF; 1 mM PMSF; 1% protease inhibitor from Sigma, St. Louis; 1% TritonX-100) at 4°C. Twenty micrograms of MBP-RIC1-conjugated agarose
beads were added to the protoplast extracts, and incubated at 4 °C for 3 hrs. The beads were washed using a washing buffer (25 mM HEPEs pH 7.4; 1 mM EDTA; 5 mM MgCl₂; 1 mM DTT; 0.5% TritonX-100) three times at 4 °C (5 mins each). GTP-bound GFP-ROP2 or –ROP6 that was associated with the MBP-RIC1 beads was used for analysis by western blotting with an anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Prior to the pull-down assay, a fraction of total proteins was analyzed by immunoblot assay to determine total GFP-ROP2 or –ROP6 (GDP-bound and GTP-bound GFP-ROP2 or -ROP6). The amount of GTP-bound GFP-ROP2 or -ROP6 was normalized to that of total GFP-ROP2 or –ROP6. The level of GTP-bound ROP2 or ROP6 relative to the control (0 nM NAA at 0 mins) was calculated by dividing the amount of normalized GTP-bound ROP2 or ROP6 from each treatment by the normalized amount from the control, which is defined as “1”.

**Co-immunoprecipitation assay**

The Co-IP is described as in (Liu et al., 2008). 1) Antibody-Protein A beads are preparation (For GFP antibody, we use anti-GFP-beads(santa cruz biotechnology) directly): Antibody is bound to Protein A beads to improving recovery of antigen-antibody complex within short incubation time. Aliquot 5 ul Protein A-sepharose beads (GE healthcare) into 1.5 ml tube. Wash with 1 ml Binding buffer, and leave beads in about 10 ul liquid. Add antibody to the slurry (10 ug anti-GFP antibody). Incubate on ice for >2 hrs. While protein extracts is centrifuged (see
below), wash Protein A beads with 1 ml Binding buffer and leave about 30 ul liquid.
Aliquot 10 ul slurry of beads into 3 spin columns (1 ml column, BioRad) (keep slurry
at the side of tube wall till extract is added). Keep columns in 2 ml microfuge
collecting tubes.

2) Prepare plant extracts:

NEB buffer: 20 mM HEPES, pH7.5, 40 mM KCl, 1 mM EDTA.

NEB+T buffer: NEB buffer containing 1% Triton X-100.

Wash buffer: 20 mM HEPES, pH7.5, 40 mM KCl, 0.1% Triton X-100.

Weigh 0.5 g tissue in a mortar on ice. Add 1mM PMSF and protease inhibitor cocktail
to 0.5 ml NEB buffer and add buffer to tissue. Grind with pestle till tissue
homogenized (about 150 rounds). Add 1 ml NEB+T buffer. Grind again about 20
round (forcefully but with small motion to avoid foam). Filter through 2 layers of
microcloth and a 40 um nylon cell strainer. Force extract through a #27 needle with a
5 ml syringe five times to sheer nuclei. Transfer extracts into a 2 ml tubes and spin at
20,000g for 10 min at 4 C in a microfuge. Save 50 ul supernatant for gel and add 1 ml
to Protein A-antibody beads in spin column (prepared in step 1).

3) IP: Add 1 ml extract to the Protein A-antibody beads. Mix by rotating at 4 C for 20
min (this can be longer if the protein is stable). Spin at 2000 RPM for 20 sec to collect
flow through (freeze 50 ul in liquid N2 for gel). Switch to new tube and spin again to
remove all extracts. Wash column by adding 1 ml Wash Buffer and spinning 10 sec.
Repeat wash with 800 ul, 600 ul, 600 ul Wash Buffer. Empty the collecting tube after
each wash. Spin again to remove all liquid from column.

4) Elute: Put spin column in a new collecting tube (1.5 ml). Add 30 ul SDS sample buffer (or 2% SDS). Incubate at room temperature for 5 min. Spin to collect elute. Load elute back on column again. Incubate 2 min. Spin to collect elute. Analyze 15 ul elute by western blotting (together with input and flow through).
3.6 References:


Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L.,


3.7 Figures and Legends

Figure 3.1 TMK controls polar growth in arabidopsis
Figure 3.2 Polarity of embryo is defect in *tmk1234* mutant
Figure 3.3 TMK mediated auxin promotion of pavement cell interdigitation
Figure 3.4 Both ROP2 pathway and ROP6 pathway are inactive in *tmk1234* mutant.
Figure 3.5 TMK mediate auxin activation of both ROP2 and ROP6
Figure 3.6 Polar localization of TMK1 in different cells.
Figure 3.7 TMK kinase domain can interact with SPK1 N terminal
Figure 3.8 A working model for ABP1/TMK auxin receptor on PM
Figure 3.1 TMK controls polar growth in Arabidopsis

A: The domain organization of TMK. Two LRR domains are found at the extracellular region. A specific bridge located between two LRR domains. A functional kinase domain is found at C-terminal.

B: Cotyledon phenotype in tmk1234 mutant. The 1-week-old seedlings are tested. The tmk1234 mutant showed strong cotyledon polarity defect. 46% of seedlings only have one cotyledon in tmk1234 mutant. It also generates specific leaf cup that is similar to in pin1-1 mutant. Red arrows indicate the leaf cup in plants.
Figure 3.2 Polarity defect in the embryo of \textit{tmk1234} mutant.

A: Embryos in wild type plant. Organized cell division pattern will reflect the polarity of embryo. In upper cell, the first division is precisely determined by auxin gradient that will affect the polarity of embryo.

B: The cell division pattern is totally disorganized in \textit{abp1-1} mutant that will cause the embryo lethal in \textit{abp1} knock out allele (Chen et al., 2001b).

C: The first division of upper cell in \textit{tmk1234} mutant is disorganized. The orientation of cell division is abnormal as indicated by red arrows (left and middle). This disorganized first division will further cause the total abnormal embryo division pattern (right).
Figure 3.3 TMK mediate auxin promotion of interdigitation in pavement cells.

A: Auxin increased the interdigitation of wild-type PCs. Seedlings was cultured in liquid MS with or without 20 nM NAA, and cotyledon PCs were imaged 4 days after stratification.

B: Interdigitation defect in tmk1234 mutant cannot be rescued by auxin application, which is similar in abp1-5 mutant.

C: Quantitative analysis of PC interdigitation showed in A and B. The degree of interdigitation in PCs was quantified by determining the density of lobes for each PC. Data are mean lobe number per mm2 ± SD (n>400 cells from three individual plants). The tmk1234 mutant had a significantly lower density of lobes than Col-0 wild type (t-test, p<0.01), and NAA significantly increased the mean density of lobes in Col-0 wild type (t-test, p<0.001) and but not in tmk1234 (t-test, p>0.1).
Figure 3.4 Both ROP2 pathway and ROP6 pathway are inactive in \textit{tmk1234} mutant

\textbf{A}: GFP-RIC4 distribution pattern in PCs of wild type and \textit{tmk1234} mutant. GFP-RIC4 was localized to the cell cortex preferentially in lobe tips or lobe emergent sites of wild type PCs but was mostly diffuse in the cytosol in \textit{tmk1234} PCs.

\textbf{B}: Quantitative analysis of the cortical GFP-RIC4 signal. Analysis was performed for the cortical signal (PM signal divided by signal in the cytosol). The cortical signal of GFP-RIC4 was greatly decreased in \textit{tmk1234} mutant (n>40, t-test p<0.001).

\textbf{C}: YFP-RIC1 localization pattern in PCs of wild type and \textit{tmk1234} mutant. YFP-RIC1 formed dot-like structure along cortical MTs (left) as shown previously (Fu et al., 2005); This localization pattern is dependent on ROP6 (Fu et al., 2009). In \textit{tmk1234} mutant, YFP-RIC1 lost its association with MTs as in \textit{rop6-1}, implying that ROP6 is inactivated in the \textit{tmk1234} mutants (n>20).

\textbf{D}: Quantitative analysis of YFP-RIC1 MT bundles in wild type and mutants. Analysis was performed for YFP-RIC1 MT length divided by cell size. The association between YFP-RIC1 was greatly decreased in \textit{tmk1234} mutant (n>20, t-test p<0.001).
Figure 3.5 TMK mediate auxin activation of both ROP2 and ROP6

A: Auxin dosage responses of ROP2 activation in wild type and tmk1234 mutant. Protoplasts were isolated from leaves of transgenic GFP-ROP2 (upper panel) or GFP-ROP2/tmk1234 (lower panel) seedlings and treated with the indicated concentrations (1 nM to 100 nM) of NAA for 2 min. GTP-bound active GFP-ROP2 was coimmunoprecipitated with MBP-tagged RIC1 and detected by immunoblot analysis with anti-GFP antibody. An aliquot of total GFP-ROP2 (GDP and GTP forms) was included as the loading control (Total ROP2).

B: Quantitative analysis of data from A. The relative ROP2 activity level was determined as the amount of GTP-bound ROP2 divided by the amount of total GFP-ROP2. The relative ROP activity in different treatments was standardized to that from mock-treated control, which was arbitrarily defined as “1”. In wild type seedlings, auxin can activated ROP2 rapidly, but not in tmk1234 mutant.

C: Auxin dosage responses of ROP6 activation in wild type and tmk1234 mutant. Similar procedure is used as in A.

D: Quantitative analysis of data from C. Same method is used as in B. In wild type seedlings, auxin can activated ROP6 rapidly, but not in tmk1234 mutant.
Figure 3.6 Polar localization pattern of TMK1 in different cells.

A: TMK1-GFP localization in young pavement cells. The cotyledon of 2 DAG seedlings is used for imaging by confocal microscope. Most PCs in this stage are beginning to produce lobes. TMK1-GFP prefers to localize to the tip of lobes in this stage.

B: TMK1-GFP localization in old pavement cells. The cotyledon of 4 DAG seedlings is used for imaging by confocal microscope. PCs in this stage have growing lobes and indentations. TMK1-GFP will localize to both lobe tip and indentation sites, which is consistent with the finding that TMK mediated auxin activation of both ROP2 and ROP6.

C: TMK1-GFP localization in developing embryo. The embryo at heart stage is imaged by confocal microscope. TMK1-GFP polar localized to the one side of cell that is similar to PIN1 localization in embryo.

D: TMK1-GFP localization in root vascular bundle cells. The root of 4 DAG seedlings is imaged by confocal microscope. TMK1-GFP localized to the basal side of vascular bundle cells that is also similar to PIN1 localization.
Figure 3.7 TMK1 kinase domain can interact with N-Terminal of SPIKE1

A: Domain organization of SPIKE1. SPIKE1 contains three DHR domains. DHR2 at C-Terminal is confirmed to have GEF activity.

B: TMK1 kinase domain is proved to interact with N-Terminal of SPIKE1 by yeast two-hybrid assay. The kinase domain (567aa-816aa) of TMK1 is fused to BD as bait. Truncated SPIKE1 proteins are fused with AD as prey. The left line is yeast grown on Leu-Trp- selection media, and using beta-galactose assay to detect interaction. The right five lines are on Leu-Trp-Ura-His- selection media that contain different concentration of 3-AT. Both assay indicated that TMK1 kinase domain can interact with 1-136aa of SPIKE1.
Figure 3.8 A working model for ABP1/TMK auxin receptor complex on PM.

ABP1 and TMK are forming a receptor complex on plasma membrane for auxin perception. When auxin is perceived, it will promote the self phosphorylation of TMK to activate kinase activity. Active TMK will phosphorylate RhoGEFs and release its GEF activity. This active GEF will further activate downstream RhoGTPase.
Conclusions

ABP1 is characterized as an auxin binding protein and regulate many auxin related responses in plant cells, however its role as auxin receptor is still not confirmed since no clear downstream pathways are indentified till now (Badescu and Napier, 2006). We placed ABP1 function at the level of auxin perception and upstream of Rho GTPase mediated cell and tissue polarity. In leaf epidermal pavement cells, auxin can rapidly activate ROP2 at the lobe protruding site, and ROP6 at indentation site, respectively. These activations are ABP1-dependent. This finding is complemented to the TIR1/AFB pathway in nucleus that mediates the auxin regulation of gene expression. We further addressed PIN1 auxin efflux carrier polarity in epidermal cells that is specially located at the apex of lobe, which is essential for activation of ROP2 at this site. And furthermore PIN1 polarity is depending on ROP2 activity, which indicate that PIN1 promoted auxin export, auxin mediated activation of ROP2, and ROP2 promoted PIN1 polar localization together forms a positive feedback loop at the lobe site that promote continuous lobe outgrowth. These findings provide a new understanding of the molecular mechanism that regulate PIN polarity that is a basic concern in plant. Finally we showed that PIN1 mediated positive feedback will accumulate auxin at outside of cell that will activate ROP6 pathway in neighboring cell form indentation. Therefore, auxin is considered as a coordinator to facilitate the outgrowth in one cell, and growth restriction in the other. These findings explain an elegant mechanism that promotes the formation of specific jigsaw-puzzled shape in leaf epidermal cells. (Xu et al., 2010)
One big question remains is how ABP1 transmit auxin signal to downstream Rho GTPase signaling because ABP1 does not contain any functional domains. Therefore we investigated auxin insensitive mutants depending on ABP1-specific phenotype, and found a group of receptor like kinases (RLKs) are in the ABP1 complex on plasma membrane. Mutation of these genes caused the similar growth defect, auxin insensitivity, and deactivation of RhoGTPase pathway as in abp1 mutant. We also confirmed that these RLKs are physically interacted and genetically linked with ABP1, which demonstrated that ABP1 together with these RLKs form an auxin receptor complex on PM. We further confirm that these RLKs can directly interact with RhoGEFs that provide a possibility that these RLKs can activate RhoGEFs and further activate RhoGTPases. These findings addressed that ABP1 together with RLKs are auxin receptor on PM and also reveal the mechanism how this receptor control downstream events.

References