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Physical and Genetic Interactions of Proteins Required for Asymmetric Cell Division in Maize

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in

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by

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ABSTRACT OF THE THESIS

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Professor Laurie G. Smith, Chair

In *Zea mays* stomatal complex formation, *pangloss* (*pan*) and *brick* (*brk*) mutants have been identified that have aberrant subsidiary cell morphology due to abnormal polarization of the subsidiary mother cell (SMC). Current knowledge of SMC division signaling pathway involves PAN1, PAN2 and actin patch accumulation at the guard mother cell (GMC)-SMC contact site. PAN1 and PAN2 are inactive leucine rich repeat receptor-like kinases thought to be involved in signal transduction. BRK1, BRK2 and

BRK3 are different components of the SCAR/WAVE complex that regulates an actin nucleator called Arp2/3 complex. In this study, the relationship between PAN and BRK were studied in regards to SMC polarization. *Pan1 and Pan2* both interact genetically with *Brk1* and *Brk3*, suggesting common roles in the SMC polarization-signaling cascade. Our previous model suggested BRK works downstream of PAN1 and PAN2, presumably as a promoter of the actin patch formation. However, in this study BRK1 appears to be upstream of PAN1, PAN2 and the actin patch, and is at least partially responsible for polarizing PAN1. I propose alternative roles for BRK in SMC polarization such as possible functions in vesicle trafficking and/or inhibition of receptor endocytosis.

INTRODUCTION

Asymmetric Cell Division

Proper cell divisions are essential for an organism's development, growth and repair. There are two types of cell division: symmetric and asymmetric. In symmetric cell division, the daughter cells are identical to the original mother cell. In contrast, asymmetric cell division creates two daughter cells that are different from the mother cell and is often associated with development of new cell lineages. In *Arabidopsis thaliana*, processes such as division of the zygote and root development are due to different cell lineages generated by asymmetric cell division (Petricka et al. 2009). Unlike animal cells, plant cells are "caged" by their cell walls. The cell wall forms in between daughter cells, locking these two cells in the position to develop to their appropriate fates. Thus, it is important that there is a coordinated signaling mechanism to initiate cell division in plants, so that the daughter cells obtains the appropriate size and/or shape for global tissue patterning and organ development.

Asymmetric cell division is generally preceded by three steps: (1) Cells can be specified and destined for asymmetric division by transfer of signals. For example, SHR is a transcription factor involved in root development of *A. thaliana.* SHR moves from the stem cell to its adjacent daughter cells to initiate development of the endodermis. Movement of SHR allows two root layers to form, the cortex and the endodermis (De Smet and Beekman, 2011). (2) Polar localizations of proteins, cytoplasm and nucleus can occur, such as localization of BASL. BASL is a key regulator in asymmetric cell division in *A. thaliana* that accumulates at the cell periphery and in the nucleus before division. After asymmetric cell division, BASL persists at the periphery as its nuclear expression

diminishes (Dong et al., 2009). Moreover, in lateral root development, correct migration and positioning of the lateral root founder nuclei are required for lateral root initiation, illustrating the importance of nuclear polarization (De Smet and Beekman, 2011). (3) cell fate determinants might be distributed asymmetrically to daughter cells after division. In *A. thaliana*, apical and basal root cell lineages are determined through expression of different transcription factors. Apical cells express WOX2 and PIN1 while basal cells express WOX8 and PIN7 (De Smet and Beekman, 2011).

Stomatal Complex Development in *Zea mays*

In addition to embryogenesis and root development, formation of the stomatal complex in plants is another example of asymmetric cell division. Stomata are pores in the epidermis of leaves that allow water and gas exchange. In the model dicot *Arabidopsis*, intrinsic transcriptional factors of the basic helix-loop-helix class (such as SPCH, MUTE and FAMA) and extrinsic factors (such as SDD1 and EPF1) are required to promote stomatal divisions and specification (Petricka et al. 2009). Maize is an excellent model to study stomatal development and asymmetric cell division because there is a gradient in a single leaf that encompasses the entire developmental sequence that produces stomata. Most mature cells are present at the tip of the leaf, while the base consists of cells that are the youngest (Nelson and Langdale, 1992).

In maize, a series of asymmetric and symmetric divisions occur to generate a four-cell stomatal complex (Figure 1). The formation of this complex begins with an asymmetric division to develop the guard mother cell (GMC) from undifferentiated cells. Next, there is an unknown extrinsic signal from the GMC to the two flanking subsidiary

mother cells (SMC) that triggers SMC polarization (Stebbins and Shah, 1960). Markers of this polarization towards the GMC include dense actin patches and SMC nuclear migration to the GMC-SMC contact site. Following SMC polarization, cytokinesis of the SMC proceeds. The result of this asymmetric division generates a larger pavement cell and a subsidiary cell. Lastly, the GMC divides symmetrically to form a guard cell pair flanked by two subsidiary cells, to finalize the formation of the stomatal complex.

The polarization of the SMC toward the GMC is essential to successfully form the stomatal complex. While the identity of the predicted extrinsic polarizing signal(s) sent by the GMC and perceived by the SMC polarization is unknown, two proteins, PAN1 and PAN2 are required for SMC polarization (Carwright et al., 2009. Zhang et al., submitted, 2012).

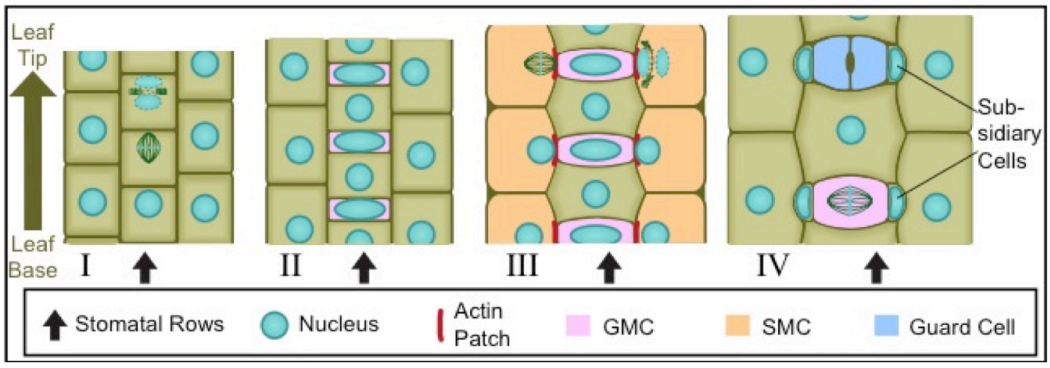

Figure 1. Formation of the stomatal complex in maize. The stomatal complex is formed through series of symmetric and asymmetric cell divisions. Formation of the maize stomatal complex can be divided into four stages. Undifferentiated cells (stage 1) undergo asymmetric cell division to generate a narrow GMC and a wider pavement cell (stage 2). SMC are two cells that flank the GMC (orange). At stage 3, actin patches form and the SMC nuclei polarize towards the GMC-SMC contact site. The SMC divides asymmetrically to generate a small subsidiary cell and a larger pavement cell as shown in stage 4. Lastly, the GMC divides symmetrically to yield a guard cell pair to complete the formation of the stomatal complex. Figure adapted from Facette and Smith, 2012.

PANGLOSS1 and PANGLOSS2, inactive LRR-RLKs involved in SMC division

PAN1 and PAN2 are leucine rich repeat receptor-like kinases (LRR-RLKs) found in maize. In *Arabidopsis*, BRI1 is a well-studied LRR-RLK that is involved in signal

transduction as a ligand-dependent membrane protein (Clouse, 2011). BRI1 is an active kinase that binds to the hormone brassinosteroid and subsequently phosphorylates other proteins to initiate a downstream signaling cascade responsible for physiological responses such as cell elongation, flowering and leaf morphogenesis (Clouse, 2011). BRI1 interacts with another LRR-RLK, BAK1, and this interaction is required for brassinosteroid responses (Li et al., 2002). Unlike BR1 however, both PAN1 and PAN2 are missing amino acid residues that are essential for kinase activity and have been shown *in vitro* to be inactive (Cartwright et al. 2009, Zhang et al., 2012). Although PAN1 and PAN2 are inactive, recessive *pan* mutants, *pan1* and *pan2*, show abnormal subsidiary mother cell morphology as a result of abnormal polarization prior to division (Figure 2; Cartwright et al. 2009). PAN1 and PAN2 polarly localize to the GMC-SMC contact site before SMC division prior to actin patch formation or nuclear polarization. This suggests that PAN1 and PAN2 might be involved in signal transduction of recruiting SMC to GMC. Furthermore, PAN1 has been found to interact with Type I plant Rho family GTPases (ROPs), which are signal transducers usually associated with cell polarity (Humphries et al. 2011). Loss of Type I ROP results in similar subsidiary defects that are seen in *pan1*. Recently, PAN2 has been demonstrated as the first observable polarizing component of the SMC polarizing signaling cascade (Zhang et al., 2012). Our current understanding of the model involves PAN2 as the first component to recruit PAN1, which would further recruit ROPs for additional signaling cascade for SMC polarization.

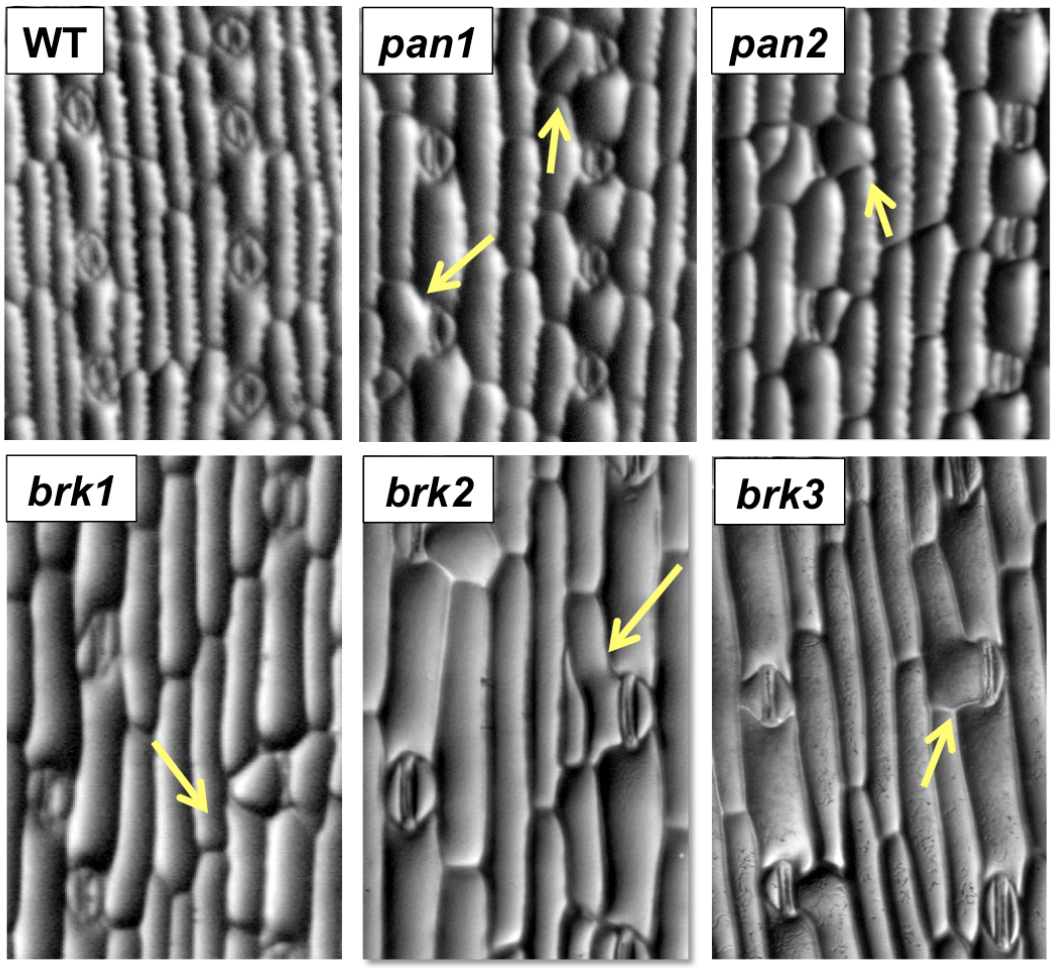

Figure 2. Subsidiary cell defects in *pan1*, *pan2*, *brk1*, *brk2* and *brk3* plants
Cyanoacrylate glue impressions of wild type and single mutant plants. Abnormal subsidiary cells are marked by yellow arrows

Three BRICK proteins involved in SMC polarization

In addition to *pan*, three *brick* mutants (*brk1, brk2* and *brk3*) have similar aberrant subsidiary cell divisions indicating their possible role in SMC division (Figure 2; Gallagher and Smith, 2000; Frank, 2003). The *Brk1* gene codes for a maize gene homologous to mammalian HSPC300 (Frank and Smith, 2002, Djakovic et al., 2006). HSPC300 is a part of a larger protein complex called WAVE/SCAR that regulates an actin nucleator called Arp2/3 (Eden et al, 2002). The Arp2/3 complex uses a pre-existing filament to form a new daughter filament in a y-shape 70° branch angle (Goley and

Welch, 2006). Furthermore, in addition to HSPC 300, the SCAR complex contains three other components, PIR121/SRA1, NAP1 and ABI (Pollitt and Insali, 2009). Previous studies suggested that all three *Brk* genes act in a common pathway after analyses of abnormal subsidiary cell frequencies in single and double mutants (Frank, 2003). Conclusively, *Brk2* maps near the SRA1 gene and SRA1 protein is absent in *brk*2 mutants (Facette, unpublished). Sequencing confirmed *Brk3* as NAP1, showing *Brk1, Brk2* and *Brk3* as components of the SCAR complex (Frank, 2003; Smith, unpublished).

The dynamic behaviors of actin polymerization and depolymerization allow cells to remodel the actin cytoskeleton to use its force in processes such as endocytosis and cytokinesis. There is evidence that actin filaments are used for clathrin-dependent endocytosis, vesicle budding and endosome trafficking in plants (Samaj et al., 2004). The phragmostplast is also guided to the site of new cell plate formation in actin-dependent manner (Smith 2001). It has been proposed that the actin patch at the GMC-SMC contact site is responsible for transferring subsidiary cell fate determinants to the daughter nucleaus to differentiate as a subsidiary cell (Gallagher and Smith, 2000). Together, these results suggest the role of actin patch at the GMC-SMC contact site as a component to help SMC polarization and division by delivering cell fate determinants or by promoting cell plate formation for new subsidiary cells.

Actin polymerization has also been studied to be responsible for cell morphology. In *A. thaliana,* disruptions in actin polymerization by mutations in Arp2/3 homolog result in distorted trichome expansion, loss of lobes in pavement cells and curled hypocotyl cells (Mathur et al., 2003). Similarly, *brk* mutants lack wave-like lobe formations in maize epidermal cells when compared to the wild type (Figure 2D). These lobes are

established via localized actin accumulation, and *brk* mutants lack F-actin accumulation at the tips of emerging lobes when compared to the wild type (Dyachock et al. 2008). Furthermore, it is known that actin filaments are responsible in bringing SMC nucleus to the GMC, but microtubules are responsible for anchoring the nucleus to the polar site (Smith, 2001; Cleary, 1995). Despite these data, the precise function of the actin patch is still unknown.

A dense actin patch is one of the markers of SMC polarization to the GMC-SMC contact site. It is plausible that BRK is involved in formation of this actin patch, by promoting actin branching via the Arp 2/3 complex. In *Arabidopsis,* components of the Arp 2/3 complex have been found to physically interact with, and be activated by, ROPs (Uhrig et al., 2007, Basu et al., 2008). These data, coupled with our observations of SMC polarization suggest the following model: (1) PAN2 accumulates at the GMC-SMC contact site followed by (2) PAN1 localization, (3) ROP interaction with PAN1, (4) activation of BRK1 via ROP and subsequent Arp2/3 complex activation, (5) leading to actin patch formation and finally SMC nuclear migration. This model places BRK downstream of PAN1 and PAN2, to promote Arp 2/3 initiated actin patch formation. (Figure 4).

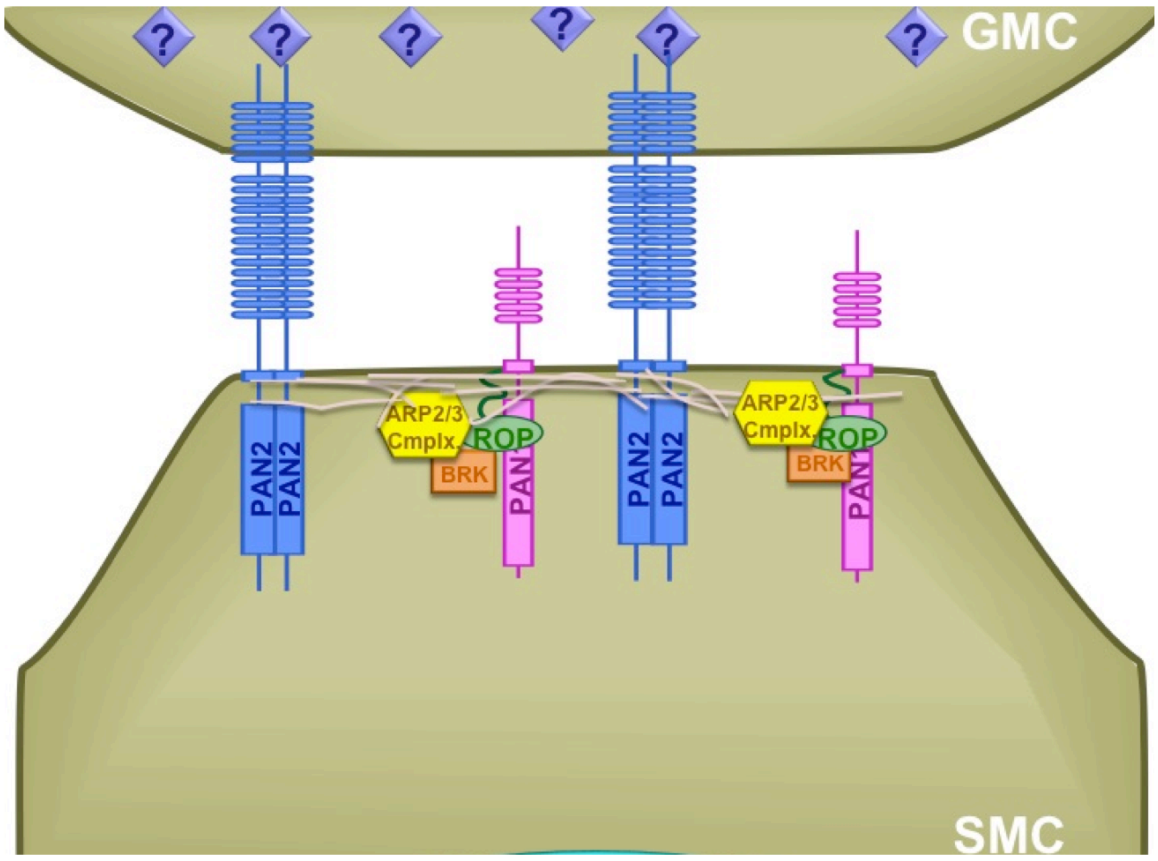

Figure 3. Signaling components of SMC polarization. Previous observations of known signaling components suggest this model for SMC polarization signling pathway: 1) PAN2 accumulates at the GMC-SMC contact site by unknown signal from the GMC (blue diamonds). This results in 2) PAN1 localization followed by 3) PAN1 interaction with ROP. This is followed by 4)ROP activation of BRK1 and Arp2/3 complex to promote 5) actin patch formation and SMC nuclear migration.

If BRK is exclusively downstream of PAN1 and PAN2 in the SMC polarization pathway, loss of BRK should not affect PAN1 or PAN2 localization. Surprisingly, there is a reduction of PAN1 accumulation at the GMC-SMC contact site in *brk* mutants (Figure 3, Sutimantanapi, 2012). This suggests that BRK's role in SMC polarization might be to promote PAN localization, rather than (or in addition to) promoting actin patch formation as a result of G-protein signaling through PAN and ROP. This calls for a closer examination of the genetic interactions between the *pan* and *brk* mutants.

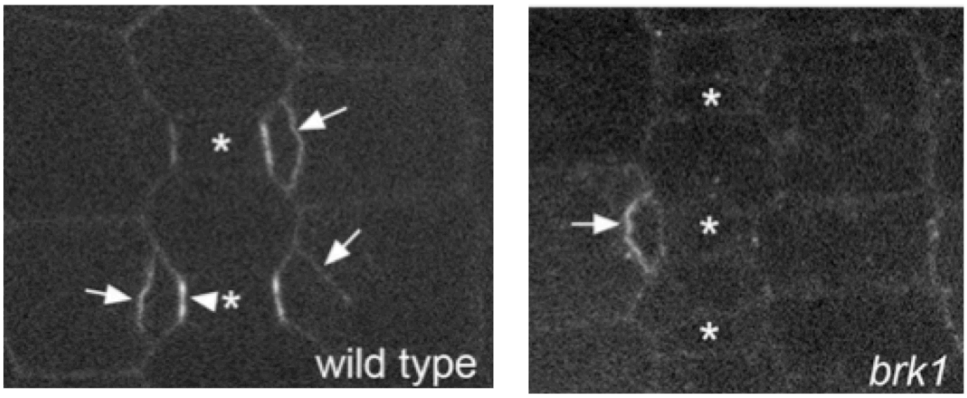

Figure 3. PAN1 and PAN2 expressions are unchanged in *brk1* and *brk3* mutants.
A. PAN1-YFP expression PAN1-YFP plants in wild type and *brk1* mutant background. White arrows show PAN1 localization in the phragmoplast and GMC-SMC contact site in wild type. PAN1 localization at the GMC-SMC contact site is depleted in *brk1*. Asterisks mark guard mother cells. Figure adapted from Sutimantanapi, 2012.

The overarching goal of this research is to identify and characterize known and unknown components of the signaling pathway that promotes asymmetric cell division of the SMC. To address this, I took a multi-pronged approach: (1) I tested physical interactions between PAN1, PAN2, and other candidate signaling proteins; (2) I described genetic interactions between PAN and BRK mutants by comparing SMC polarity defects in single and double mutants; (3) I described for the first time the subcellular location of BRK1 using live cell imaging, and also compared it's localization in reference to PAN1, PAN2, and actin. (4) I examined the effects of pharmacological and genetic disruptions on BRK localization to elucidate its placement within the polarization sequence. Together, these data suggest that *Pan* and *Brk* interact genetically, cementing their involvement in SMC polarization signaling cascade. Furthermore, in comparing BRK1 localization to other known components of the SMC division signaling, results suggest BRK1 acts prior to PAN1 and PAN2 SMC division pathway.

MATERIALS AND METHODS

Plant Materials

Wild type, *pan1, pan2, brk1, brk3, pan1;brk1, pan1;brk3, pan2;brk1 and pan2;brk3* double mutants were used. The *pan1-EMS* allele was previously described by Cartwright et al. (2009). The *pan2-L2* allele was previously described by Zhang et al. (2012). The *brk1* and *brk3* alleles were described in Frank et al. (2003). Double mutant plants were generated by using these alleles. PAN1-YFP was previously described by Humphries et al. (2011). PAN2-YFP was previously described by Zhang et al. (2012). BRK1-CFP, ABD2-YFP, and HIS-YFP were obtained from the "Characterizing sub-cellular compartments in maize using fluorescent protein tagged lines" project (Mohanty et al, 2009; http://maize.jcvi.org/cellgenomics/).

Yeast Two Hybrid Analysis

For use in the GAL4-based yeast two-hybrid interaction system, cDNA fragments encoding intracellular portions of PAN1 and PAN2 were cloned into Yeast 2 hybrid vectors via Gateway cloning. Primers P1SOL_pENTR_F and P1_STOP_pENTR_R were used to amplify the cDNA segment encoding amino acids 308-662 of PAN1 from pDONR-PAN1. pDONR-PAN1 was obtained from M. Facette and was generated using methods described by Zhang et al. (2012). Primers P2SOL_pENTR_F and P2STOP_pENTR_R were used to amplify the cDNA segment encoding amino acids 675-1075 of PAN2 from pSK-PAN2. Intracellular regions of additional proteins were cloned from maize RNA obtained from M. Facette. Primer sequences are outlined in Table 1. PCR products were cloned to the entry vector pENTR/D-TOPO (Invitrogen). Gateway

recombination was used to transfer the inserts from these entry clones to destination vectors pASGW-attR and pACTGW-attR (Nakayama et al., 2002). Transformation of these plasmids into yeast strain AH109 was carried out as described by Gietz and Woods (2002). At least 4 independent co-transformed colonies from two separate experiments were tested for each construct to determine their ability to grow in the absence of histidine.

Table 1. List of primers used for yeast two-hybrid assay

Gene	Primer Name	Sequence
PAN1	P1Sol_pENTR_F	cacc gca GGaGACGAGGGGAAGGAGTCCGGCAA
	P1STOP_pENTR_R	TCAGCCGATCCGGTCGAGGCTCTCG
PAN2	P2Sol_pENTR_F	cacc gca AGGATCTCACGGCAGTTTTCTAGCTC
	P2STOP_pENTR_R	CTAGATCGACGAAAGATCCTCGTA
GRMZM2G120657	120657_F_intra	caccGCCATGAAACGTTGGGAGGCGAG G
	120657_R	TCTGCTCAAGTGTAGCCGAGACG
GRMZM2G032132	032132_F_intra	caccATCCTCCACGCCAAGAGCACG
	032132_R	TAAGCTGTGGAGCTGCGTTAGCAT
GRMZM2G073928	032132_F	caccATGTGGCGTGAGAAGCAGGAGAA GG
	073928_r	GCTGTTCTTGGCCTCTTGCAGCATCTC
GRMZM2G306028	306028_R	CGAGTGCATCCTGGAGACCTTTTC
	306028_F_T01	caccATGGAGTTCTTCACCGAGTACGG
GRMZM2G102088	102088_F	caccATGATGGCGGAAGGCCCCAATTTC G
	102088_R	GTCGGTAGTCATCGGTTGGGACATGC
GRMZM2G107575_P01	CalcB_pENTR_F	caccATGGGGTGCTTCCATTCCACGGCG AAG
	CalcB_pENTR_R	CGTGACGAGATCGTCGACTTCCGAGT T
GRMZM2G149031	149031_F_intra	caccGATGAGGCGTACTTCGCGACG
	149031_R_intra	AACTAGGGCTGTTCTCAATCC
GRMZM2G159105	159105_F_intra	caccAGCCCAGACCGACATAAGTTTCAA C
	159105_R	CGCCACAAGACCACTAAACACTTGGC
GRMZM2G048294	48294_F_intra	caccATTGAGTGCAAGAAGCGGAAG
	48294_R	GTCCTTCTCCTCCTTGTCTTCTTTCAG

Protein gel blot analysis

Immunoblotting experiments used maize leaf tissues enriched in dividing cells (the basal 3 cm of leaves remaining on 3- to 4-week-old maize plants after removal of all leaves with fully or partially expanded sheaths). “Symmetric division” fraction contained regions 0-1.5cm from the basal root. “Asymmetric division” fraction contained regions from 1.6 – 2.75cm from the basal root. Membrane fractions of extracts from these tissues were prepared, separated via SDS-PAGE, and analyzed via immunoblotting as previously described (Cartwright et al., 2009). PAN1 was detected with affinity-purified anti-PAN1 at 1mg/ml diluted 1:250. PAN2 was detected with affinity-purified anti-PAN2 at 1µg/ml.

Yeast protein extracts were prepared for immunoblotting (Supplementary Figure 7) from co-transformed colonies as described in the Clontech Yeast Protocols Handbook (Protocol No. PT3024-1, Version PR973283, July 2009 (http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17602&minisite=10020&secItmId=14852). Separation, transfer and detection of proteins was carried out as described by Cartwright et al. (2009) using rabbit anti-GAL4 binding domain (SC-577, Santa Cruz Biotechnology) diluted 1:600, rabbit anti-HA Epitope Tag polyclonal antibody (Pierce PA1-985) diluted 1:500, and affinity purified anti-PAN2 at 1µg/ml.

Double mutant analyses

For scoring of mutant phenotypes, mature leaf 3 was collected from 1-2 weeks old plants. Imprints of lower epidermal layer were made using cyanoacrylate glue and examined using a stereoscopic microscope. At least three plants were examined for each genotype. Over 150 cells were counted in random chosen locations.

Inhibitor studies

Short-term inhibitor studies on BRK1-CFP;ABD2-YFP and PAN1-YFP were done with leaf tissue excised from the basal 1-3 cm of unexpanded leaves that were 1-2 weeks old. For latrunculin B treatment, 5mM stock solution of latrunculin B was made in DMSO (EMD 428020). Excised tissue was placed in various concentration of DMSO diluted to 1uM and 10uM from stock solution in water with 0.1mg/ml propidium iodide and 0.01% Triton.

For long-term inhibitor studies to generate the growth curve with latrunculin B, wild type seeds were sterilized by immersing in 100% bleach with 0.01 % Triton and vacuum infiltrating for 15 minutes, then rinsed and lightly dusted with Captan, an anti-fungal powder (Lilly Miller). Seeds were placed on damp 0.8mm thick Whatman paper on 60mm x 15 mm polystyrene petri dishes (0875713A, Fisher Scientific). 5mM stock solution of latrunculin B was used in various dilutions. These dishes were left in RT in a container with layers of wet paper towels and covered with plastic wrap. Seeds were checked every couple of days and 1 ml of the solution was applied based on the dampness of the Whatman paper. Fully expanded regions of leaf 2 were stained using propidium iodide to stain nuclei and cell walls. Tissues were mounted on water and visualized using 60x confocal microscope as described below.

For long-term inhibitor studies to count the frequencies of abnormal subsidiary cell divisions, seeds were germinated in ½ MS agar in 5ml polystyrene round-bottom tubes (BD Falcon). Varying concentrations of inhibitors were included in liquid agar by using 5mM stock solution of latrunculin B, 5mM stock solution of CK636 (Sigma-

Aldrich C7374) and 10mM stock solution of CK548 (Sigma-Aldrich C7499). Tubes were placed in 23°C room under growth light. Fully expanded regions of leaf 2 was stained using propidium iodide to stain nuclei and cell walls. Tissues were mounted on water and visualized using 60x confocal microscopy as described below.

Confocal Microscopy

Fluorescence was visualized using a Nikon TE-200U confocal microscope system equipped with a 60X 1,2 NA water immersion objective, a Yokogawa Nipkow spinning disk confocal head, and a Roper Cascade 512b EM CCD camera using on-chip gain and reading off at 5 Mhz. YFP was excited with 514nm and were viewed with a Chroma HQ570/65nm emission filter. CFP was excited with a 440nm laser and viewed with a Chroma HQ525/50nm emission filter. Propidium iodide was excited with an Argon/Krypton laser (568 nm line) and visualized with a Chroma HQ620/60 filter. The confocal system was controlled using MetaMorph software vs. 7.0r1 (Universal Imaging Corporation, Downington, PA). Z-Projections of image stacks were assembled using Metamorph. Image processing was carried out using Image J vs. 1.36b (http://rsb.info.nih.gov/ij/) or Adobe Photoshop vs. 8.0.

RESULTS

Soluble regions of PAN1 and PAN2 do not physically interact, but PAN2 interacts with itself.

Since PAN1 and PAN2 are both identified as inactive LRR-RLKs that co-localize at the contact site of GMC and SMC, these two proteins might physically interact (Cartwright et al., 2009; Zhang et al., 2012). Figure 4 shows the results of yeast two-hybrid system that tested interactions of intracellular domains of PAN1 and PAN2. PAN1 and PAN2 show no positive interaction in both prey and bait combinations, although presence of both proteins was confirmed by immunoblotting (Figure 4B). It was shown, however, that PAN2 interacts with itself as a homodimer, shown by growth on media without histidine (Bottom right, Figure 4A). This result is further supported by co-immunoprecipitation results of PAN2-YFP and endogenous PAN2 pull-down shown in Zhang et al. (2012).

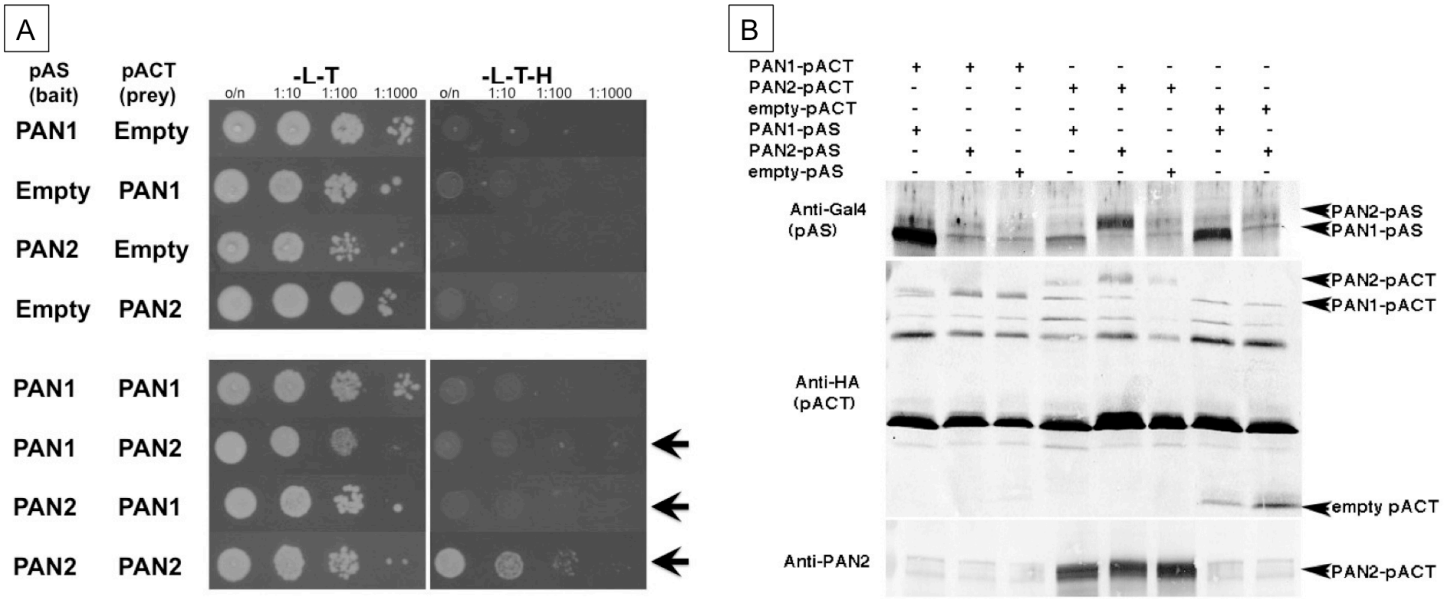

Figure 5. Soluble regions of PAN1 and PAN2 do not form a heterodimer but PAN2 form a homodimer in yeast-2-hybrid screening.

A. Yeast two-hybrid assay with PAN1 and PAN2. PAN1 and PAN 2 soluble regions were cloned into GAL-4 binding domain (pAS, bait) and activating domain (pACT, prey) vectors and co-transformed on –Leu/-Trp media for presence of the two vectors. Positive transformants were selected to screen for interaction on –Leu/-Trp/-His media. No interaction is shown between PAN1 and PAN2. Intracellular regions of PAN2 with itself shows positive interaction. Serial dilutions of yeast two-hybrid transformants are shown above

B. Immunoblot of fusion constructs used in yeast two-hybrid assay. Fusion constructs in yeast two-hybrid experiment of PAN1 and PAN2 were detected by using three different antibodies; anti-Gal4 for pAS vector, anti-HA for pACT vector and anti-PAN2 for PAN2**.** Blot confirms the expression of fusion proteins.

Since PAN1 and PAN2 do not interact with one another, they may have other signaling partners. In hope to find other signaling partners of PAN1 and PAN2, numerous yeast two-hybrid experiments were conducted with potential candidates. These candidates were selected based on proteomic comparisons of membrane proteins isolated from tissues undergoing stomatal divisions (Zhang et al. 2012). The candidate proteins were depleted in *pan1*, *pan2* and/or *pan1;pan2* double mutant plants relative to wild type, and therefore could be potential signaling partners of PAN1 and PAN2. Except for positive interaction seen with PAN2 mentioned earlier, no other interactions were found with four potential candidates (Figure 5). Two proteins, a LRR-RLK (GRMZM2G120657) and MAPKKK (GRMZM2G102088) showed auto-activation in the yeast two-hybrid system when tested with PAN1-pACT and PAN2-pACT. Intracellular regions were clone for three additional candidates, but these have not been tested for interaction.

Table 2. No other interactions were found with PAN1 and PAN2 through yeast-2-hybrid screening.
Six potential candidates found from separate proteomics analyses of maize membrane proteins in *pan*1 and *pan2* mutants were tested for interaction through yeast-2-hybrid screening. None of the tested candidates showed interaction with PAN1 or PAN2 in vitro.

TESTED PROTEIN (in pAS)	Maize Accession Number	PAN1-pACT	PAN2-pACT
PAN1	GRMZM5G836190	-	-
PAN2	GRMZM2G034572_P01	-	+
LRR-RLK	GRMZM2G120657	Auto activation	Auto activation
LRR-RLK, similar to AtCRN	GRMZM2G032132_P01	-	-
LRRK	GRMZM2G073928	-	-
MAP Kinase, similar to AtMAPK17	GRMZM2G306028_P02	-	-
MAPKKK, Raf subfamily	GRMZM2G102088_P02	Auto activation	Auto activation
Calcineurin B like	GRMZM2G107575_P01	-	-
Protein Kinase C Substrate	GRMZM2G149031_P01	Untested	Untested
Lysm-RLK	GRMZM2G159105_P01	Untested	Untested
LRR-RLK, similar to AtBRI1	GRMZM2G048294_P01	Untested	Untested

PAN and BRK interact genetically

Since *pan* and *brk* mutants show similar aberrant subsidiary cell division, double mutant analyses were performed to see if they interact genetically. Single mutants, *pan1, pan2, brk1* and *brk3* showed 10-24% aberrant subsidiary cell divisions (Figure 6). In double mutant plants, much higher frequencies of abnormal subsidiary cells were observed than sum of two single mutants. This shows that the *pan;brk* double mutants have a synergistic phenotype.

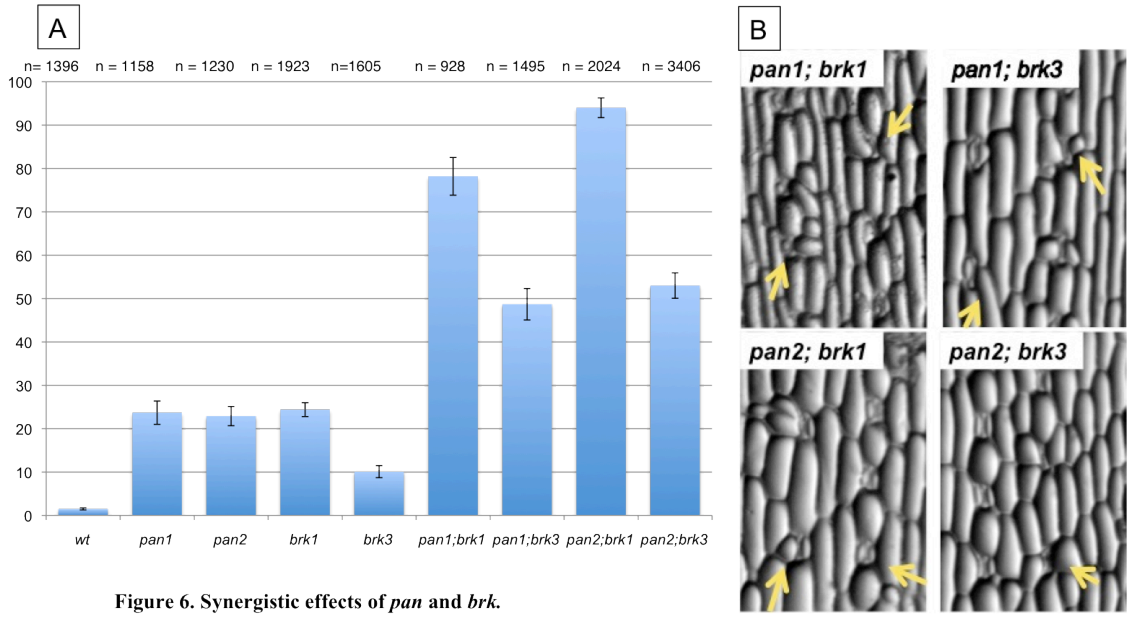

Figure 6. Synergistic effects of *pan* and *brk*.
A. Abnormal subsidiary cells from mature maize leaves of wild type, single and double mutants were counted. Double mutants show much higher percentages of abnormal subsidiaries than addition of abnormal subsidiary cells from single mutants. N represents number of subsidiary cells counted. Error bars represent standard error of the mean.
B. Cyanoacrylate glue impressions from *pan1;brk1, pan1;brk3, pan2;brk1* and *pan2;brk3* plants

The decrease in PAN1 patch intensity in *brk1* is not due to decreased protein abundance

In studies with PAN1-YFP transgenic plants in *brk* background, it was shown that PAN1 patch was depleted at the GMC-SMC contact site when compared to the wild type (Figure 3, Sutimantanapi, 2012). In order to distinguish whether this is due to decreased PAN1 accumulation at the GMC-SMC contact site or decreased PAN1 in protein expression, membrane proteins were extracted from first two division zones of wild type

and three single mutant plants (*pan1* or *pan2, brk1 and brk3)* from the basal 0.5 - 3 cm of unexpanded maize leaves. This segment of the maize tissue contains cells that are actively dividing and expanding to form stomata, processes that are involved with PAN1 and PAN2 localization. First zone includes cells from stage 1 of stomatal complex formation, when cells are dividing symmetrically (labeled symmetric division). Second zone includes cells from stages 2-4, where asymmetric cell division occurs (labeled asymmetric division). It was important to separate maize tissue into these two zones to ensure that any changes in PAN1 and PAN2 protein expression were specific to the area of asymmetric cell division. As shown in Figure 7, PAN1 and PAN2 expression levels are not reduced in *brk1* and *brk3* when compared to wild type in both division zones. This suggests that PAN1 and PAN2 might be improperly recruited to the GMC and SMC contact site in *brk* mutants.

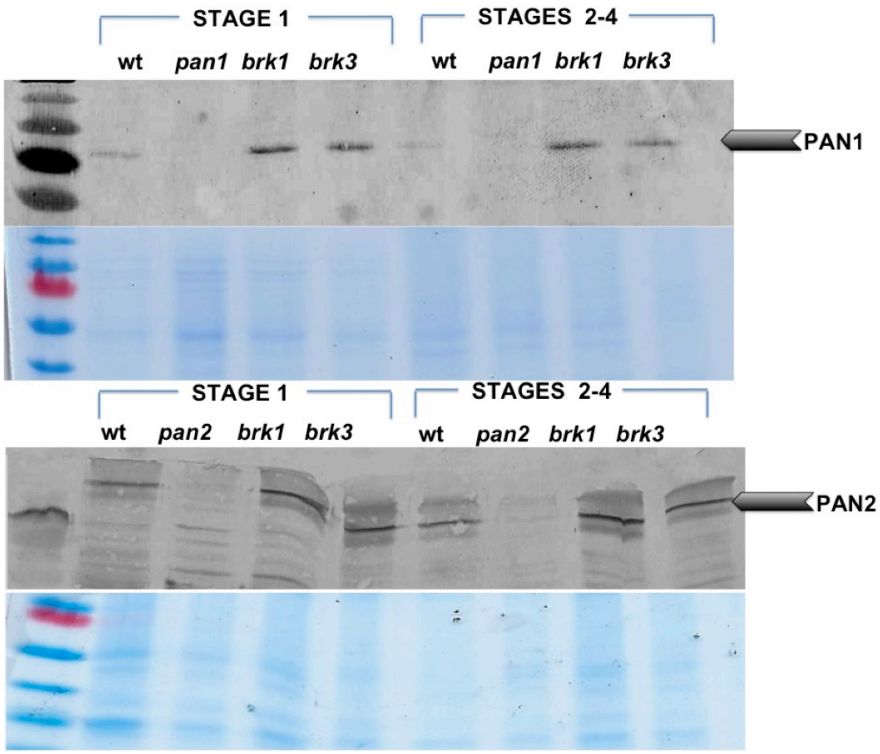

Figure 7. PAN1 and PAN2 expressions are unchanged in *brk1* and *brk3* mutants.
Immunoblot of maize membrane protein extracts from cells at stage 1 and from cells at stages 2-4. PAN1 and PAN2 expression levels are not reduced compared to wild type in b*rk1* and *brk3* mutants during stage 1 and stages 2-4. Coomassie blue staining shown below each immunoblot as loading control.

BRK1 localizes at the GMC and SMC contact site before SMC division.

To understand more about the role of BRK1 in SMC polarization, BRK1-CFP transgenic plants were used to observe BRK localization. Bright cell corners are seen before BRK1 patch formation in undifferentiated cells, and these dots persist throughout formation of the stomatal complex (yellow arrows, Figure 8A). This finding is consistent with bright corners of BRK1-YFP seen in *A. thaliana* in root tips (Dyachok et al., 2008). As shown in Figure 8B, BRK1 patches form at the GMC-SMC contact site before nuclear polarization, similar to PAN1 and PAN2 (Cartwright et al., 2009; Zhang et al, 2002). These patches remain at the contact site until SMC division and disappear after GMC division. Additionally, BRK1 patches are shown at lobe tips of elongating epidermal

cells, where F-actin patches also accumulate (Frank, 2003).

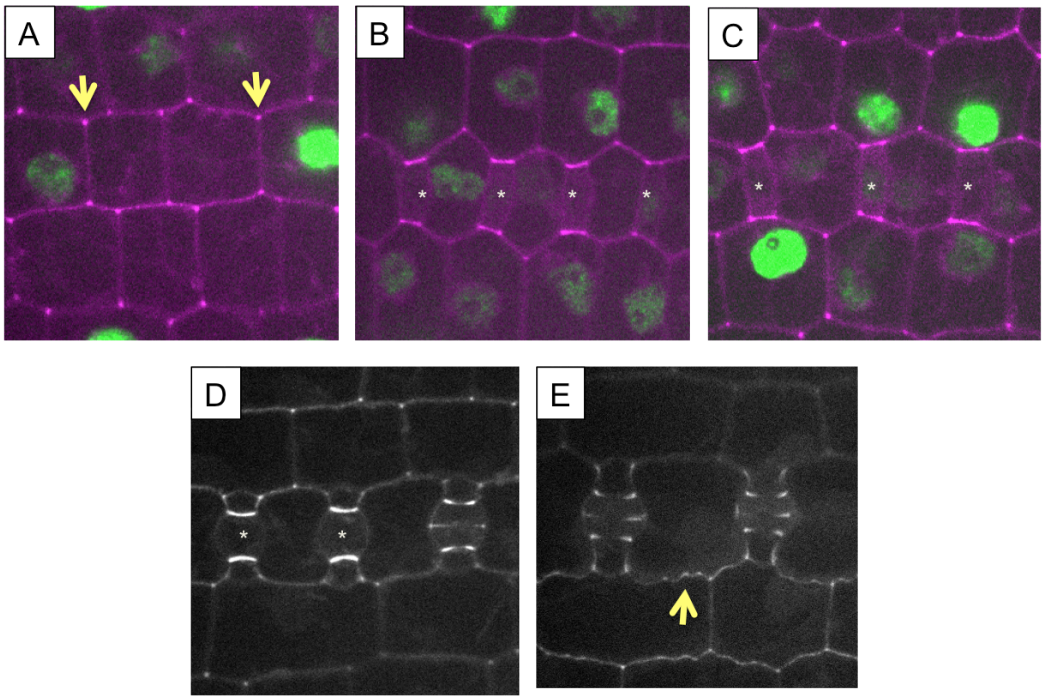

Figure 8. BRK1 localizes at cell corners and at the GMC and SMC contact site
Successive stages of SMC division during stomata formation. Images A-C show merged images of BRK1 localization in BRK1-CFP (magenta); histone-YFP (green) transgenic plants. Images D-E illustrate BRK localization in BRK1-CFP transgenic plants. Asterisks mark guard mother cells.
A. BRK1 localizes at cell corners before BRK1 patch formation
B-D: BRK1 patch forms at GMC-SMC contact site before nucleus polarization and persists through SMC division
E: BRK1 localizes at lobe tips of epidermal cells.

Mammalian homolog of BRK1, HSCP300, has been studied to regulate actin polymerization by activating the Arp 2/3 complex (Eden et al, 2002). Effective actin nucleation by the Arp 2/3 complex requires pre-existing actin filaments therefore, it was hypothesized that BRK might be involved in generating new actin filaments to previously established actin patches (Machesky et al., 1999). To investigate the relationship between BRK1 and actin, BRK1-CFP;Actin Binding Domain2 (ABD2)-YFP transgenic plants were observed for localization of BRK and actin patches. As shown in Figure 9, BRK1 patches localize before actin patches, suggesting that BRK1 works upstream of actin

patch formation.

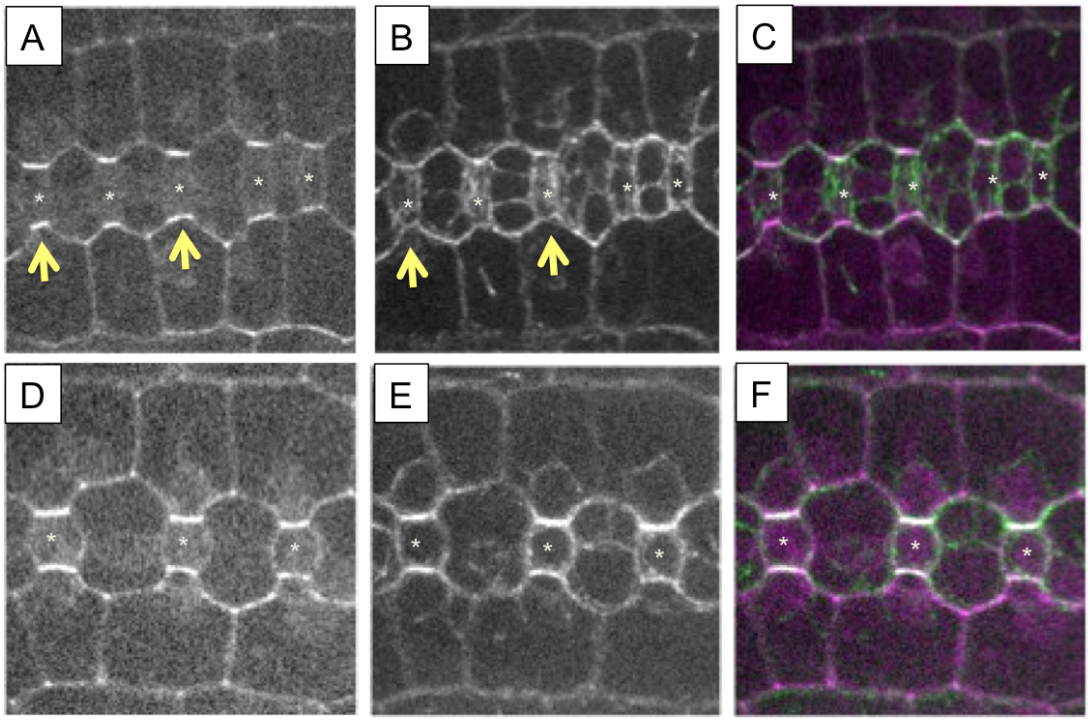

Figure 9. BRK1 localizes at GMC-SMC contact site before actin patch formation
Images from transgenic plants expressing both BRK1-CFP;ABD2-YFP.
A and D show BRK localization from BRK1-CFP transgenic plants. Leftmost images show BRK1 localization from BRK1-CFP. Middle images show actin localization from ABD2-YFP. Right images show merged images of BRK (magenta) and actin (green). Top panel shows that BRK patches form prior to actin patch formation (yellow arrows). Bottom panel shows presence of both BRK and actin patches. Asterisks mark guard mother cells.

To understand more about the interactions between BRK1, actin and SMC polarization, long-term and short-term inhibitor studies were performed to observe whether the actin patch was required for the establishment and the maintenance of BRK1, PAN1 and PAN2 patches. Short-term treatments using latrunculin B (latB) which is expected to disrupt actin microfilaments (Spector et al., 1989; Collins et al., 2005), were used to test if actin is required for BRK1 and PAN1 patch maintenance. BRK1-CFP/ABD2-YFP transgenic plants were immersed in various latB concentrations ranging from 0uM to 10uM for 0 to 60 minutes. ABD2-YFP was used as control to ensure that

latB treatment was working. Cortical actin microfilaments appeared shorter and thicker after treatment of 5uM latB in 60 minutes, shown by its increase in intensity compared to control (Table 4). Actin patches at SMC nucleus polarization sites started to disappear at 5uM in 60 minutes and were not present at 10uM for 60 minutes (Figure 10A-E, Table 3). However, even at the highest concentration of latB, BRK1 patch intensity remained similar to the control and remained at the GMC-SMC contact site. Thus, actin patches are not required for the maintenance of BRK1 to the GMC-SMC contact site. Interestingly, PAN1-YFP patches were also unaffected and seen at the same intensity when compared to control at the GMC-SMC contact site with latB treatment (Figure 10F). Since PAN1 patches are depleted in *brk1* mutants (Figure 3, Sutimantanapi, 2012), this suggests that actin patches (and cortical F-actin) are not required for maintenance of PAN1 at the patch site, and the function of BRK1 is either in establishing PAN1 patches, or perhaps BRK1 (and SCAR/WAVE complex members) have an actin-independent role in maintaining PAN1 patches.

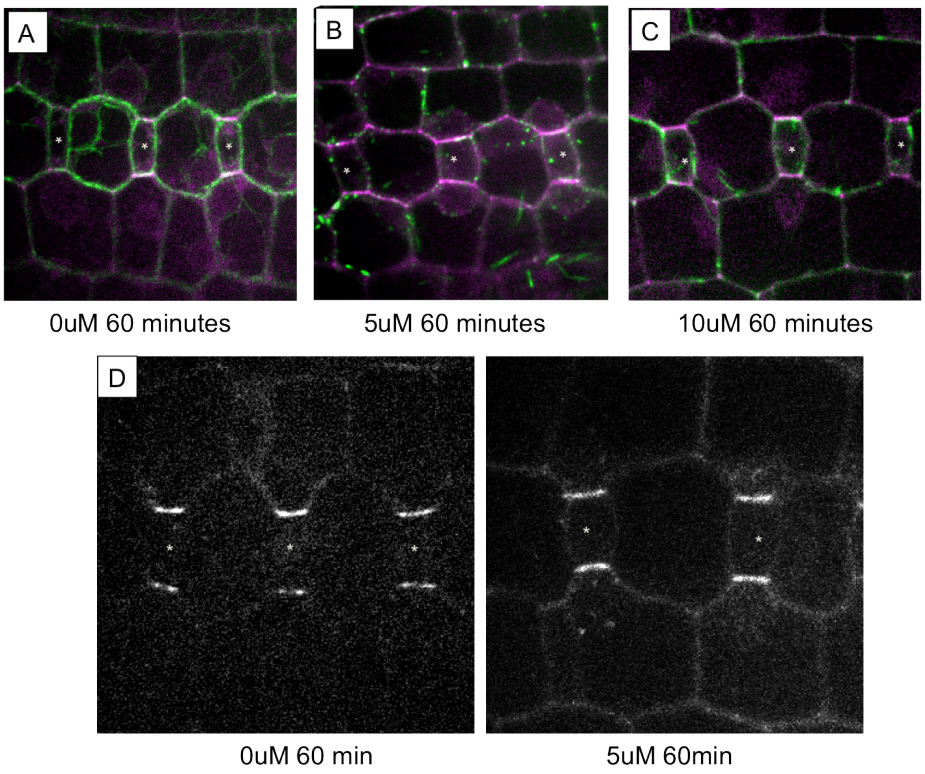

Figure 10. BRK and PAN1 are unaffected by latrunculin B treatment

A-C: Top panel shows merged images of BRK-CFP (magenta) and ABD2-YFP (green). Actin filaments begin to disappear with latB treatment of 5uM at 60 minutes. Actin patches disappear at 10uM of latB at 60 minutes. BRK patches remain at both concentrations. Asterisks mark guard mother cells. Control contained 0.2% DMSO.

D. PAN1 is unaffected by latrunculin B treatment. Images show PAN1 localization from PAN1-YFP transgenic plants. PAN1 patches appear to be at same intensity at 5uM of latB treatment 60 minutes when compared to control. Asterisks mark guard mother cells.

Table 3. Quantification of patch presence on BRK1-CFP;ABD2-YFP transgenic plants treated with latB

+++ indicate presence of BRK1 or actin patches at all GMC-SMC contact site with polarized SMC nuclei in the field of view. + being no patches in the field of view. Actin filaments begin to disappear at 5uM at 60 minutes, while BRK1 patches remain unaffected.

	BRK patch	Actin filaments	Actin patch
control	+++	+++	+++
1uM 60min	+++	+++	+++
5uM 60min	+++	+	++
10uM 60min	+++	+	+

Table 4. Quantification of fluorescence intensity on BRK1-CFP;ABD2-YFP transgenic plants treated with latB

+++ indicate standard of intensity seen from control. + indicate less intensity and + ++++ indicates higher intensity of florescence. Actin filaments are brighter with latB treatment. BRK1 intensity Is unaffected

	BRK patch	Actin filaments	Actin patch
control	+++	+++	+++
1uM 60min	+++	+++	+++
5uM 60min	+++	++++	++
10uM 60min	++	+++++	not present

For long-term treatments, wild type, *pan1* and *pan2* seeds were grown in media containing pharmacological inhibitors. Arp2/3 is activated by the SCAR complex (Eden et al., 2002). If Arp2/3 complex were involved in SMC division, inhibiting this complex would presumably mimic the *brk* phenotype and affect SMC polarization. PAN1-YFP, PAN2-YFP and BRK1-CFP seeds were also to be used in this treatment with Arp2/3 inhibitors to study whether the Arp 2/3 complex was required for the establishment of PAN1, PAN2 and BRK1 patches. To generate a growth curve to use as a standard, wild type seeds were germinated in various concentrations of latB and two Arp 2/3 inhibitors (CK 548 and CK636). Treatment with these inhibitors caused an increase in abnormal subsidiary cell frequencies at intermediate concentrations, such as 50nM of latB and 50uM of Arp 2/3 inhibitors. Surprisingly, the number of abnormal subsidiaries decreased at higher concentrations (Figure 11A and B). Therefore, three concentrations were selected to observe effects of these inhibitors in wild type, *pan1* and *pan2* mutants. Seeds were germinated on ½ MS-agar with latB, CK548 or CK636. This treatment on ½ MS-agar showed no apparent inhibitor dependent trend that was followed in all three genotypes. Unlike the standard curve, wild type seeds on CK548 and CK636, and *pan2* seeds on CK548 and CK636 showed a decrease in abnormal subsidiary cell frequencies at intermediate inhibitor concentration, but an increase at higher concentrations (Figure 11C-E). However, wild type in latB, *pan*1 in CK548 and CK636 and *pan2* in latB showed an increase in abnormal subsidiary cell divisions with increasing inhibitor concentrations. Paired, two-tailed Student's t-tests failed to show statistical significance of the biological replicates. The long-term treatment data had inconsistent results and thus, additional experiments with transgenic seeds were not conducted.

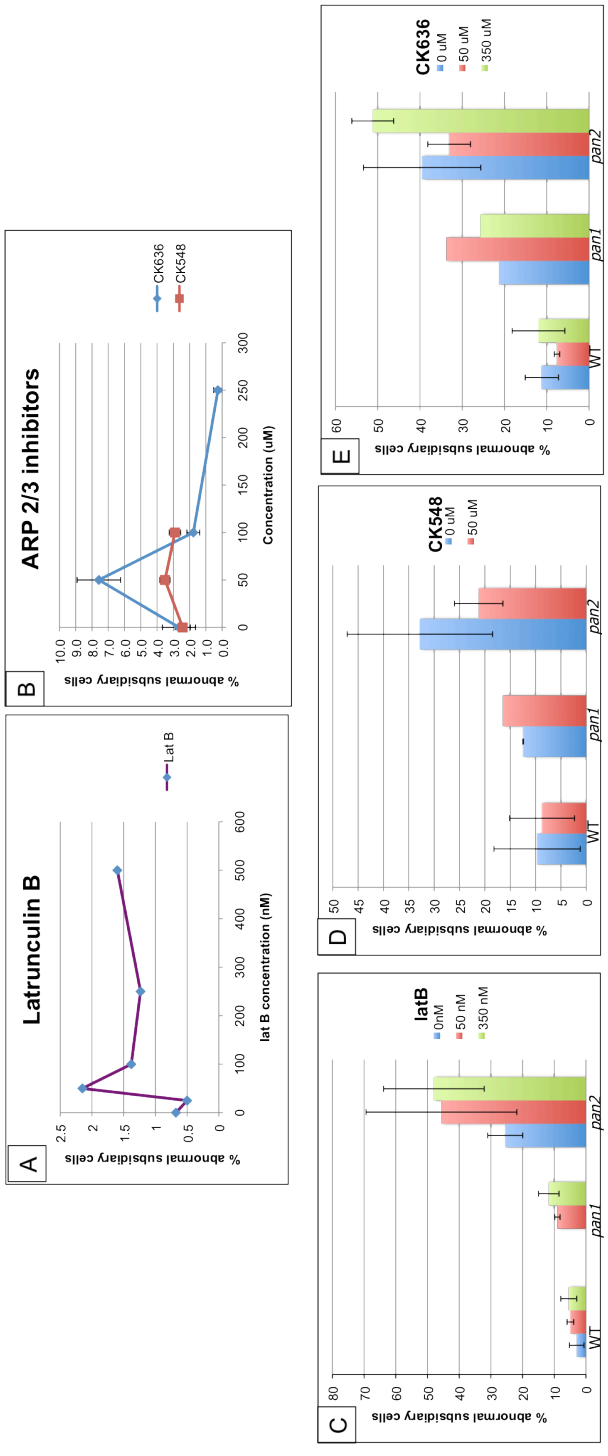

Figure 11. Long term inhibitor treatments with latrunculin B, CK636 and CK548
A and B. Latrunculin B (latB) and Arp 2/3 inhibitor (CK636, CK548) growth curves
Wild type seeds were grown on various concentrations of lat B, CK636 and CK548 to generate a standard growth curve. An increase in the percentage of abnormal subsidiary cells was seen in intermediate concentrations (50nM of latB, 50uM of CK636 and 50uM of CK548), followed by a decrease at higher concentrations of inhibitors. Error bars represent standard error of the mean.
C-E. Various effects on latrunculin B, CK548 and CK636 on wild type, *pan1* and *pan2* seeds
Wild type, *pan1* and *pan2* seeds were germinated in various latrunculin B, CK 548 and CK636 concentrations. Control contained same amount of DMSO as the highest inhibitor concentration. Error bars represent standard error of the mean. Paired, two-tailed Student's t-tests failed to show statistical significance of the biological replicates.

BRK1 works upstream of PAN1 and PAN2

Since BRK1 appears at the same site as PAN1 and PAN2, additional localization analyses were performed with transgenic plants to generate the order in which these proteins appear in the SMC division pathway. The timing of appearance for three proteins is very close; however, in several cases BRK1-CFP patches could be observed in cells, which did not have PAN1-YFP and PAN2-YFP patches (yellow arrows, Figure 12, 13), while the opposite (PAN1- or PAN2-YFP patches without BRK1-CFP patches) was never observed. This result indicates that BRK1 might work upstream of PAN1 and PAN2 in SMC division.

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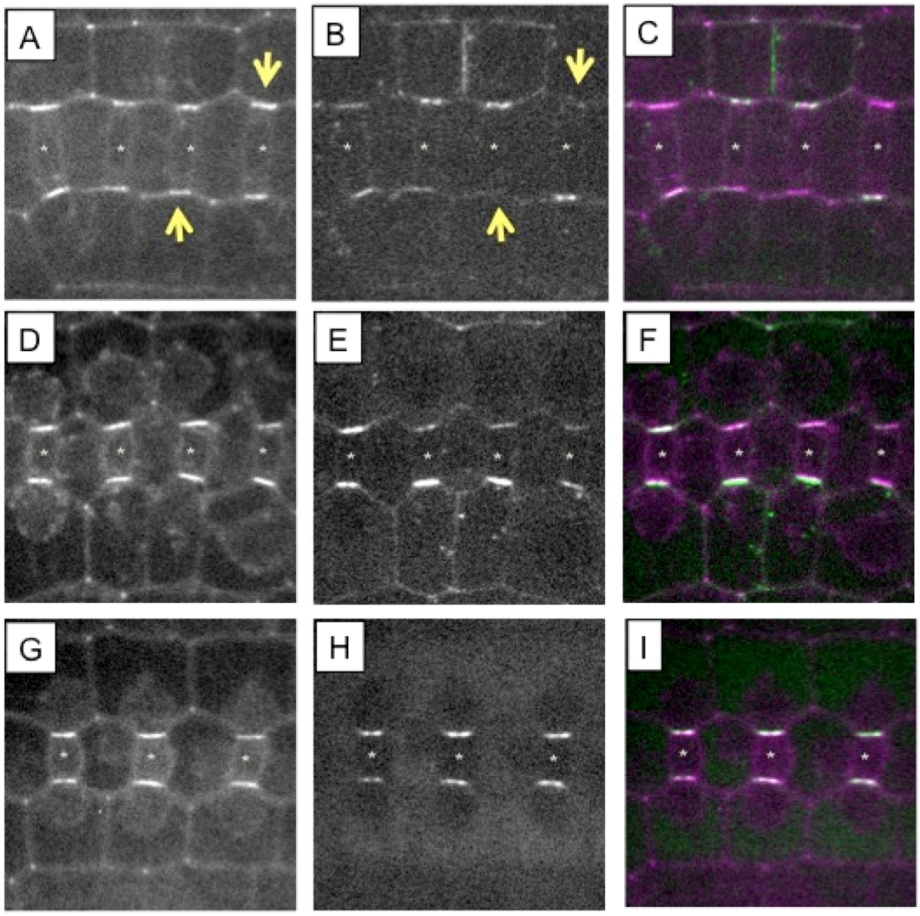

Figure 12. BRK localizes at GMC-SMC contact site before PAN1 patch formation in wild type
A-I: Images from transgenic plants expressing both BRK1-CFP;PAN1-YFP. Leftmost images show BRK1 localization from BRK1-CFP. Middle images show PAN1 localization from PAN1-YFP. Right images show merged images of BRK1 (magenta) and PAN1 (green). BRK patches forms prior to PAN1 patch formation (yellow arrows). Asterisks mark guard mother cells.

Table 5. Quantification of PAN1-YFP and BRK1-CFP patch appearance based on SMC nuclei polarization.

There are instances in which only BRK1-CFP patch is seen with unpolarized SMC nuclei but not PAN1-YFP on its own.

	PAN1 patch only	BRK1 patch only	PAN1 and BRK1 patches
Unpolarized SMC nuclei	0	137	153
Polarized SMC nuclei	0	0	128

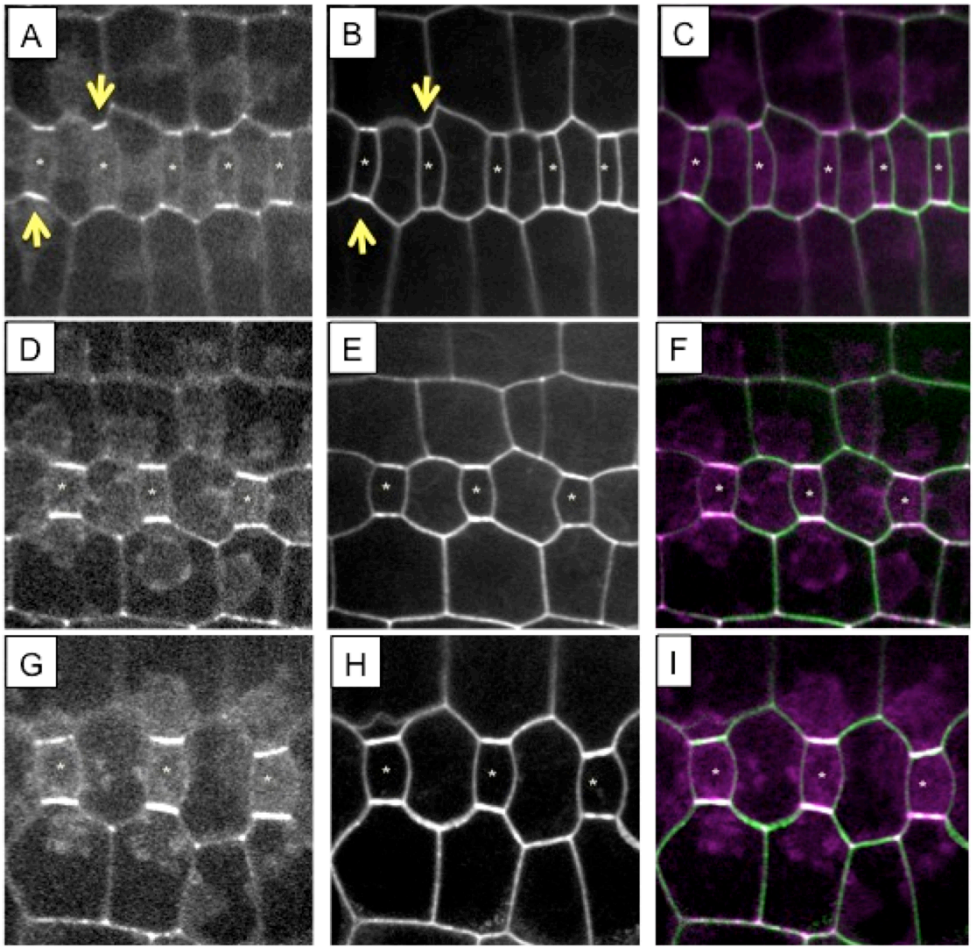

Figure 13. BRK localizes at GMC-SMC contact site before PAN2 path formation in wild type
A-I. Images from transgenic expressing both BRK1-CFP;PAN2-YFP. Leftmost images show BRK localization from BRK1-CFP. Middle images show PAN2 localization from PAN2-YFP. Right images show merged images of BRK1 (magenta) and PAN2 (green). BRK1 patches form prior to PAN2 patch formation (yellow arrows) . Asterisks mark guard mother cells.

Table 6. Quantification of PAN2-YFP and BRK1-CFP patch appearance based on SMC nuclei polarization

There are instances in which only BRK1-CFP patch is seen with unpolarized SMC nuclei but not PAN2-YFP on its own.

	PAN2 patch only	BRK1 patch only	PAN2 and BRK1 patches
Unpolarized SMC nuclei	0	105	104
Polarized SMC nuclei	0	4	112

Previous studies have shown that PAN1 interacts with ROP, and ROP has also been found to directly interact and activate the SCAR complex (Humphries et al., 2011; Uhrig et al., 2007; Basu et al., 2008). From observing that BRK1 appears before PAN1 and PAN2 using transgenic plants, it was hypothesized that that BRK1 might have multiple roles in the SMC division pathway, working upstream and downstream of PAN1 and PAN2. To examine a possible feedback loop between BRK1, PAN1 and PAN2, BRK1-CFP patches were observed in *pan1* and *pan2* mutants. The intensity of BRK1 patches was similar in *pan1* and *pan2* mutants when compared to the wild type (Figure 14). In *pan1* and *pan2* mutants, BRK1 patches were present before and after SMC nuclear polarization and remained at the GMC-SMC contact site until SMC division. These results indicate that PAN1 and PAN2 are not involved in the recruitment of BRK1 and place BRK as the most upstream component of the SMC division pathway.

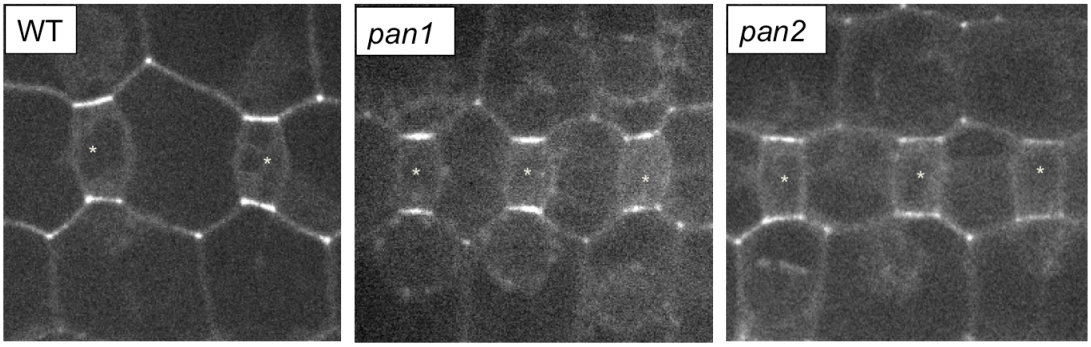

Figure 14. BRK localization is unaffected in *pan1* and *pan2* mutants
Images show BRK localization in BRK1-CFP transgenic plants in wild type, *pan1* and *pan2* mutant backgrounds. BRK localization is unaltered in mutants compared to the wild type.

DISCUSSION

I. Relationship between PAN1 and PAN2

PAN1 and PAN2 are inactive leucine rich receptor like kinases that localize at the GMC-SMC contact site before SMC asymmetric cell division (Cartwright et al., 2009; Zhang et al., 2012). Since PAN1 was initially identified as an inactive kinase, it was previously hypothesized that PAN1 might interact with an active partner such as PAN2. However, the recent discovery of PAN2 as an inactive kinase, coupled with yeast two-hybrid presented here and co-IP data from Zhang et al. prove otherwise (2012), suggesting PAN1 and PAN2 are not signaling partners, and do not interact. However, negative yeast two-hybrid results do not rule out the possible interaction between PAN1 and PAN2. This interaction may be transient or intermittent, not allowing growth to occur in yeast.

No other potential candidates proved to be possible interactors of PAN1 and PAN2 through yeast two-hybrid screening. Since soluble regions of these proteins were cloned to yeast two-hybrid vectors, it is possible other regions of the protein (extracellular domain or ligand binding domain) or signal specific ligands are needed for positive interaction. Two of the candidates showed auto-activation in the yeast system when placed in pAS. These proteins showed growth with PAN1-pACT and PAN2-pACT in absence of histidine, but also grew in the interaction of an empty pACT vector. Future tasks include performing different type of interaction assays such as co-immunoprecipitation to find *in vivo* interactors. Additionally, bimolecular fluorescent complementation (BiFC) or increasing concentration of 3-AT in the media can be used as methods to prevent the auto-activation seen the yeast two-hybrid system.

II. BRK as the first component of the SMC division pathway

In double mutant analysis, *Pan* and *Brk* interacted genetically and *pan;brk* double mutants display a synergistic effect on abnormal subsidiary cell formation. This suggests that the relationship between PAN and BRK may be more complex than previously thought. It seems unlikely that BRK is strictly downstream of PAN1 and PAN2. Rather than having a linear relationship in which one protein directly effects the expression of the other in an upstream-downstream manner, BRK and PAN appear to work in combination for SMC polarization.

Previous study showed depletion of PAN1 localization in *brk1* but this study showed that PAN1 and PAN2 expression levels were not reduced by western blots. These western blots show that PAN1 and PAN2 expression levels might be higher in *brk1* and *brk3* than to the wild type, although this may be due to small increases in *brk1* and *brk3* protein loading as seen in the Coomassie-stained gel in Figure 7. Additional replicates of this experiment with better loading controls are needed to confirm the possible increase in expression of PAN1 and PAN2 in *brk* mutant plants. Regardless of these small loading errors, it appears that neither PAN1 nor PAN2 are depleted in *brk* mutants, suggesting that the observed decrease in PAN1 at SMC-GMC patches is likely due to a decrease in protein recruitment to the patch site rather than a decrease in protein synthesis.

Inhibitor studies were conducted to observe the importance of the Arp2/3 complex in either maintenance or establishment of PAN1, PAN2 and actin patch formation. Short-term treatments showed that actin is not required in the maintenance of PAN1 and BRK1 patches. With this finding, the role of actin patch at the GMC-SMC contact site remains elusive. Although it was expected that high concentration of latB

would be needed to penetrate the waxy cuticle for this treatment, to use 5mM to see an effect was surprising. With latB being a much more potent pharmalogical agent than Arp 2/3 inhibitors, short-term treatments were not conducted with Arp 2/3 inhibitors. It may be possible to perform this experiment by removing the cuticle, which may decrease the amount of inhibitor needed to see an effect; however, this method greatly increases the chance of injuring the tissue. Moreover, the use of ABD2-YFP lines enabled us to monitor the effects of latB treatment. Since these ARP2/3 inhibitors have not been tested in plants and in animals there is no direct observable effect on actin, there is no clear way to monitor the efficacy of these inhibitors. Therefore, I opted not to pursue these treatments.

It was hoped that the long-term inhibitor treatment would provide better insight as to the role of the Arp2/3 complex in the establishment of PAN1, PAN2 and in generating the *brk* phenotype. Unfortunately, results from the long-term treatment were contradictory and inconclusive. Two different germination methods were used, which could have contributed to such differences. The method of using ½ MS-agar appeared to work better; however, mold was an issue for both methods although seeds were dusted with antifungal powder. Intermediate concentration of latB has also been found to have various effects on root elongation and hypocotyl curvature in *A. thaliana* when compared to higher concentration of latB, which suggests the likeliness of conflicting results (Collings et al., 2006; Yamamoto and Kiss, 2002). In this treatment, low germination percentage was also an issue (such as *pan1* in ½ MS-agar treatment). In future, it might be helpful to note beforehand which families have high germination frequencies so that more samples can be obtained.

By using various transgenic plants, BRK1 was concluded to appear before both PAN1 and PAN2 and becomes the first known first signaling component of the SMC division pathway. Moreover, since BRK patches appear unaffected in both *pan1* and *pan2* mutants, while PAN1 is depleted in BRK, this strongly suggests that the primary role of BRK occurs prior to that of PAN1 and PAN2. However, since it was difficult to compare BRK1-CFP and PAN2-YFP timing because the polar accumulation of PAN2 is hard to assess due to PAN2-YFP fluorescence around the cell periphery. Although BRK1 seems to localize before PAN2, it is possible that PAN2 polarization occurs before. This is especially relevant since it is known that PAN2 is genetically upstream of PAN1 (Zhang et al., 2012). Observing the PAN2 patches at GMC-SMC contact site in *brk1* mutants will help confirm whether BRK acts upstream of PAN2. If BRK1 is indeed working upstream of PAN2, then PAN2 patches would be depleted in *brk1*.

III. Possible roles of BRK1 to polarly localize PAN1 and PAN2 to GMC-SMC contact site

Our previous model proposed BRK1 as a downstream component of PAN1 and PAN2, promoting actin patch formation in the SMC division pathway. With recent findings from Zhang et al. placing PAN2 upstream of PAN1, the SMC signaling pathway appeared to begin with signal initiation by PAN2 recruiting PAN1, followed by SMC polarization and actin patch formation, and BRK localization to the same spot (2012). This was concluded because BRK1 regulates Arp2/3 complex activity, which promotes actin nucleation and this actin branching may be responsible for GMC-SMC contact site actin patch formation.

However, it is surprising that results from this study place BRK1 as the upstream component of the SMC division pathway before PAN2, PAN1 and actin patch formations and this might require a modification of the model presented in Figure 3. However, this does not mean that BRK1's function is unrelated to the dense actin patches that form prior to SMC nuclear migration. There may be different actin patches or filaments that are unseen from the current actin patch seen at the GMC-SMC contact site. The ABD2-YFP transgenic line used in this study did not visualize actin filaments shown at the phragmoplast (Sutimantanapi, unpublished data). Therefore, it is possible that actin patches associated with BRK1 localization during earlier stages of SMC polarization may not be shown.

With BRK1 as the first component of the SMC signaling pathway, the role of BRK1 can be speculated by looking at various actin-dependent processes for polar accumulation of proteins. It is unknown how PAN1 and PAN2 are recruited to the GMC-SMC contact site in the first place and it seems likely that BRK1 may be involved with this recruitment. One hypothesis is that BRK1 might promote actin nucleation needed for clathrin-mediated endocytosis of PAN1 and PAN2 (Figure 14A). Arp 2/3 complex has been found to localize at sites of clathrin-mediated endocytosis. Although the role of actin in clathrin-mediated endocytosis is not clear, it may assist with invaginating the membrane, pinching off and/or driving off the vesicles from the plasma membrane (Goley and Welch, 2006). In such cases, PAN1 and PAN2 at around the cell periphery can be recruited and recyled to GMC-SMC contact site via endocytosis. This can be tested by using fluorescence recovery after photobleaching (FRAP) on PAN1-YFP and PAN2-YFP transgenic plants.

If not part of endocytosis, BRK1 might assist to promote the delivery of PAN1 and PAN2 to the GMC-SMC contact site by actin-mediated vesicle trafficking (Figure 14B). In tip-growing cells, such as pollen tubes, actin cables are used to deliver vesicles with cell wall and plasma membrane components. It is possible that BRK1 promotes actin branching needed to direct recycled PAN1 and PAN2 and/or newly synthesized PAN1 and PAN2 from the Golgi. Interestingly, ROP localizes to the plasma membrane of elongating pollen tubes, which is thought to control the Ca^{2+} influx for efficient vesicle fusion to the growth site (Samaj et al., 2004). In *A. thaliana,* ROP7 has been found to interact with SCAR through BiFC and yeast two-hybrid screening (Uhrig et al. 2007). With the recent finding of PAN1's interaction with Type 1 ROPs in maize (Humphries et al, 2011), it might be possible that BRK1, ROP and PAN1 work together to coordinate SMC polarization. Future tasks include co-immunoprecipitation of BRK1 to confirm interaction with ROP in maize tissues, as well as observing BRK1-CFP localization in *rop* mutants.

Lastly, it is possible that BRK organizes actin network needed to lock PAN1 and PAN2 at its localized site (Figure 14C). PINFORMED1 (PIN1) is a well-studied transmembrane protein that generates directional auxin movement (Petricka et al., 2009). Proper localization of PIN is required for auxin movement and have effects such as cell differentiation in early embryos or generation of puzzle like cell morphology in *A. thaliana* leaf epidermis (Krecek et al., 2009; Li et al., 2011). In recently published data, cortical actin nets generated by ROP2-RIC4 pathway inhibit PIN1 endocytosis at the plasma membrane (Nagawa et al., 2012). It is possible that BRK is also responsible for creating this actin net to keep PAN1 and PAN2 localized at the GMC-SMC contact site.

Results from this study place BRK1 at the top of SMC polarization signaling cascade before PAN2, PAN1 and the actin patch. However, the function of BRK1 in SMC polarization remains unknown, and additional experiments are required to fully understand its function. Future work will focus on the possible roles of BRK1 in promoting actin-mediated localization of PAN1 and PAN2 to the GMC-SMC contact site or in generating the actin net to inhibit receptor endocytosis.

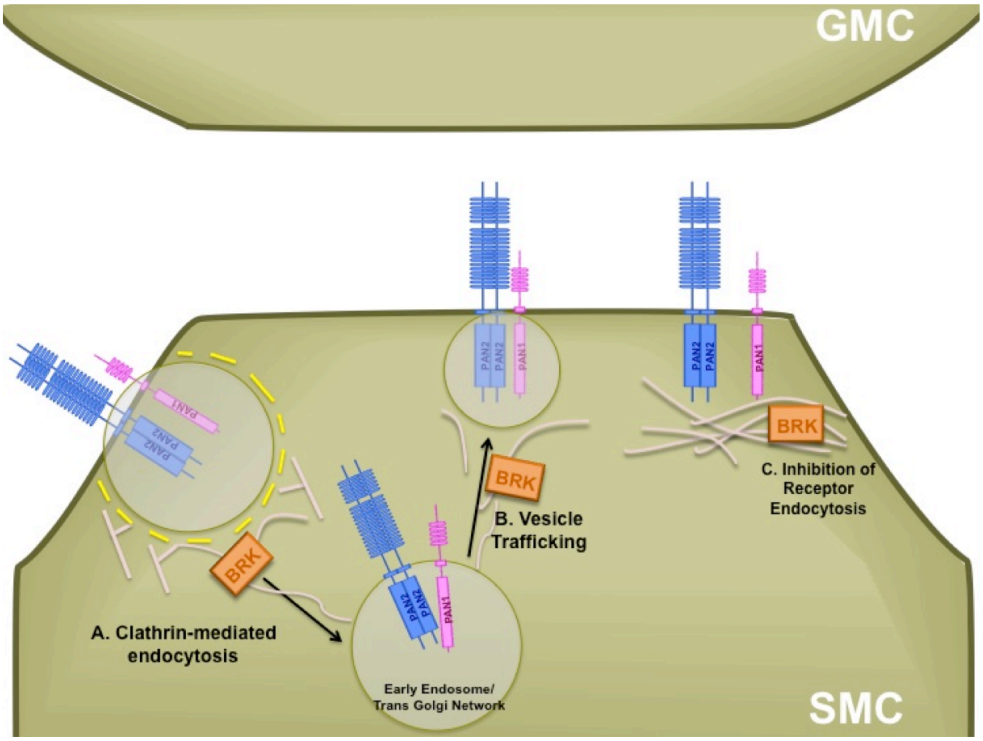

Figure 15. Possible roles of BRK1

A. BRK1 is required for removing PAN1 and PAN2 from non-patch locales via clathrin-coated endocytosis, which requires actin and the ARP complex.
B. BRK1 is required for direct delivery of PAN1 and PAN2 to the patch site via actin-mediated vesicle trafficking.
C. BRK1 is required for forming an "actin net" which inhibits PAN1 and PAN2 endocytosis from the patch site.

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