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IN BRIEF

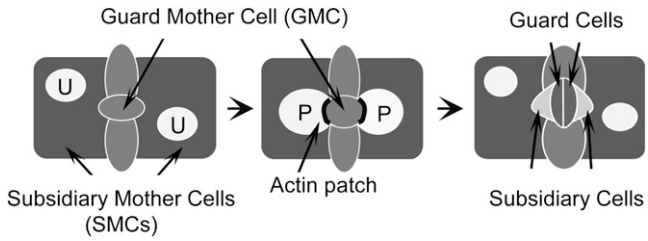
A New Role for ROP GTPases in the Polarization of Cell Division

During plant development, symmetrical division is generally responsible for cell proliferation, whereas asymmetrical division gives rise to new cell types. In either case, the location of the plane of division within the dividing cell is vital to producing daughter cells correctly (reviewed in Rasmussen et al., 2011). The division plane in an asymmetrically dividing cell is related to the cell's polarity, the establishment of which is only beginning to be understood. Stomatal development in maize (*Zea mays*) has proven suitable for studying this process: an asymmetrical division produces the guard mother cell (GMC), and subsidiary cells are the products of asymmetrical divisions of the subsidiary mother cells (SMCs) (see figure). Recently, maize PANGLOSS1 (PAN1) was identified as playing a role in SMC polarity (Cartwright et al., 2009). Now, **Humphries et al. (pages 2273–2284)** have shown that Rho-related GTPases of plants (ROPs) are also involved.

ROPs are plant-specific members of the Rho GTPase family that is present in all eukaryotes. ROPs act as molecular switches regulating a variety of processes, including polarized cell growth (reviewed in Nibau et al., 2006; Yang and Fu, 2007). Several receptor-like kinases have been shown to interact with ROPs or their regulators. These features prompted the authors to ask whether ROPs might function with PAN1 (a Leu-rich repeat receptor-like protein with an inactive kinase domain) in establishing polarity for asymmetric cell division in SMCs.

Humphries et al. focused on ROP2 and ROP9, nearly identical type I ROPs. Mutants lacking both proteins were not recovered but some subsidiary cells were defective in plants lacking ROP2 and containing only one wild-type copy of *rop9*. Furthermore, mutation of either gene enhanced the *pan1* phenotype, leading to even higher numbers of altered subsidiary cells. In addition, a chemical inhibitor of ROP resulted in dose-dependent increases in defective subsidiary cells in the wild type and in *pan1*. Humphries et al. found that the altered subsidiary cell phenotypes are likely due to abnormal SMC polarity, supporting the notion that the ROPs and PAN1 promote correct polarization of SMCs.

The authors showed that ROP appears at GMC contact sites within SMCs after PAN1 does and that this ROP localization is PAN1 dependent. Furthermore, Humphries et al. provide evidence that the polarized localization of ROP is crucial to its function in the orientation of SMC divisions and that ROP and PAN1 interact physically, either directly or indirectly. In sum, the authors have convincingly established that ROPs are involved in the polarization of asymmetric SMC division, acting downstream of the PAN1 receptor-like protein. This work thus adds a new function to the list of processes in which ROPs participate, namely, polarization of cell division. It also supports the idea that Leu-rich repeat receptor-like kinases (or receptor-like proteins) are important in the regulation of ROPs, in this case by affecting ROP's spatial localization.



Maize stomatal development. SMCs on either side of the GMC become polarized, with their nuclei moving toward the GMC and an actin patch forming at the contact site between the GMC and SMC, before the SMCs divide asymmetrically to form the subsidiary cells and the GMC divides symmetrically to form the guard cell pair. U, unpolarized SMC nucleus; P, polarized SMC nucleus. [*Reprinted from Figure 1 of Humphries et al. [2011]*.)

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ROP GTPases Act with the Receptor-Like Protein PAN1 to Polarize Asymmetric Cell Division in Maize [W] [OA]

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Plant Rho family GTPases (ROPs) have been investigated primarily for their functions in polarized cell growth. We previously showed that the maize (*Zea mays*) Leu-rich repeat receptor-like protein PANGLOSS1 (PAN1) promotes the polarization of asymmetric subsidiary mother cell (SMC) divisions during stomatal development. Here, we show that maize Type I ROPs 2 and 9 function together with PAN1 in this process. Partial loss of ROP2/9 function causes a weak SMC division polarity phenotype and strongly enhances this phenotype in *pan1* mutants. Like PAN1, ROPs accumulate in an asymmetric manner in SMCs. Overexpression of yellow fluorescent protein-ROP2 is associated with its delocalization in SMCs and with aberrantly oriented SMC divisions. Polarized localization of ROPs depends on PAN1, but PAN1 localization is insensitive to depletion and depolarization of ROP. Membrane-associated Type I ROPs display increased nonionic detergent solubility in *pan1* mutants, suggesting a role for PAN1 in membrane partitioning of ROPs. Finally, endogenous PAN1 and ROP proteins are physically associated with each other in maize tissue extracts, as demonstrated by reciprocal coimmunoprecipitation experiments. This study demonstrates that ROPs play a key role in polarization of plant cell division and cell growth and reveals a role for a receptor-like protein in spatial localization of ROPs.

INTRODUCTION

The generation of cell polarity is critical for a wide variety of cellular processes, including polarized cell growth, cell migration, asymmetric cell division, and targeted secretion. Members of the Rho family of small GTPases (e.g., Rho, Rac, and Cdc42) are found in all eukaryotes and play important roles in establishing and maintaining cell polarity (Schmitz et al., 2000; Jaffe and Hall, 2005; Ridley, 2006). Rho family GTPases act as molecular switches regulated by noncovalent binding of GTP, cycling between a GTP-bound active form and a GDP-bound inactive form. A variety of downstream effectors bind to the active form of these GTPases to mediate their effects on cellular processes important for cell polarity.

Although plants lack direct orthologs of Rho, Rac, and Cdc42, they have a distinct group of GTPases in the Rho family, termed ROPs (Rho of plants) or RACs (Zheng and Yang, 2000; Christensen et al., 2003). Whereas ROPs are involved in a variety of processes, they have been investigated primarily for their roles in polarized cell growth (reviewed in Nibau et al., 2006; Yang and Fu, 2007). In *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*), a subset of related ROPs is localized to the plasma membrane at the growing tips of pollen tubes and root hairs, and interference with the function of this subset of ROPs causes arrest or depolarization of tip growth, demonstrating a central role for ROPs in this process (Kost et al., 1999; Li et al., 1999; Molendijk et al., 2001; Jones et al., 2002; Gu et al., 2003). In addition to promoting root hair elongation, ROPs influence the site of root hair emergence from the epidermal cell surface (Molendijk et al., 2001; Jones et al., 2002). ROPs also regulate the spatial distribution of growth in epidermal pavement cells, which form interlocking lobes via polarized diffuse growth at multiple sites at their margins (Fu et al., 2002, 2005). The Rho family GTPase Cdc42 is essential for asymmetric cell division in animals (reviewed in Siller and Doe, 2009), but a similar role for ROPs has not been demonstrated in plants, where cytokinesis and its spatial regulation are accomplished very differently compared with animals (reviewed in Rasmussen et al., 2011).

Depolarization of ROP localization via its overexpression leads to depolarized growth, indicating that tip localization of ROP is essential for its function in polarized growth (Kost et al., 1999; Li et al., 1999; Molendijk et al., 2001; Jones et al., 2002). Multiple regulators of ROP activity play important roles in its polarized localization. For example, presumptive activators of ROP in the plant-specific PRONE family of guanine nucleotide exchange factors (GEFs) are localized at pollen tube tips, and their mislocalization causes ROP-dependent depolarization of pollen tube growth (Gu et al., 2006; Zhang and McCormick, 2007). Two classes of ROP negative regulators, RopGAPs (GTPase activating proteins) and RhoGDIs (guanine nucleotide dissociation inhibitors), have

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been implicated in restricting ROP activity to the tips of pollen tubes and root hairs (Carol et al., 2005; Klahre et al., 2006; Klahre and Kost, 2006; Hwang et al., 2008, 2010). However, little is known about how these ROP regulators are themselves spatially regulated to determine sites of ROP activity.

The development of the stomatal complex in maize (*Zea mays*) provides an example of the role that cell polarity plays in directing the orientation of cell division. Stomatal complexes are composed of a pair of guard cells flanked by a pair of subsidiary cells, which regulate stomatal opening. This complex is produced by a coordinated set of asymmetric divisions (Figure 1A). The first produces a guard mother cell (GMC), which will eventually divide symmetrically to produce the guard cell pair. Before the GMC divides, however, its lateral neighbors (subsidiary mother cells [SMCs]) become polarized with respect to the GMC, evident by the migration of the SMC nucleus to the GMC interface and by formation of a cortical F-actin patch at this site. SMCs subsequently divide asymmetrically to produce the subsidiary cells. We previously characterized a mutant, *pan1*, with defects in SMC polarity that lead to aberrantly oriented SMC divisions (Gallagher and Smith, 2000; Cartwright et al., 2009). PAN1 is a leucine-rich repeat (LRR) receptor-like protein with an inactive kinase domain, which becomes localized asymmetrically within the SMC at the GMC contact site shortly after GMC formation (Cartwright et al., 2009). Thus, PAN1 is thought to participate in a pathway that promotes cell polarity signaling from the GMC to the SMC. However, other components of this pathway remain to be identified.

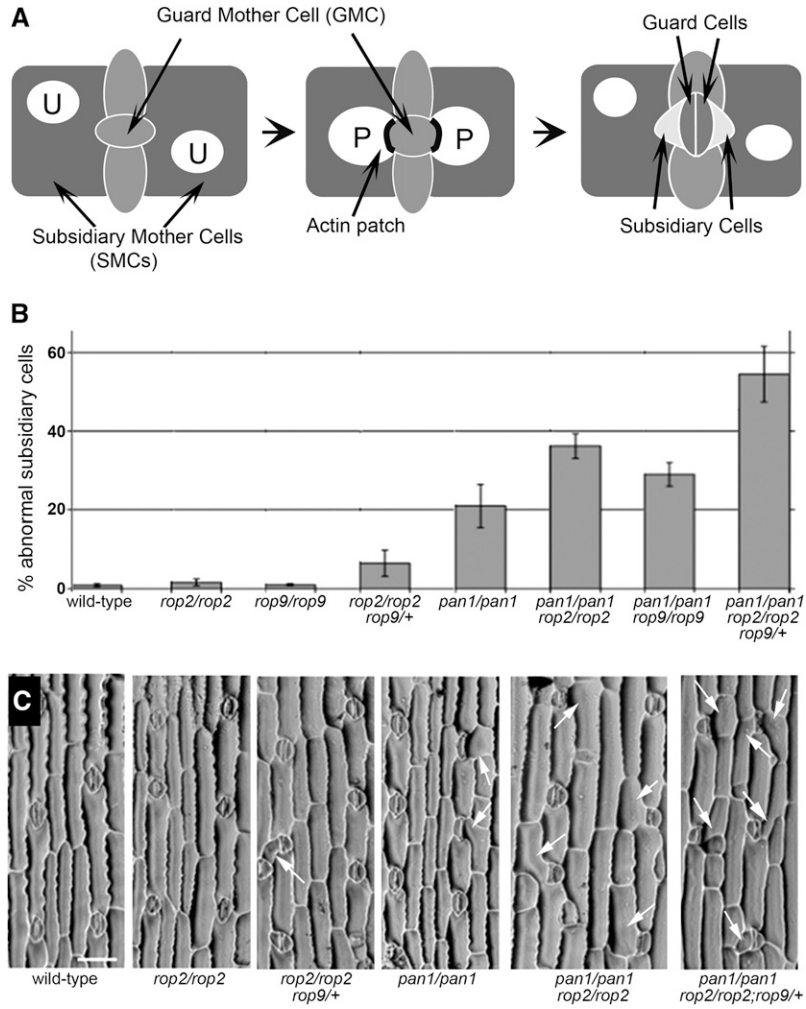

Figure 1. Subsidiary Cell Defects in *pan1* and *rop* Mutants.

(A) Schematic representation of stomatal development in maize. SMCs initially flank the GMC in an unpolarized state (left panel). An actin patch subsequently forms at the contact site between the GMC and SMC, and the SMC nuclei polarize toward the GMC (middle panel). SMCs then divide asymmetrically to form a subsidiary cell, and the GMC divides symmetrically to form the guard cell pair (right panel). U, unpolarized SMC nucleus; P, polarized SMC nucleus.

(B) Quantification of abnormal subsidiary cells in the leaf epidermis of *pan1* and *rop* mutants ($n > 500$ per genotype). Error bars represent SD.

(C) Representative images of the epidermis from a subset of the mutants analyzed. Arrows indicate abnormal subsidiary cells. Bar = 100 μm.

ROPs or their regulators have been shown to interact with a variety of receptor-like kinases (RLKs). A ROP protein was isolated as a component of a complex containing the *Arabidopsis* LRR-RLK CLAVATA1, a regular of shoot apical meristem maintenance (Trotochaud et al., 1999). A Cys-rich RLK of *Arabidopsis* (NCRK) and members of another RLK family (RLCK Class VI) in *Medicago* and *Arabidopsis* interact directly with ROPs via their kinase domains, and the kinase activity of RLCK is activated by GTP-ROP in vitro (Molendijk et al., 2008; Dorjgotov et al., 2009). PRONE family ROP-GEFs interact with two distinct LRR-RLKs that promote tip growth: tomato (*Solanum lycopersicum*) and *Arabidopsis* pollen receptor kinase (PRK) family members in pollen tubes (Kaothien et al., 2005; Zhang and McCormick, 2007) and *Arabidopsis* FERONIA (FER) in root hairs (Duan et al., 2010). These findings imply the participation of ROPs in diverse LRR-RLK signaling pathways.

Given the known roles of ROPs in polarized cell growth and their previously established associations with RLKs, we hypothesized that maize ROPs may play a role in the polarization of SMC divisions, perhaps in conjunction with PAN1. Although little is known about the in vivo functions of the nine ROPs in maize, ROP2 has a role in the male gametophyte, as *rop2* mutant pollen transmits at a reduced frequency under competitive conditions (i.e., the presence of wild-type pollen; Arthur et al., 2003). Like *rop2*, the nearly identical *rop9* is also expressed in pollen (Christensen et al., 2003), raising the possibility of a redundant role similar to *rop2*. We investigated the role of maize ROP2 and ROP9 in asymmetric SMC divisions. A combination of genetic, protein localization, and biochemical observations demonstrates that these ROPs function downstream of PAN1 to promote the premitotic polarization of SMCs.

RESULTS

Maize ROP2 and ROP9 Act with PAN1 to Promote Premitotic Polarization of SMCs

To investigate the role of ROP in the polarization of SMC divisions in maize, *rop* mutants were analyzed for subsidiary cell defects in the leaf epidermis. The *Arabidopsis* ROPs (e.g., *ROP1*, *ROP2*, and *ROP4*), known to play a role in polarized cell growth, are in a group known as the Type I ROPs, based on sequence and apparent plasma membrane–targeting mechanisms (reviewed in Berken and Wittinghofer, 2008). The maize genome encodes only three Type I ROPs: ROP2, ROP4, and ROP9 (Christensen et al., 2003). Based on this knowledge and the availability of *rop2* and *rop9* (but not *rop4*) mutant alleles causing pollen transmission defects, we chose to focus on maize ROP2 and ROP9, which are almost identical, differing at only one amino acid. Plants homozygous for mutations in *rop2* or *rop9* alone showed no significant increase in defective subsidiary cells compared with the wild-type (Figures 1B and 1C). Although *rop2/rop2;rop9/rop9* double homozygotes were never recovered, possibly due to an important shared function for the two genes in the male gametophyte, *rop2/rop2; rop9/+* mutants were recovered and had 7.4% defective subsidiary cells (a significant increase compared with the wild type, Student's *t* test, $P < 0.01$; Figures 1B and 1C). *rop2/+; rop9/rop9* mutants did not show subsidiary cell defects, possibly due to much higher expression of the *rop2* gene compared with *rop9* in the leaf division zone (Christensen et al., 2003). Combinations of *rop* and *pan1* mutants were also analyzed to investigate possible genetic interactions. Indeed, mutations in either *rop2* or *rop9* enhance the effect of the *pan1* mutation on SMC divisions, with *rop2* again having a stronger effect (Figures 1B and 1C). Further enhancement of the *pan1* phenotype was observed in *pan1/panl; rop2/rop2;rop9/+* triple mutants, which showed a nearly threefold increase in aberrant subsidiary cells compared with *pan1* single mutants (Figures 1B and 1C).

To confirm that the phenotype observed in the *rop* mutants was indeed caused by a loss of ROP function, we took an independent approach to examine the effects of ROP inhibition on maize subsidiary cell formation. NSC23766 inhibits mammalian Rac by blocking its interaction with a GEF, targeting specific residues of Rac involved in GEF binding (Gao et al., 2004). All of these residues are conserved in all nine maize ROPs, including ROP2 and ROP9 (see Supplemental Figure 1 online). We therefore predicted that NSC23766 would inhibit the functions of maize ROP2 and ROP9 (along with other ROPs). Wild-type plants grown in various concentrations of inhibitor (25 to 100 μM, similar to the concentration range used in prior studies for inhibition of Rac in vivo; Gao et al., 2004; Hable et al., 2008) displayed a dose-dependent effect, with 4 to 11% defective subsidiary cells (Figure 2). These findings strengthen the conclusion that ROPs are involved in the formation of subsidiary cells in maize. Moreover, consistent with the finding that *rop* mutations enhanced the *pan1* aberrant subsidiary cell phenotype (Figure 1C), NSC23766 treatment of *pan1* mutants caused a dose-dependent increase in the frequency of aberrant subsidiary cells (Figure 2A).

Previous analysis of the maize *pan1* mutant demonstrated that the subsidiary cell defects can be traced back to premitotic SMC polarity defects. To investigate SMC polarity defects in *rop* mutants, nuclear positioning and actin patch formation were analyzed as markers of SMC polarity. In the wild type, 92% of SMCs displayed a correctly polarized nucleus, and most of these had a normal actin patch (Figures 3A and 3B). Similar to previous results (Cartwright et al., 2009), 68% of SMCs in *pan1* mutants had polarized nuclei, and many SMCs with both polarized and unpolarized nuclei had delocalized or absent actin patches. Consistent with their weak SMC formation defect, *rop2/rop2;rop9/+* mutants displayed mild defects in nuclear polarization and actin patch formation (Figures 3A and 3B). However, in the *pan1/pan1;rop2/rop2;rop9/+* mutants, only about half of the SMCs displayed polarized nuclei, and the majority of SMCs with either polarized or unpolarized nuclei had delocalized or absent actin patches. These results suggest that the subsidiary cell defects of *rop* and *rop;pan* double and triple mutants, like *pan1* mutants, are due to defects in SMC polarization and that PAN1 and ROP2/ROP9 function cooperatively to polarize premitotic SMCs correctly.

After GMC formation, SMCs protrude slightly toward the GMC as it elongates (see Supplemental Figure 2A online). Given the known role of ROPs in polarized cell growth, we considered whether the primary function of ROPs in SMCs might be to promote the formation of this protrusion, with SMC division defects in *rop* mutants occurring somehow as a secondary consequence. To investigate this possibility, the degree of protrusion of SMCs

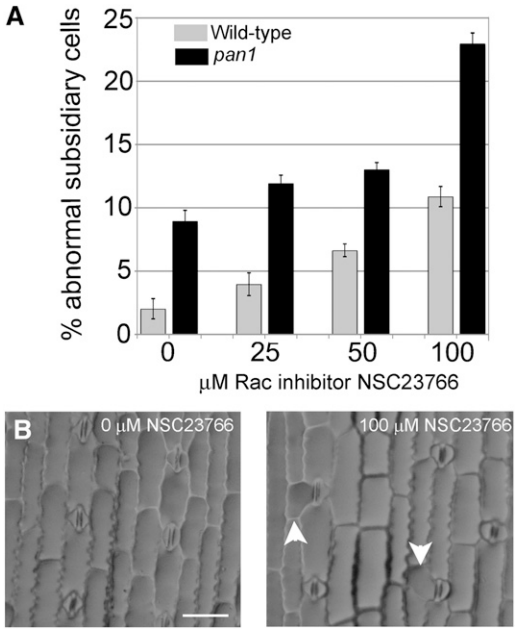

Figure 2. Rac Inhibitor NSC23766 Causes Dose-Dependent Defects in Subsidiary Cell Formation in Maize.

(A) Quantification of abnormal subsidiary cells observed in wild-type (gray) and *pan1* (black) plants treated with varying concentrations of NSC23766 or a control treatment (n > 350 per treatment). Error bars represent SD.

(B) Leaf epidermis of wild-type plants treated with 1% DMSO (left) or with 100 μM NSC23766 in 1% DMSO (right). White arrowheads point to abnormal subsidiary cells in the drug-treated sample. Bar = 100 μm.

toward GMCs was measured in wild-type, *pan1*, *rop2/rop2;rop9*/+, and *pan1/pan1;rop2/rop2;rop9*/+ mutants. As shown in Supplemental Figure 2B online, no significant difference was observed between any of these genotypes ($P > 0.1$), arguing against the protrusion hypothesis and instead suggesting that ROPs 2 and 9 contribute directly to premitotic SMC polarization.

ROPs Exhibit PAN1-Dependent Polarized Localization in Maize SMCs

Although all maize ROPs are closely related, a 15–amino acid region, which is 100% identical in the three Type I maize ROPs but only 33 to 60% identical in other maize ROPs (see Supplemental Figure 3 online), was targeted for antibody production. To investigate the specificity of the affinity-purified antibody raised against this peptide, its reactivity to bacterially expressed ROP2 and ROP7 (a Type II ROP) was compared by immunoblotting. While ROP2 was strongly recognized by the antibody, ROP7 (which is 46.6% identical to ROP2 in the peptide region) appears to be only faintly recognized (Figure 4A), indicating that this antibody will chiefly recognize maize Type I ROPs 2, 4, and 9.

When used to probe the membrane fraction of extracts from the zone of the developing maize leaf where cell division occurs (the division zone), the ROP antibody recognized a prominent band at the expected size for ROP (~25 kD; Figure 4B). To determine the relative contributions of maize ROP2 and ROP9 to this signal, various quantities of division zone membrane proteins from wild-type, *rop2/rop2*, *rop9/rop9*, and *rop2/rop2;rop9*/+ leaves were probed with the ROP antibody. The *rop2* and *rop9* single mutants display a slightly reduced signal compared with the wild type, and the *rop2/rop2;rop9*/+ mutant showed a further reduction (Figure 4B). This confirms that the 25-kD band corresponds to ROP, that endogenous ROP2 and ROP9 are recognized by this antibody, and that Type I ROPs are depleted but not eliminated in *rop2/rop2;rop9*/+ mutants as expected. The signal remaining in the *rop2/rop2;rop9*/+ mutants is likely due mainly to the presence of ROP9 expressed from the wild-type allele and ROP4, which is also expressed at this developmental stage (Christensen et al., 2003).

Immunolocalization using the ROP antibody revealed ROP localization in SMCs at the interface with GMCs (arrowheads, Figure 4D); no specific staining signal was observed anywhere else in epidermal cells within the division zone. This pattern of localization is similar to that observed previously with the PAN1 antibody (Cartwright et al., 2009). We previously showed that the lengths of adjacent GMCs can be used as a measure of SMC developmental age, with GMC length increasing steadily over time as SMCs polarize and eventually divide (Cartwright et al., 2009). To investigate the timing of ROP localization in SMCs, ROP and PAN1 immunostaining patterns were compared in relation to nuclear position and the length of the adjacent GMC (these comparisons were necessarily indirect because double staining was precluded by the fact that both PAN1 and ROP antibodies were raised in rabbits). To ensure that an equivalent spectrum of SMC stages was being examined, nuclear position and adjacent GMC lengths were assessed in the entire population of SMCs analyzed in separate ROP and PAN1 immunostaining experiments and found to be equivalent (see "total analyzed," Figure 4E). The average length of adjacent GMCs for SMCs with PAN1 patches was 5.2 μm compared with 5.87 μm for those with ROP patches (a significant difference, Student's t test, $P < 0.01$), indicating that ROP patches form later than PAN1 patches (Figure 4E, blue bars "with patch"). Moreover, 41% of SMCs with PAN1 patches had a polarized nucleus compared with 58% with ROP patches (Student's t test, $P < 0.001$, Figure 4E, green bars "with patch"). This indicates that both PAN1 and ROP patches form prior to nuclear polarization and further supports the conclusion that ROP patches form later than PAN1 patches.

To investigate whether PAN1 is required for the formation of ROP patches, ROP localization was examined in *pan1* mutants. Strikingly, ROP patches were never observed in *pan1* mutants (Figure 4D), although immunoblot analysis demonstrated that ROP protein is still present in the membrane fraction of *pan1* mutants with no obvious reduction in overall abundance (Figure 4C, lanes 1 versus 4). This suggests that ROP proteins are distributed differently in *pan1* mutants and we are only visualizing the most concentrated sites of ROP protein accumulation via immunolocalization. Our findings that ROPs colocalize with PAN1 at GMC contact sites, require PAN1 to accumulate at this site, and are detected there later than PAN1 strongly argue that ROPs function downstream of PAN1 in SMC polarization.

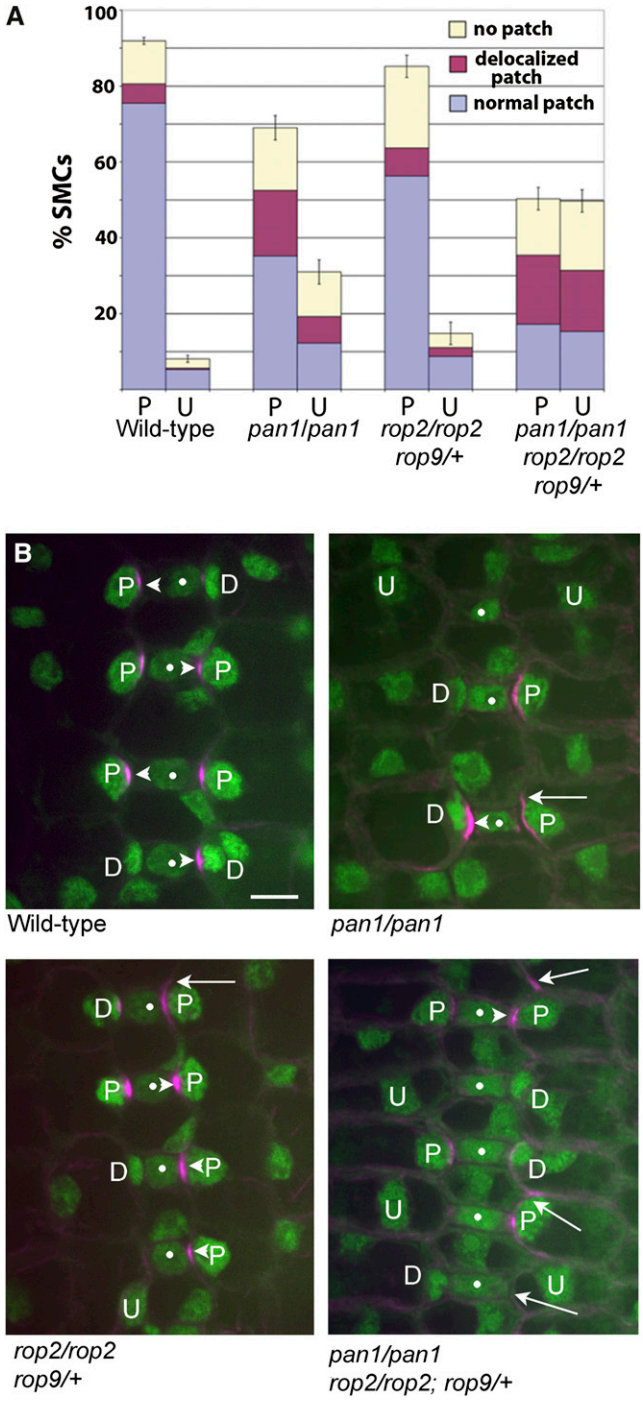

Figure 3. Analysis of Polarity Defects in SMCs of the Wild Type versus *pan1* and *rop* Mutants.

(A) Quantification of the proportion of SMCs with polarized (P) or unpolarized (U) nuclei and actin patch status ($n > 300$ cells per genotype). Error bars represent SD. Each mutant genotype analyzed displays a statistically significant increase in unpolarized nuclei (Student's *t* test, $P < 0.01$) and delocalized or absent actin patches ($P < 0.01$) compared with the wild type.

(B) Representative images of F-actin and propidium iodide–stained nuclei (false-colored magenta and green, respectively) in developing stomata of the indicated genotypes. White dots mark GMCs; arrowheads point to normal actin patches in SMCs; arrows indicate examples of sites in SMCs where an actin patch is either missing or delocalized. U, unpolarized nuclei; P, polarized nuclei; D, divided SMCs. Bar = 10 μm.

ROPs Show Altered Triton X-100 Solubility in *pan1* Mutants Compared with the Wild Type

ROP activation has been associated with recruitment to nonionic detergent-resistant membrane (DRM) domains of the plasma membrane through posttranslational modifications (Sorek et al., 2007). To investigate the influence of PAN1 on partitioning of ROPs within the membrane, wild-type and *pan1* leaf division zone membrane fractions were prepared and subjected to Triton X-100 solubilization. In the wild type, the majority of the ROP protein remained associated with the membrane fraction, while a smaller proportion was solubilized in Triton X-100 (Figure 4C). By contrast, in *pan1* mutants, the majority of ROP protein was solubilized by Triton X-100 with a smaller proportion remaining associated with the membrane fraction. These results indicate that PAN1 promotes the association of Type I ROPs with DRMs in the maize leaf division zone.

Polarized Localization of YFP-ROP2 Is Associated with Correctly Oriented SMC Divisions

To characterize ROP localization further, transgenic lines were generated in which ROP2 was N-terminally tagged with yellow fluorescent protein (YFP) and expressed from its native promoter. In multiple, independent, transgenic lines, YFP-ROP2 often localized nonuniformly within SMCs, appearing concentrated at GMC contact sites, similar to the localization observed via ROP antibody staining (arrowheads, Figure 5A). However, even in cells exhibiting such polarized YFP-ROP2 localization, a lower level of signal was always observed around the entire SMC, which was not observed with ROP antibody staining, consistent with the conclusion from immunolocalization experiments that only the most concentrated sites of ROP accumulation are observable by that method. Sometimes, however, YFP-ROP2 appeared only slightly enriched at GMC contact sites, or not at all, displaying instead a relatively uniform distribution around the SMC surface (arrows, Figure 5B). Moreover, some YFP-ROP2 transgenic plants exhibited subsidiary cell defects in the leaf epidermis similar to those observed in *pan* and *rop* mutants (Figure 5C).

With the knowledge that overexpression of wild-type ROP in tip-growing cells can lead to its delocalization and depolarized growth (Kost et al., 1999; Li et al., 1999; Molendijk et al., 2001; Jones et al., 2002), we hypothesized that this subsidiary cell defect is due to overexpression and consequent delocalization of YFP-ROP2. To test this possibility, individual siblings within a family of transgenic plants (offspring of the same parents) were analyzed for YFP-ROP2 levels via immunoblotting with anti-ROP antibody, examined via microscopy for YFP-ROP2 localization in SMCs, and analyzed for subsidiary cell defects to determine whether these features were correlated. Indeed, in the plant family presented in Figure 5, siblings displaying the most frequent subsidiary cell

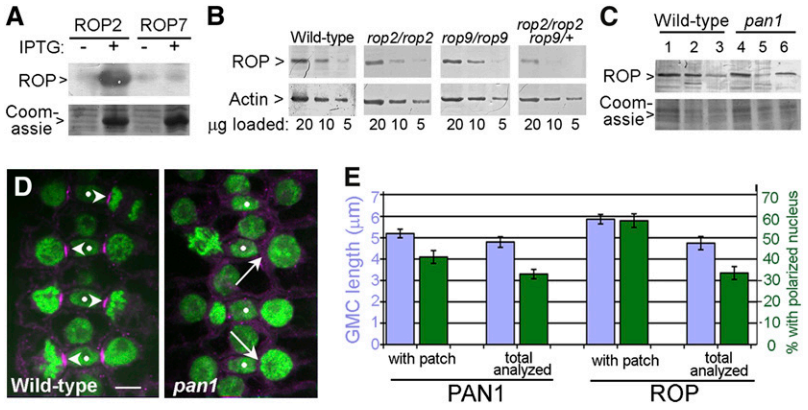

Figure 4. Analysis of ROP Proteins.

(A) Immunoblot of His-tagged maize ROP2 and ROP7 probed with the ROP antibody. The Coomassie blue–stained gel (below) shows induction of both fusion proteins after induction by isopropyl β-D-1-thiogalactopyranoside.

(B) Immunoblots of decreasing quantities of wild-type and *rop* mutant membrane proteins isolated from maize leaf division zones probed with the ROP antibody and an actin antibody as a loading control.

(C) Immunoblot of membrane proteins from the maize leaf division zone: total (lanes 1 and 4), Triton X-100–insoluble (lanes 2 and 5), and Triton X-100–soluble (lanes 3 and 6) probed with the ROP antibody. Coomassie blue staining of a duplicate gel is shown as a loading control.

(D) Immunofluorescent staining with ROP antibody of developing stomata showing ROP localization and propidium-stained nuclei (false-colored magenta and green, respectively). Arrowheads indicate SMC/GMC contact sites with ROP accumulation in the wild type; arrows indicate SMC/GMC contact sites lacking ROP accumulation in the *pan1* mutant. When SMCs with adjacent GMCs having a length:width ratio > 2 were analyzed, ROP patches were observed in 163/202 wild-type SMCs (80.7%) compared with 0/175 *pan1* mutant SMCs. Bar = 10 µm.

(E) Analysis of the timing of PAN1 and ROP patch formation in SMCs. Maize leaf division zones were labeled for PAN1 and ROP in separate immunofluorescence experiments. The appearance of ROP or PAN1 patches in the flanking SMCs was recorded, and GMC lengths were measured (blue), as was the polarization of the SMC nucleus (green). Regardless of the presence or absence of a PAN1 or ROP patch, nuclear position and adjacent GMC lengths were assessed in the entire population of SMCs analyzed in the separate immunostaining experiments (total analyzed) to ensure that an equivalent spectrum of SMC stages was being examined; $n > 100$ SMCs for each staining experiment. Error bars represent SD.

defects (#2, 5, and 7 with 10 to 11% abnormal subsidiary cells) had the highest levels of YFP-ROP2 protein (greatly exceeding the levels of endogenous Type I ROPs) and the highest proportion of SMCs with depolarized YFP-ROP2 localization (Figures 5D to 5F). Conversely, the plant with the lowest YFP-ROP2 levels (#4, with levels similar to endogenous Type I ROPs) also had the lowest proportion of depolarized YFP-ROP2 and the weakest subsidiary cell phenotype, with only 2% abnormal subsidiaries. A Pearson's correlation coefficient of 0.9 indicates a strong correlation between the percentage of SMCs with partially or fully depolarized YFP-ROP2 and the percentage of abnormal subsidiary cells within this family. A correlation between YFP-ROP2 levels, polarization of YFP-ROP2 in SMCs, and subsidiary division defects was also observed in a second, independent transgenic line (data not shown). Although transgene copy number was not determined, the patterns of transgene inheritance we observed are consistent with variation in transgene copy number as the explanation for variable YFP-ROP2 levels among members of these families. These results indicate that correctly polarized localization of ROP2 promotes correctly oriented SMC divisions.

Polarized Localization of PAN1 in SMCs Is Insensitive to Perturbation of ROPs

Whereas it appears that ROP localization depends on PAN1, a question remained as to whether some sort of feedback mechanism exists in which PAN1 localization is also dependent on ROPs. Although it was not possible to test the effects on PAN1 localization of complete ROP elimination, we examined PAN1 localization in a variety of situations where ROP is either partially depleted or depolarized. First, PAN1 localization was examined in *rop2/rop2; rop9*/+ mutants. By whole-mount immunofluorescence, PAN1 localization in *rop2/rop2; rop9*/+ SMCs appeared indistinguishable from that in the wild type, showing strongly polarized localization (Figures 6A and 6B). As a complementary approach, we also examined the effects of ROP depletion on localization of PAN1 fused at its C terminus to YFP expressed from its native promoter in stable transgenic plants. As shown in Supplemental Figure 4 online, PAN1-YFP localization in wild-type plants showed all the hallmarks of PAN1 localization previously demonstrated via immunofluorescence with PAN1 antibody. As shown for PAN1 localization determined via immunofluorescence, PAN1-YFP localization in SMCs of *rop2/rop2; rop9*/+ mutants appeared identical to that in wild-type SMCs (Figures 6C and 6D). PAN1-YFP was also unaffected by treatment with the Rac inhibitor NSC23766 (see Supplemental Figure 5 online). To investigate whether ROP depolarization affects PAN1 localization, plants overexpressing YFP-ROP2 (as determined by immunoblot analysis) were fixed and stained with the PAN1 antibody. Even in SMCs with depolarized YFP-ROP2, PAN1 patches formed normally (Figures 6E and 6F). These observations demonstrate that PAN1 localization at GMC contact

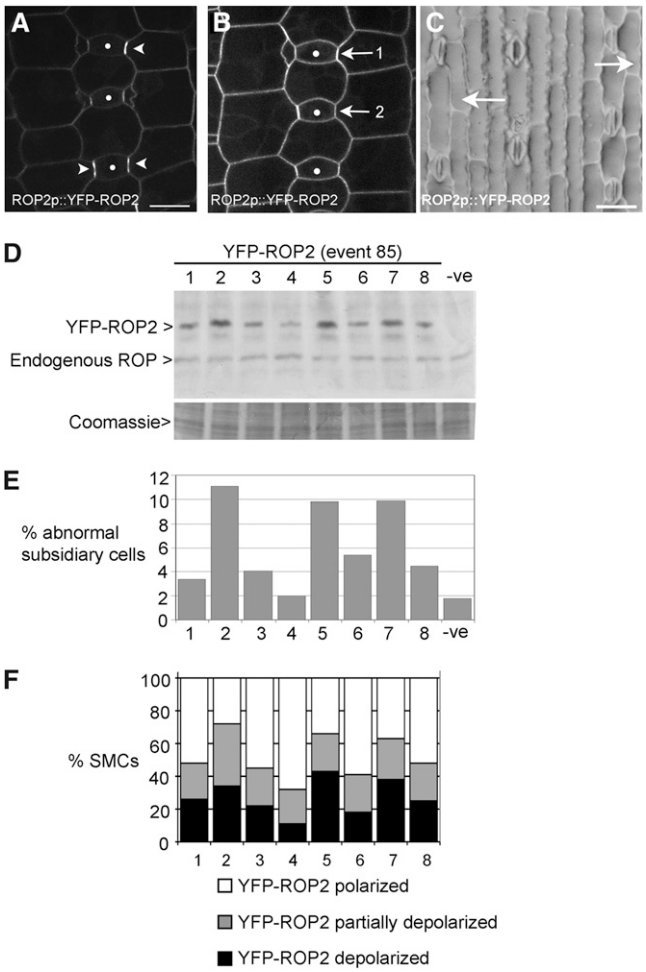

Figure 5. Analysis of YFP-ROP2 Transgenic Lines.

(A) An example of polarized YFP-ROP2 localization in SMCs with dots marking GMCs and arrowheads pointing to YFP-ROP2 patches at GMC contact sites. Bar = 10 μm.

(B) An example of YFP-ROP2 localization in a high-expressing plant, where YFP-ROP2 was judged as partially depolarized (arrow 1) or fully depolarized (arrow 2).

(C) Representative image of the leaf epidermis of a high-expressing YFP-ROP2 plant, with arrows indicating abnormal subsidiary cells. Bar = 100 μm.

(D) Immunoblots of maize leaf division zone membrane proteins probed with ROP antibody showing variable YFP-ROP2 levels in eight different individuals within a single family accompanied by constant levels of endogenous ROP protein. A negative control plant lacking the YFP-ROP2 transgene (-ve) confirms the identity of the YFP-ROP2 band in transgenic plants. Coomassie blue staining of a duplicate gel demonstrates equal loading.

(E) Quantification of abnormal subsidiary cells in the leaf epidermis of the same eight plants analyzed in **(D)** ($n > 400$ subsidiary cells per individual).

(F) Quantitative analysis of YFP-ROP2 localization in SMCs (with adjacent GMCs having a length: width ratio > 2) of the same eight plants analyzed in **(D)** and **(E)**. In each SMC ($n > 100$ for each plant), YFP-ROP2 localization was scored as polarized, partially depolarized, or fully depolarized (see **[A]** and **[B]** for examples of each class).

sites within SMCs is insensitive to depletion or depolarization of ROPs, further supporting the conclusion that ROPs act downstream of PAN1 in SMC polarization.

PAN1 and ROP Are Physically Associated in Maize Leaf Extracts

A variety of observations reported here suggesting a functional link between PAN1 and ROP proteins prompted us to investigate whether these proteins are physically associated. Using the PAN1 antibody to immunoprecipitate endogenous PAN1 protein and the ROP antibody to probe the immunoprecipitates on immunoblots, ROPs were found to coimmunoprecipitate with PAN1 from membrane fractions of wild-type maize leaf division zones (Figure 7A). Although present at wild-type levels in membrane fractions of *pan1* mutants, ROPs were not detected in PAN1 immunoprecipitates from *pan1* mutants, which lack detectable PAN1 protein (Cartwright et al., 2009), demonstrating that ROP is not bound nonspecifically by the PAN1 antibody or beads. In the reciprocal experiment, ROP antibody was used to immunoprecipitate endogenous ROP proteins, and PAN1 was found to coimmunoprecipitate (Figure 7B). Although coimmunoprecipitation appears less efficient in this experiment, when no ROP antibody was added to the wild-type lysate, PAN1 was not found in the precipitate, demonstrating that PAN1 is not bound nonspecifically by the beads. These results demonstrate a physical association between endogenous PAN1 and ROP proteins in extracts from maize leaf division zones.

DISCUSSION

Plant ROPs promote polarized growth in various cell types (reviewed in Nibau et al., 2006; Yang and Fu, 2007). In this study, we present several indications that ROPs function downstream of the receptor-like protein PAN1 to polarize correctly the asymmetric divisions of maize SMCs. This demonstrates a role for ROPs in polarization of plant cell division and provides new insights into mechanisms by which ROP activity can be spatially regulated.

Functional redundancy among members of the ROP gene family and multiple roles for ROPs at various stages of the plant life cycle preclude complete elimination of ROP function in SMCs. However, partial knockdown of maize Type I ROPs in *rop2/rop2;rop9*/+ mutants resulted in weak SMC polarity defects and a low frequency of aberrantly oriented SMC divisions. Treatment with the Rac inhibitor NSC23766 had a similar effect. Furthermore, *rop2* and *rop9* mutations (individually and in combination) strongly enhanced *pan1* SMC division polarity defects, indicating that ROPs and PAN1 function cooperatively to polarize SMC divisions, potentially acting in a common pathway. However, if ROPs function solely with PAN1 to polarize SMC divisions, then reduction of ROP function would not be expected to enhance SMC polarity defects in *pan1* mutants, which lack detectable PAN1 protein (Cartwright et al., 2009), so the PAN1 pathway is already completely knocked out in these mutants. Thus, the enhancement of the *pan1* phenotype observed in *pan1;rop* double and triple mutants implies that ROPs have

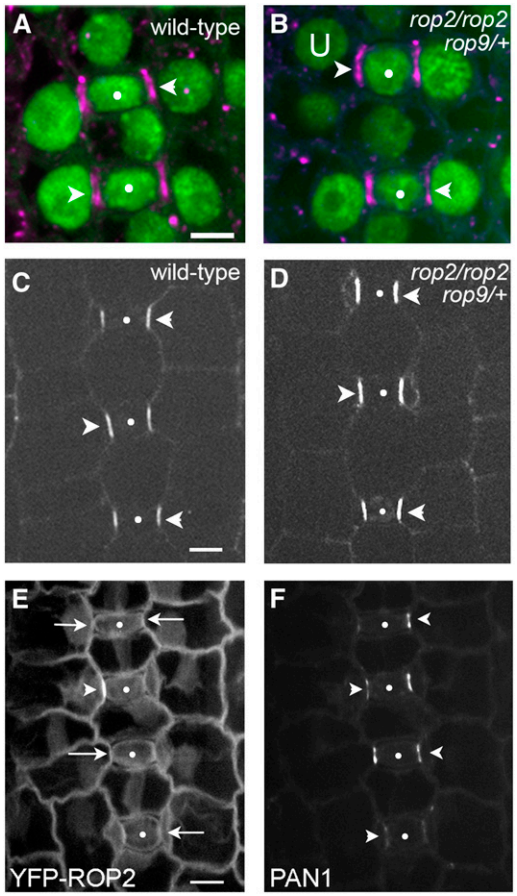

Figure 6. PAN1 Localization in the Wild-Type and *rop* Mutant or YFP-ROP Transgenic SMCs.

GMCs are marked with white dots; white arrowheads point to examples of PAN1 patches in SMCs at sites of GMC contact (or YFP-ROP2 patch in **[E]**). **(A)** and **(B)** Immunofluorescence staining with anti-PAN1 reveals PAN1 patches in both the wild type **(A)** and the *rop2/rop2; rop9*/+ mutant **(B)** SMCs. Top left arrowhead in **(B)** indicates an example of a PAN1 patch in an SMC with unpolarized nucleus (labeled U). SMCs flanking GMCs of this size almost always have polarized nuclei, suggesting that this SMCs nucleus is unpolarized due to ROP depletion, but it nevertheless has a normal PAN1 patch. A total of 188/202 (93.1%) wild-type and 168/182 (92.3%) *rop2/rop2;rop9*/+ mutant SMCs had PAN1 patches revealed by immunofluorescence.* Bar = 10 μm.

(C) and **(D)** PAN1-YFP localization appears indistinguishable in SMCs of the wild type **(C)** and *rop2/rop2; rop9*/+ mutant **(D)**. A total of 267/275 (97.1%) wild-type and 245/255 (96.1%) *rop2/rop2;rop9*/+ mutant SMCs had PAN1-YFP patches.* Bar = 10 μm.

(E) and **(F)** Transgenic plants with high levels of YFP-ROP2 protein as determined by immunoblotting were fixed and processed for PAN1 immunofluorescence. In **(E)**, YFP-ROP2 shows polarized localization in one SMC (arrowhead) but not in most SMCs in this field (arrows). In **(F)**, PAN1 immunofluorescence in the same field of cells shows PAN1 patches in cells with and without YFP-ROP2 patches. PAN1 patches were observed in 105/115 (89.5%) of SMCs in transgenic plants with high levels of YFP-ROP2 compared with 132/145 (91.0%) in nontransgenic plants.* Bar = 10 μm.

*For these quantitative analyses, SMCs adjacent to GMCs with a length to width ratio >2 were analyzed because virtually all SMCs at this stage in the wild type have polarized nuclei and PAN1 patches.

PAN1-independent functions in SMC polarization, potentially relaying signals from other receptors or performing altogether different functions that promote SMC polarization.

Immunolocalization with an antibody preferentially recognizing maize Type I ROPs revealed strongly polarized localization of these ROPs in SMCs at the site of contact with GMCs, the same site where PAN1 itself accumulates. YFP-ROP2 expressed from its native promoter at levels comparable to those of endogenous Type I ROPs was similarly polarized in most SMCs. Higher levels of YFP-ROP2 expression were associated with its delocalization in SMCs, and this correlated with the occurrence of aberrantly oriented SMC divisions similar to those observed in *rop2/rop2; rop9*/+ mutants and NSC23766-treated plants. Consistent with earlier studies investigating ROP function in polarized cell growth, our findings imply that polarized localization of maize ROPs is important for their role in the SMC polarization.

Our finding that ROP localization at GMC contact sites of SMCs requires PAN1 strongly supports the conclusion that ROP2 and ROP9 function in a common pathway with PAN1. Whereas it was not possible to analyze PAN1 localization in the complete absence of ROP function, we found no alteration in PAN1 localization within SMCs of *rop2/rop2;rop9*/+ mutants (where ROP4 is presumably still present and localized normally), NSC23766-treated plants, or in plants overexpressing YFP-ROP2, even in cells with depolarized YFP-ROP2 localization. Together, these observations suggest that ROPs act downstream of PAN1 in SMC polarization, with PAN1 playing an essential role in ROP localization at GMC contact sites. Given the complete lack of detectable ROP patches in SMCs of *pan1* mutants, it is curious that the *pan1* mutant phenotype is nevertheless enhanced when combined with *rop* mutations. It is possible that ROP patches are still present in SMCs of *pan1* mutants but at a level too low for detection via immunofluorescence, and these weak ROP patches are further reduced in the *pan;rop* double and triple mutants. Alternatively, ROPs might have functions outside (as well as within) the GMC contact site that help to polarize SMC divisions. Indeed, recent work has

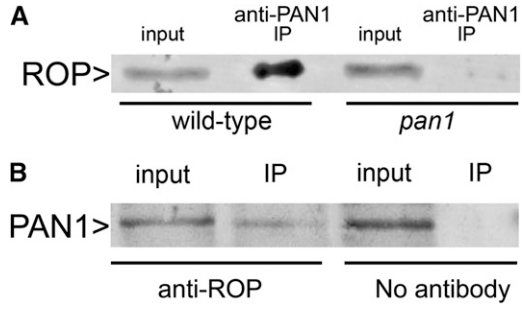

Figure 7. PAN1 and ROP Coimmunoprecipitation.

(A) Immunoblots probed with ROP antibody show that ROPs are immunoprecipitated with anti-PAN1 from solubilized membrane fractions of the wild type but not *pan1* mutant leaf division zones. IP, immunoprecipitate.

(B) Immunoblots probed with anti-PAN1 show that PAN1 is immunoprecipitated when anti-ROP antibody is added to the lysate but not when no antibody is added.

indicated that *Arabidopsis* ROPs function together with the DOCK family ROP GEF SPIKE1 at ER exit sites to promote polarized growth of epidermal pavement cells and trichomes (Zhang et al., 2010).

Our demonstration that PAN1 is required for polarized ROP localization in SMCs adds to our understanding of how ROPs can become localized at specific sites on the cell surface, a poorly understood aspect of ROP behavior that is important for its function in cell polarity. Insights in this area have come previously from studies analyzing the interaction of LRR-RLKs of the tomato and *Arabidopsis* PRK family with PRONE family ROP-GEFs. Several observations support a model in which interaction of PRK receptors with ligand(s) at the pollen tube tip leads to phosphorylation of a PRONE family ROP-GEF, relieving its autoinhibition and allowing it to activate ROPs locally (Kaothien et al., 2005; Zhang and McCormick, 2007). Additionally, the LRR-RLK FER was recently identified as a regulator of tip growth in root hairs, where it interacts with a PRONE family ROP-GEF to regulate accumulation of reactive oxygen species at the growing tip (Duan et al., 2010). While these interactions between RLKs and PRONE family ROP-GEFs clearly imply spatial regulation of ROP activity by LRR-RLKs, our results directly demonstrate a role for a receptor-like protein in spatial localization of ROPs.

Another observation demonstrating a link between PAN1 and ROP functions is the increased Triton X-100 solubility of membrane-associated ROPs in *pan1* mutants compared with wild-type plants. Prenylation has long been known to mediate association of both active (GTP-bound) and inactive (GDP-bound) forms of *Arabidopsis* Type I ROPs with the plasma membrane (Kost et al., 1999; Caldelari et al., 2001), and more recently, palmitoylation (*S*-acylation) has been associated with ROP activation within the plasma membrane. Whereas endogenous ROP6 partitions between Triton-soluble and insoluble membrane fractions, a constitutively active ROP ($AtROP6^{CA}$) was found exclusively in the DRM fraction, and this was associated with an increase in its *S*-acylation (Sorek et al., 2007). Transient *S*-acylation could provide a mechanism for recruitment of active ROPs to DRM domains (London and Brown, 2000). The higher proportion of ROPs partitioned to the DRM fraction in the wild type compared with *pan1* suggests a role for PAN1 in targeting of ROPs to DRM domains, potentially by facilitating *S*-acylation of ROPs.

Further evidence of a functional link between PAN1 and ROP is our finding that endogenous Type I ROPs coimmunoprecipitate with endogenous PAN1 and vice versa. This result suggests that PAN1 and ROP associate in a complex, but not necessarily through a direct interaction. In fact, glutathione *S*-transferase pull-down experiments failed to reveal a direct binding interaction between PAN1 and ROPs (data not shown); however, this does not exclude the possibility of a direct interaction. Indeed, two other RLKs have been shown to interact directly with ROPs (Molendijk et al., 2008; Dorjgotov et al., 2009). Interestingly, both interact preferentially with the GTP-bound active form of ROP, suggesting that these receptors are downstream effectors of ROP rather than upstream regulators of its localization or activity. Furthermore, two of the PRK family LRR-RLKs discussed earlier (SI-PRK1 and SI-PRK2) were also recently shown to interact directly with a ROP protein, binding preferentially to the GTP-bound form (Löcke et al., 2010). Accordingly, PRKs have been suggested to interact with ROPs both indirectly (via a PRONE ROP-GEFs to activate ROPs locally as discussed earlier) and directly, potentially functioning as ROP effectors as part of a feedback loop regulating pollen tube growth (Löcke et al., 2010). Further work will be required to determine whether maize ROPs interact with PAN1 directly or indirectly (e.g., via a PRONE family ROP-GEF) to mediate SMC polarization.

How do ROPs act to promote the polarization of SMC divisions? In cells undergoing polarized growth, ROPs promote the localized accumulation and fusion of vesicles needed for growth at least in part via local regulation of F-actin dynamics (reviewed in Yang and Fu, 2007; Yang, 2008; Fu, 2010). Similarly, in maize SMCs, ROPs act with PAN1 to promote the localized accumulation of F-actin at the GMC interface. This may be achieved via ROP activation of maize homologs of proteins that stimulate F-actin assembly, such as RIC4 (a protein mediating ROP stimulation of F-actin assembly in *Arabidopsis*), the SCAR/WAVE complex (a regulator of the actin-nucleating ARP2/3 complex), or formins (actin nucleators), all of which are regulated by Rho family GTPases in eukaryotic cells (Yang, 2008; Campellone and Welch, 2010). However, the functional significance of localized F-actin accumulation in maize SMCs is unclear. The small protrusion of the SMC that forms as it polarizes toward the GMC is unaffected in *rop* and *pan1* mutants, so the division polarity defects in these mutants cannot be attributed to polar growth defects. Nevertheless, SMC F-actin patches may be involved in polarized vesicle trafficking, which is essential for polarization of many eukaryotic cell types (reviewed in St Johnston and Ahringer, 2010). ROPs also promote migration of the SMC nucleus to the GMC interface and/or tethering of the nucleus at that site. Since nuclear polarization in SMCs is an actin-dependent process (Kennard and Cleary, 1997; Panteris et al., 2006), ROPs may also promote nuclear polarization at least partly via regulation of F-actin dynamics. However, ROPs may also promote SMC polarization via mechanisms that do not directly involve actin. Notably, in *Caenorhabditis elegans* embryos and *Drosophila melanogaster* neuroblasts, the Rho family GTPase Cdc42 promotes the premitotic polarization of asymmetrically dividing cells via direct interaction with the polarity protein PAR6 (Siller and Doe, 2009; Nance and Zallen, 2011). Although plants have no obvious homolog of PAR6 or other proteins that work with it, this underscores the possibility that ROPs may act via actin-independent as well as actin-dependent mechanisms to promote premitotic SMC polarity.

METHODS

Plant Material

The *pan1-Mu* allele used in this study contains a *Mu1* transposon insertion in the coding region and was previously described; maize (*Zea mays*) plants homozygous for this mutant allele contain no detectable PAN1 protein (Cartwright et al., 2009). The *rop2-m1* mutant allele used in this study contains a *Mu1* insertion 26 bp upstream of the ATG; pollen containing this allele had a 20-fold lower level of *rop2* mRNA compared with the wild type (Arthur et al., 2003). The Trait Utility System for Maize methodology (Bensen et al., 1995) was used to obtain a *Mu8* insertion in the first intron of the *rop9* gene (44 bp from the donor splice site). All *rop* mutations were backcrossed at least three times to the inbred line W22 before use in the experiments described. Primers used to identify mutant

and wild-type alleles in plants from segregating families are described in Supplemental Table 1 online.

PAN1-YFP, YFP-ROP2, CFP-β tubulin, and CFP-PIP2 constructs were generated via triple-template PCR, cloned into the binary vector pAM1006, and transformed into maize as described at http://maize.jcvi.org/cellgenomics/protocol.shtml and by Mohanty et al. (2009). All constructs contain the entire genomic region of each gene (including introns) together with 5′ and 3′ genomic sequence to drive transgene expression in plants as detailed at http://maize.jcvi.org/cellgenomics/protocol.shtml.

Analysis of Aberrant Subsidiary Cells

Subsidiary cell defects in the leaf epidermis were examined by creating impressions in cyanoacrylate glue of leaf 3. The proportions of aberrantly divided subsidiary cells were determined via double-blind analysis.

NSC23766 Treatment

Seeds were germinated in Petri dishes on Whatman GB003 blotting paper wetted with 1% DMSO (control) or varying concentrations of the Rac inhibitor NSC23766 (Calbiochem) diluted to 25 to 100 μM from a 10 mM stock in DMSO. After 4 d, 1% DMSO or Rac inhibitor was reapplied. After 8 d, cyanoacrylate glue impressions were made of fully expanded regions of leaf 2 and examined in a double-blind analysis for subsidiary cell defects. In PAN1-YFP transgenic plants, the basal 1 cm of leaf 2 was examined after 8 d of growth for YFP localization in SMCs where the adjacent GMC had a length:width ratio ≥ 2.

Analysis of SMC Polarity Markers

To analyze actin localization, the basal 1 cm of leaf 3 from 8- to 10-d-old plants was fixed and stained with Alexafluor 488-phalloidin (Invitrogen/Molecular Probes), counterstained with propidium iodide for nuclear visualization, mounted in Vectashield (Vector Labs), and imaged via confocal microscopy as previously described (Cartwright et al., 2009). Images were analyzed in a double-blind manner for actin patches and nuclear positions in SMCs.

Generation and Purification of Anti-ROP Antibody

A peptide corresponding to amino acids 124 to 138 of maize ROP2 (DDKQFFVDHPGAVPI) was synthesized, conjugated to KLH, and used for polyclonal antibody production in rabbits by Genemed Synthesis. The peptide was coupled to Sulfolink resin and affinity purified using Gentle Ag/Ab binding and elution buffers according to the manufacturer's protocols (Pierce/ThermoFisher Scientific).

Protein Extraction and Analysis

Immunoblotting experiments presented in Figures 4B and 5D used the basal 2 cm of leaves remaining on 2- to 3-week-old plants after removal of all leaves with fully or partially expanded sheaths. Membrane fractions of extracts from these tissues were prepared, separated via SDS-PAGE, and analyzed via immnoblotting as previously described (Cartwright et al., 2009) using anti-ROP at 1 μg/mL or anti-actin (MAB1501R; Millipore) at 0.5 μg/mL.

To analyze Triton X-100 solubility of ROP proteins (Figure 4C), membrane fractions prepared as described above were extracted for 1 h in 1% Triton X-100 in buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 10% glycerol, and 1% Sigma-Aldrich protease inhibitor for plants and centrifuged at 110,000*g* for 40 min. The resulting pellet and supernatant were defined as the Triton X-100 insoluble and soluble fractions, respectively, and analyzed via immunoblotting as described above for ROP protein content alongside an aliquot of the total membrane fraction removed prior to addition of Triton X-100.

Immunoprecipitations were performed using protein extracts from wild-type and *pan1* mutant leaf tissues (the basal-most 1 to 3 cm of partially expanded adult leaves 8 to 10). Membrane fractions were prepared as described above and detergent solubilized as described previously (Chinchilla et al., 2007). Solubilized proteins were precleared with protein A–agarose (Roche), and overnight immunoprecipitation was performed with 3 μg of PAN1 (Cartwright et al., 2009) or ROP affinity-purified antibody added to 250 μL of precleared, solubilized protein following the manufacturer's instructions (Roche). Five microliters of the input sample (removed prior to addition of antibody), along with proteins immunoprecipitated from 60 μL worth of the input sample, were analyzed via SDS-PAGE and immunoblotting with PAN1 or ROP antibodies as described above.

Bacterial Expression of ROPs

The coding regions of maize *rop2* and *rop7* genes were amplified by PCR using the primers listed in Supplemental Table 1 online and cloned into pET28a (Novagen) using the *Bam*HI/*Sal*I sites for *rop2* and *Bam*HI/*Xho*I sites for *rop7*, generating N-terminal His tag fusions. Fusion proteins were induced with isopropyl β-D-1-thiogalactopyranoside as previously described (Cartwright et al., 2009).

Immunolocalization

Immunolocalization of ROP and PAN1 was performed as described previously (Cartwright et al., 2009) using affinity-purified primary antibodies at 2 μg/mL and tyramide-based signal amplification with Invitrogen TSA Kit 12 (Alexa Fluor 488) according to the manufacturer's protocols or TSA kit 14 (Alexa Fluor 568) for localization of PAN1 in the presence of YFP-ROP2.

Confocal Microscopy and Image Processing

Confocal microscopy was performed using a custom-assembled spinning disk microscope system described previously (Walker et al., 2007). Image processing was performed using Metamorph version 7.0r1, NIH Image J version 1.37, or Adobe Photoshop version 8.0, applying only linear adjustments to pixel values.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: PAN1 (ACI95776.1), ROP2 (AAD34356), and ROP9 (AAO41290). Accession numbers corresponding to protein sequences aligned in Supplemental Figures 1 and 3 online are listed in their respective legends.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Critical Residues for Rac-Inhibitor NSC23766 Binding.

Supplemental Figure 2. Measurement of SMC Protrusion at the GMC Contact Site.

Supplemental Figure 3. Alignment of Maize ROP Proteins in the Region Used for Immunization to Generate the ROP Antibody Used in This Study.

Supplemental Figure 4. PAN1-YFP Coexpressed with CFP-Tubulin Revealing Cell and Nuclear Outlines in an Area with SMCs at Various Stages of Polarization.

Supplemental Figure 5. PAN1-YFP Localization in SMCs of Plants Treated with Rac Inhibitor NSC23766.

Supplemental Table 1. Primer Sequences Used in This Study.

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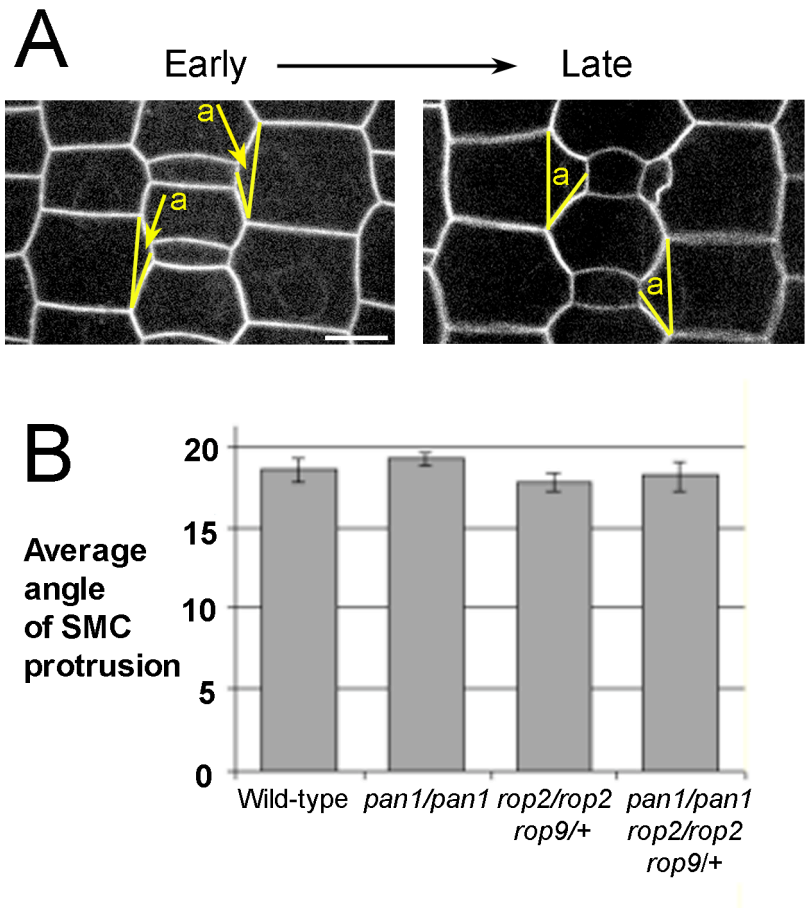
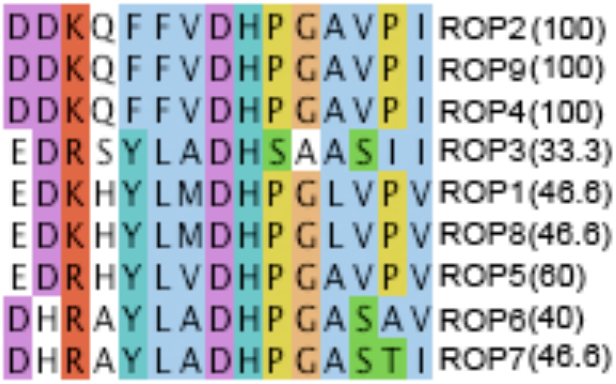
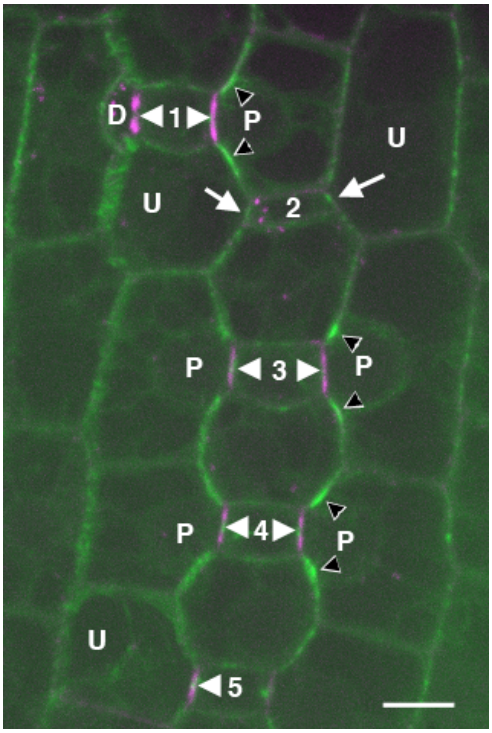
Supplemental Figure 1: Critical residues for Rac-inhibitor NSC23766 binding. Alignment of first 76-80 amino acids of Rho family GTPases from various eukaryotic species. Residues important for NSC23766 binding specificity are highlighted in red; all of these residues are present in maize ROPs 2 and 9 (along with all other maize ROPs, not shown) and all the Racs but one critical residue is missing in CDC42 and RhoA, which are not inhibited by NSC23766 (Gao et al., 2004). Genbank accession numbers: Zm (Z. mays) ROP2 (AAD34356) and ROP9 (AAO41290), Hs (H. sapiens) Rac1 (AAM21111), Dm (D. melanogaster) Rac1 (AAV85902), Ce (C. elegans) Rac1 (AAA28140), Fd (F. distichus) Rac1 (AAR14182), Hs (H. sapiens) CDC42 (AAM21109) and RhoA (AAM21117).



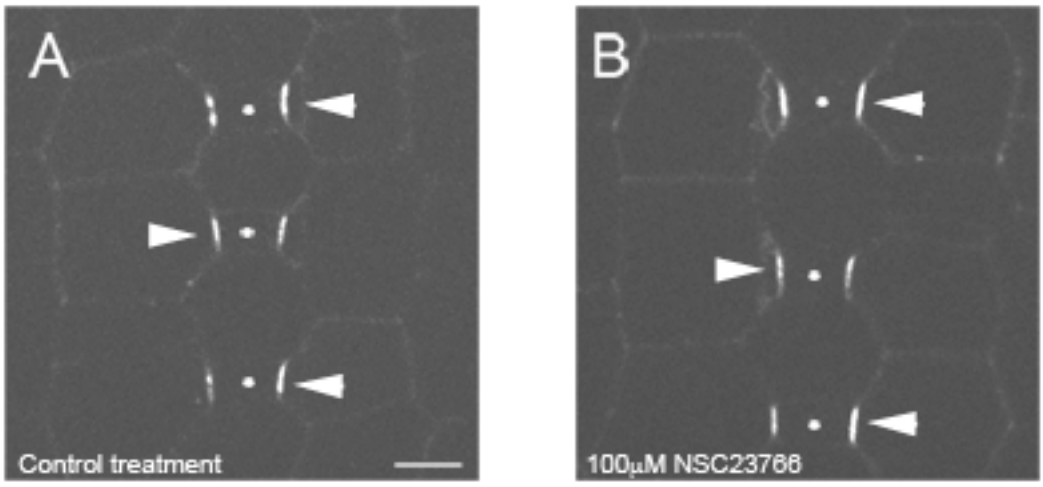
Supplemental Figure 2: Measurement of SMC protrusion at the GMC contact site.
A) Images of developing stomata from transgenic plants expressing CFP-PIP2, a plasma membrane localized protein that reveals cell outlines. White dots mark GMCs. Early and late stages are shown, illustrating the protrusion of SMCs that accompanies guard cell elongation. To measure the protrusion of SMCs toward GMCs, SMCs whose adjacent GMC had a length: width ratio >2 (similar to those in the right panel) were analyzed using the angle function of Image J. A line was drawn joining the two corners of the SMC adjacent to the GMC (see longer line in illustration). From one of these corners, a second line was drawn to the middle of the SMC-GMC interface, and the angle between these two lines was measured (a), with a greater angle (as illustrated in the "late" panel on the right) representing a more pronounced SMC protrusion. Scale bar = 10μm.
B) Results of quantitative analysis (n >200 SMCs analyzed per genotype); error bars represent standard deviation.



Supplemental Figure 3: Alignment of maize ROP proteins in the region used for immunization to generate the ROP antibody used in this study.
This 15 amino acid region is expected to be exposed on the protein surface (Berken and Wittinghofer, 2008). The % identity to ROP2 of each ROP within the peptide region is shown in parentheses. Accession Numbers: ROP2 (AAD34356), ROP9 (AAO41290), ROP4 (AAD34358.1), ROP3 (AAD34357.1), ROP1 (ADW83711.1), ROP8 (AF376054.1), ROP5(CAB96794.1), ROP6 (AAO41292.1), ROP7 (AAO41293.1).



Supplemental Figure 4: PAN1-YFP (magenta) co-expressed with CFP-tubulin (green) revealing cell and nuclear outlines in an area with SMCs at various stages of polarization.
GMCs are numbered 1-5. PAN1-YFP patches are indicated by white arrowheads, whereas white arrows point to GMC/SMC junctions with no PAN1-YFP patch. SMC nuclei are labeled U (unpolarized), P (polarized) or D (divided). Preprophase band edges are marked with black arrowheads in SMCs flanking GMCs 1, 2, and 3, As shown previously via immunolocalization with anti-PAN1 antibody (Cartwright et al., 2009) PAN1-YFP patches are absent in SMCs flanking the youngest (shortest) GMC (# 2); these SMCs also have unpolarized nuclei. PAN1-YFP patches subsequently form in SMCs at sites of GMC contact before nuclear polarization (e.g. SMC flanking GMC 5 on the left, which has a weak PAN1-YFP patch and an unpolarized nucleus). PAN1-YFP patches are consistently present in older SMCs with polarized nuclei (those flanking GMCs 3 and 4 on both sides and flanking GMC 1 on the right) and persist after SMC division (see newly formed subsidiary cell flanking GMC 1 on the left). Scale bar = 10μm.



Supplemental Figure 5: PAN1-YFP localization in SMCs of plants treated with Rac inhibitor NSC23766.
White dots mark GMCs; arrowheads mark PAN1-YFP patches in SMCs at sites of GMC contact. PAN1-YFP localization in control plants (A) and plants treated with 100μM NSC23766 (B), demonstrating that PAN1-YFP patches form normally in the presence of the inhibitor (n > 300 SMCs examined for each treatment). When SMCs with adjacent GMCs having a length to width ratio >2 were analyzed, PAN1-YFP patches were observed in 322/334 SMCs in control plants (93.6%) compared to 291/315 (92.3%) in NSC23766-treated plants. Scale bar = 10μm.

Primers used to generate YFP fusion constructs	
PAN1-YFP	**Primer sequence 5' to 3'**
P1 primer	gctcgatccacctaggctTGTTCAGGTCTTCGGGTCTT
P2 primer	cacagctccacctccacctccaggccggccGCCGATCCGGTCGAGGCTC
P3 primer	tgctggtgctgctgcggccgctggggccTGACTTCCAGCGCGACGGC
P4 primer	cgtagcgagaccacaggaGACCCCAAACTGATGGACCT
YFP-ROP2	
P1 primer	GCTCGATCCACCTAGGCTttggatccccgatttcatta
P2 primer	CACAGCTCCACCTCCACCTCCAGGCCGGCCcatcctcttccgccctcc
P3 primer	TGCTGGTGCTGCTGCGGCCGCTGGGGCCatgagcgcgtccaggttca
P4 primer	CGTAGCGAGACCACAGGAccgttaaaactggtgctttca
CFP-β-tubulin	
P1 primer	gctcgatccacctaggctCCTCTAAAGCCCCGATGGCAGAAACCT
P2 primer	cacagctccacctccacctccaggccggccCATGGCGGCAGGACGATTGTTGC
P3 primer	tgctggtgctgctgcggccgctggggccATGAGGGAGATCCTCCACATC
P4 primer	cgtagcgagaccacaggaGGTACCCGAAATACCCGAATTACCCGAACT
Primers used for rop cloning into pET28a for bacterial expression	
Zm *rop2*	
5' primer	CAGGATCCATGAGCGCGTCCAGG
3' primer	TGGTCGACTCACAAAATGGAGCACG
Zm *rop7*	
5' primer	CAGGATCCATGAGCGTGACCAAG
3' primer	TGCTCGAGCTAAGCAGCGCACG
Primer used to genotype rop alleles in maize	
Zm *rop2*	
RopB-F11	CTCCCCTCCCCACCACCACTA
RopB-R6	GCAACAATGAGGAGGAAGGAGCGT
Zm *rop9*	
Rop9-F4	GGGAGTCCCCGGATTTTGAA
Rop9-R13	GGGGAAGGCGTGAGTAGTGG
MuEnd (used for *rop2* and *rop9*)	AGAGAAGCCAACGCCAWSGCCTCYATTTCGTC

Supplemental Table 1: Primer Sequences used in this study