RanGAP1 is a continuous marker of the Arabidopsis cell division plane

Xianfeng Morgan Xu,a,1 Qiao Zhaoa-2, Thushani Rodrigo-Peirisb, Jelena Brkljacica, Chao Sylvia Hea,1 Sabine Müllerb, and Iris Meiera,3

aPlant Biotechnology Center and Department of Plant Cellular and Molecular Biology, The Ohio State University, Columbus, OH 43210; and bSchool of Biological Sciences, University of Auckland, Auckland 1142, New Zealand

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In higher plants, the plane of cell division is faithfully predicted by the preprophase band (PPB). The PPB, a cortical ring of microtubules and F-actin, disassembles upon nuclear-envelope breakdown. During cytokinesis, the expanding cell plate fuses with the plasma membrane at the cortical division site, the site of the former PPB. The nature of the “molecular memory” that is left behind by the PPB and is proposed to guide the cell plate to the cortical division site is unknown. RanGAP is the GTPase activating protein of the small GTPase Ran, which provides spatial information for nucleocytoplasmic transport and various mitotic processes in animals. Here, we show that, in dividing root cells, Arabidopsis RanGAP1 concentrates at the PPB and remains associated with the cortical division site during mitosis and cytokinesis, requiring its N-terminal targeting domain. In a fass/ton2 mutant, which affects PPB formation, RanGAP1 recruitment to the PPB site is lost, while its PPB retention is microtubule-independent. RanGAP1 persistence at the cortical division site, but not its initial accumulation at the PPB requires the 2 cytokinesis-regulating kinesins POK1 and POK2. Depletion of RanGAP by inducible RNAi leads to oblique cell walls and cell-wall stubs in root cell files, consistent with cytokinesis defects. We propose that Arabidopsis RanGAP1, a continuous positive protein marker of the plant division plane, has a role in spatial signaling during plant cell division.

Cytokinesis | Ran cycle | Preprophase band | Nuclear pore

Cell division in higher plants involves the construction of a new cell wall in the proper orientation of the division plane. Because plant cells are embedded in a rigid matrix of cell wall material, the spatial orientation of the new cell wall defines the geometry of the respective plant tissue (1). The plane of cell division is defined by the assembly of the preprophase band (PPB), a ring of microtubules and F-actin that appears during G2 phase, and the migration of the premitotic nucleus into the plane defined by the PPB. During mitosis, the site of the former PPB becomes the cortical division site (CDS), which remains “marked” in an unknown way and which is thought to guide the phragmoplast and the outgrowth of new plasma membrane.

The molecular nature of the CDS has long been enigmatic, and only recently have the first molecular markers been identified. A “negative marker” is the local depletion of cortical F-actin and of the kinesin KCA1 (2, 3). Several proteins appear and disappear with the PPB and reappear later at the CDS. These include the microtubule-associated protein AIR9, RSH, a hydroxyproline-rich glycoprotein, and T-PLATE, a protein resembling transport vesicle coat proteins (4–6). Only 1 protein is known that is recruited to the PPB and remains at the site of the future CDS throughout the cell division cycle. TANGLED was originally identified in maize, where in tan mutants cells divide in aberrant orientations, suggesting a requirement of TANGLED for proper division-plane establishment (7).

Arabidopsis TANGLED is recruited to the PPB in a microtubule- and kinesin-dependent manner, and persists at the CDS after PPB disassembly (8). Two related kinesins, PHRAGMOPLAST-ORIENTING KINESINS 1 and 2 (POK1 and POK2) were found to interact with TANGLED and a pok1 pok2 double mutant resembles the maize tan mutant in terms of misoriented division planes (9). Although the data suggest a role for kinesins and the pioneer protein TANGLED in division-plane definition, the molecular mechanism of the process remains unknown.

Ran is a small GTPase that in vertebrates controls multiple cellular processes including nucleocytoplasmic transport, spindle assembly, nuclear envelope reassembly, centrosome duplication, and cell-cycle control (ref. 10 and references therein). Crucial for its roles is the asymmetric distribution of RanGTP and RanGDP, enabled by specific locations of the RanGTPase activating protein RanGAP and the Ran nucleotide exchange factor RCC1. Although vertebrate RCC1 remains chromatin bound throughout cell cycle, RanGAP migrates from its interphase location at the outer surface of the nuclear pore to mitotic locations such as the kinetochores (11, 12). Unlike vertebrate RanGAP, Arabidopsis RanGAP1 was shown to associate with the phragmoplast and growing rim of the cell plate during cytokinesis (13, 14). The phragmoplast is a plant-specific array of microtubules, actin filaments and associated molecules that act as a framework for the future cell wall and might be analogous to the spindle midbody of animal cells (15). All subcellular targeting events of Arabidopsis RanGAP1 require an N-terminal domain (WPP domain, named after a highly conserved tryptophan-proline-proline motif), which is unique to plants.

Here, we show that Arabidopsis RanGAP1 positively labels the PPB and, like TANGLED, remains associated with the future site of division throughout cell cycle. RanGAP1 recruitment to the PPB depends on FASS/TONNEAU2, a putative regulatory subunit of protein phosphatase 2A, which is necessary for PPB assembly (16). Its persistence at the CDS depends on POK1 and POK2. Inducible depletion of Arabidopsis RanGAP in seedling roots leads to misplaced cell walls similar to the Arabidopsis tan mutant alleles. Together, our data present RanGAP as a novel continuous positive protein marker of the plant division plane, dependent on known regulators of plant cytokinesis and poised to signal spatial information during plant cell division.

Results

RanGAP1 Positively Marks the Arabidopsis Division Plane Through Mitosis and Cytokinesis. The mitotic localization pattern of Arabidopsis RanGAP1 was revealed using indirect immunofluorescent microscopy. The authors declare no conflict of interest. This article is a PNAS Direct Submission. The authors declare no conflict of interest.

1Present Address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.
2Present Address: Samuel Roberts Noble Foundation, Ardmore, OK 73401.
3To whom correspondence should be addressed. E-mail: meier.56@osu.edu.

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GAP1-GFP fusion protein in transgenic Arabidopsis performed real-time imaging of a 35S-promoter driven Ran-GAP1 localization throughout cell division, confirming the specificity of the immunofluorescence signal. To was seen in a RanGAP1 null mutant (data not shown), con-
mulating in several bright dots on chromosomes, resembling the kinetochore regions (Fig. 1 A and Fig. S1). As the cells pro-
gressed into anaphase, RanGAP1 was found enriched around the spindle midzone in addition to remaining at the CDS (Fig. 1 A and C). When the spindle midzone microtubules assembled into the phragmoplast, RanGAP1 was concentrated at the midline of the phragmoplast or the nascent cell plate and the CDS (Fig. 1 A). None of the above-mentioned staining patterns was seen in a RanGAP1 null mutant (data not shown), con-
firming the specificity of the immunofluorescence signal. To monitor RanGAP1 localization throughout cell division, we performed real-time imaging of a 35S-promoter driven Ran-
GAP1-GFP fusion protein in transgenic Arabidopsis roots. Ex-
cept that RanGAP1-GFP accumulation on the phragmoplast midline was less evident, the fusion protein showed an essentially identical localization pattern compared with endogenous Ran-
GAP1. In 43% of dividing cells, significant enrichment of RanGAP1 at the PPB and CDS was observed continuously throughout mitosis and cytokinesis (Table 1, Fig. S2, and Movie S2). Cells dividing without observable RanGAP1 concentration might either be below the detection limit of the assay, or suggest that the accumulation does not occur equally in all cells. The signal narrowed as the cells progressed from preprophase to metaphase and anaphase, similar to what has been observed for TANGLED (S). In summary, a concentration of RanGAP1 was seen at the division plane from preprophase to cytokinesis, making RanGAP1, after TANGLED, the second continuous positive protein marker of the plant division plane.

**Table 1. Number of cells observed with RanGAP1 (or its derivatives) concentrated at the PPB and CDS in different backgrounds**

<table>
<thead>
<tr>
<th></th>
<th>RanGAP1 WT</th>
<th>ton2–14</th>
<th>pok1–1;2–1</th>
<th>pok1–1;2–2</th>
<th>tan-csh</th>
<th>RanGAP1WPP/AAP-GFP</th>
<th>RanGAP1ΔC-GFP</th>
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<tr>
<td>PPB</td>
<td>23 (55)</td>
<td>0 (17)</td>
<td>13 (24)</td>
<td>11 (27)</td>
<td>10 (25)</td>
<td>0 (24)</td>
<td>15 (28)</td>
</tr>
<tr>
<td>CDS</td>
<td>28 (69)</td>
<td>0 (35)</td>
<td>0 (33)</td>
<td>0 (25)</td>
<td>17 (40)</td>
<td>0 (26)</td>
<td>14 (39)</td>
</tr>
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In each case, data collected from two independent experiments were summarized. Endogenous RanGAP1 was visualized using immunofluorescence with the anti-RanGAP1 antibody, whereas the anti-GFP antibody was used to stain both RanGAP1WPP/AAP-GFP and RanGAP1ΔC-GFP. The total number of dividing cells examined in the corresponding stages is shown in parentheses.

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Fig. 1. *Arabidopsis* RanGAP1 demarcates the PPB and CDS during mitosis and cytokinesis. (A) Concentration of RanGAP1 at the PPB (arrowheads in the topmost image), kinetochore (arrow), spindle midzone (bracket), phragmoplast midline (asterisk) and CDS (arrowheads in second, third and bottommost images) in dividing root cells, revealed by immunofluorescence. α-Tubulin (magenta) indicates the different mitotic stages listed on the left. (B) Four consecutive focal planes (increment of 0.3 μm) from a likely preprophase cell at the root tip, demonstrating near-continuous RanGAP1 enrichment at the PPB (arrowheads). (C) Four consecutive focal planes (increment of 0.3 μm) from a dividing root tip cell in anaphase, showing RanGAP1 association with both the spindle midzone (bracket) and CDS (arrowheads). DNA (magenta) was stained with SYTOX Orange in B and C. (Scale bars: 10 μm.)
protein partners. To investigate whether the WPP domain is also sufficient for RanGAP1 targeting during mitosis, localization of a fusion protein between the WPP domain and GFP (RanGAP1ΔC-GFP) was monitored by immunofluorescence. Similar to full-length RanGAP1, RanGAP1ΔC-GFP was targeted to the PPB and CDS, but not to the kinetochore region or spindle midzone (asterisk). Note that the formation of perinuclear microtubules (yellow arrow) indicated a preprophase cell in the ton2-14 mutant without a detectable microtubular PPB. RanGAP1 (green) was immunostained in root tip cells, with α-tubulin (magenta) staining to indicate the different mitotic stages listed on the left. (Scale bars: 10 μm.)

**Fig. 2.** RanGAP1 retention at the PPB is microtubule-independent, and its localization at the site of the PPB and CDS requires FASS/TONNEAU2. (A) A preprophase cell coexpressing RanGAP1-GFP and mCherry-TUB6 was imaged before and 10 min after treatment with 5 μM oryzalin. Concentration of RanGAP1-GFP at the PPB (arrowheads) remains after the disassembly of microtubules. (B) FASS/TONNEAU2 is required for RanGAP1 targeting to the site of the PPB and CDS, but not to the kinetochore (white arrow), spindle midzone (bracket) or phragmoplast midline (asterisk). Note that the formation of perinuclear microtubules (yellow arrow) indicated a preprophase cell in the ton2-14 mutant without a detectable microtubular PPB. RanGAP1-GFP (arrowheads) remains after the disassembly of microtubules (asterisk). (C) A fusion protein between the WPP domain and GFP (C-GFP) was monitored by immunofluorescence.

Inducible RanGAP Depletion in Roots Leads to Incomplete and Misplaced Cell Walls. The functional investigation of mitotic plant RanGAP is non-trivial for several reasons. First, depleting RanGAP likely affects interphase nucleocytoplasmic transport, which could conceivably lead to pleiotropic downstream effects. Second, *Arabidopsis* RanGAP is encoded by a family of 2 highly similar genes (63% identity and 80% similarity at the amino acid level) with similar expression patterns, expected to act redundantly. And third, RanGAP activity is likely essential, and a complete loss-of-function mutant is therefore expected to be lethal.

We isolated T-DNA insertion mutants for RanGAP1 and RanGAP2 and applied 2 specific polyclonal antisera to investigate protein levels (see Materials and Methods for details). Both the RanGAP1 mutant allele rgl-1 and the RanGAP2 mutant allele rg2-3 are likely null alleles based on the absence of detectable protein (Fig. S6). Both rgl-1 and rg2-3 were phenotypically normal, suggesting that the 2 copies of RanGAP are indeed functionally redundant (data not shown). A cross between rgl-1 and rg2-3 showed that the rgl-1 rg2-3 double mutant is gametophyte lethal (T.R.-P., X.M.X., and I.M., unpublished data), suggesting that RanGAP activity is essential in plants.

In light of these findings, an inducible RNAi strategy was instead explored. Two constructs were made to specifically
deplete RanGAP1 in the rg2-3 background (RanGAP1RNAi/rg2-3) or RanGAP2 in the rg1-1 background (RanGAP2RNAi/rg1-1). Identical phenotypes were achieved from both constructs (see below). The phenotype was dependent on the homozygosity of the corresponding T-DNA insertion background. A line that contained a truncated RanGAP1RNAi construct in rg2-3 that did not deplete RanGAP1 was used as a control against artifacts caused by the induction conditions (Fig. 4A). It behaved identical to the RNAi lines under uninduced conditions shown below (data not shown). Only data observed for RanGAP1RNAi/rg2-3 are described below.

After 4–9 days of induction, there was a pronounced reduction of RanGAP1 level in the roots of RanGAP1RNAi/rg2-3 plants (~70% reduction, Fig. 4A) compared with uninduced control plants, with a lesser reduction in shoots (~40% reduction, data not shown). Immunofluorescence experiments with the anti-RanGAP1 antibody showed that the level of RanGAP1 was not depleted equally in all cells, with some cells, especially toward the root tip, still showing RanGAP1 signal at the nuclear envelope (data not shown). At this stage, induced plants had shorter roots with swollen root tips (Fig. 4B). Under higher magnification, this phenotype resembled the previously reported radially swollen mutants (19, 20). No obvious shoot phenotype was detected, possibly based on the less severe RanGAP1 depletion in shoots. The swollen root phenotype might conceivably be a currently not understood interphase effect on directional cell expansion, downstream of nucleocytoplasmic partitioning or other unknown functions of RanGAP.

When the root tips were examined closely, oblique cell walls and cell wall stubs were found frequently in induced RanGAP1RNAi/rg2-3 plants, suggesting aberrant cell division events (Fig. 4C). This was observed in the root tip, still showing RanGAP1 localization patterns at the nuclear envelope (data not shown). Immunofluorescence experiments with the anti-RanGAP1 antibody, nucleus (arrow), spindle midzone (arrowhead), phragmoplast midline (asterisk) and CDS (arrowheads in second, third, and bottommost images). a-tubulin (magenta) was stained to indicate the different mitotic stages listed on the left. (Scale bars: 10 μm.)

**Discussion**

An unsolved question in the process of plant cytokinesis is how the spatial memory of the PPB position is retained into cytokinesis, when the cell plate fuses with the plasma membrane at exactly the position of the former PPB. Until recently, no positive markers were known that labeled the division site after disassembly of the PPB. *Arabidopsis TANGLED* has recently been shown to be such a marker and *tangled* mutants show disruption of cell plate guidance (8). However, the molecular mechanism of the process is still unknown. A major player of delivering spatial information in animal cells is the small GTPase Ran. The specific localization of either RCC1 or RanGAP provides local information, through establishing a high or low concentration of RanGTP, enabling in turn local processes, such as microtubule plus-end growth and microtubule attachment to kinetochores.

We have now found that in *Arabidopsis* RanGAP1 is a positive marker of the PPB to CDS memory. RanGAP1 persists at the future division site after disassembly of the PPB and consistently persists in the presence of the microtubule-depolymerizing drug oryzalin. Its association with the PPB site requires FASS/TONNEAU2, suggesting that, although RanGAP can persist after PPB disassembly, it requires microtubules for its primary targeting to the PPB. Alternatively, FASS/TONNEAU2 might act upstream of both PPB assembly and a microtubule-independent process of RanGAP targeting to the PPB. Interestingly, RanGAP1 persistence at the division site, but not RanGAP1 association with the PPB also requires the kinesins.
Pok1 and Pok2, which have been shown to be required for efficient association of Tangled with PPB and CDS (9). We found evidence that the WPP-domain of RanGAP1 physically interacts with the C-terminal coiled-coil domain of Pok1 and with full-length Pok1. Pok1 interaction and CDS association require the same 3-amino acid motif within the WPP domain (Figs. S3 and S4), indicating a close correlation between the ability to bind Pok1 (and possibly other coiled-coil proteins) and retention at the CDS.

The finding that RanGAP1 still associates with the PPB in pok1 pok2 mutants suggests that Pok1 and Pok2 are not responsible for the delivery of RanGAP1 to the PPB per se. However, the absence of a RanGAP1 signal at the CDS in pok1 pok2 mutants suggests a defect in maintaining an aspect of CDS identity necessary for the delivery of RanGAP1 to the PPB. We have observed that the WPP-domain of RanGAP1 physically associates with either Pok1 or Pok2 directly, or with another member of the large family of Arabidopsis kinesins. The Arabidopsis genome encodes for 23 kinesins that are in some way implicated in mitosis-related processes. Pok1 and Pok2 belong to a group of 8 mitotic kinesins that are specific to plants. This group contains AtPAKR1 and AtPAKR2, which associate with the phragmoplast, and several as yet uncharacterized kinesins (3). The possibility that RanGAP1 interacts in vivo with a different kinesin might explain why the original recruitment of RanGAP1 to the PPB is not disrupted in the absence of Pok1 and Pok2. Pok1 and Pok2 might then be required to deliver a factor to the CDS that is essential for RanGAP retention after PPB disappearance. It will be important to determine the currently unknown subcellular location of Pok1 and Pok2, specifically during cell division, and the affinity of RanGAP for other kinesins to begin to address these scenarios.

Interestingly, we found no effect of depleting the WIP coiled-coil protein family involved in anchoring RanGAP1 at the NE on the association of RanGAP1 with the PPB, CDS, or other mitotic locations. Nevertheless, introducing the WPP to AAP mutation that disrupts binding to the WIP family disrupted all cellular targeting events. This suggests that different, currently unknown interaction partners of the WPP domain are involved in anchoring RanGAP1 to the cell cortex, the kinetochores, and the cell plate. Of these events, only targeting to the cell cortex requires Pok1 and Pok2, suggesting that the different mitotic targeting events are again accomplished by different mechanisms.

Induced depletion of RanGAP leads to incomplete and irregularly positioned cell walls. Although we cannot formally exclude downstream effects of impaired interphase RanGAP functions, we favor the hypothesis that RanGAP plays a direct role in Arabidopsis cytokinesis, consistent with its continuous association with the division site. What could such a role be? Several lines of evidence point at important functions of the Ran cycle in microtubule biology during animal mitosis and cytokinesis. The general theme is that a high local concentration of RanGTP promotes microtubule growth. This is based on the recently recognized function of karyopherins of the importin beta type to act as inhibitors of microtubule growth by sequestering NLS-containing spindle assembly factors (SAFs), such as TPX2 and HURP. Like in nuclear import, RanGTP dissociates the karyopherin-cargo complex, thereby releasing SAFs from inhibition. Downstream events involve TPX2-dependent phosphorylation of Aurora-A kinase, which in turn is proposed to activate microtubule nucleation factors (ref. 10 and references therein). One proposition in line with these activities is that RanGAP could assist in the disassembly of the PPB by keeping local RanGTP levels low and thereby favoring depolymerization over polymerization of microtubules at this site.

In addition to microtubule stability, RanGTP has been shown to affect the polarity of microtubule motor activities. Wilde et al. (21) showed that RanGTP increases plus-end-directed motor activity and decreases minus-end directed activity. During plant cytokinesis, the microtubule arrays of the phragmoplast are oriented with the plus ends toward the growing cell plate and deliver vesicles presumably by plus-end-directed motor activity (22). The association of RanGAP1 with the CDS and growing rim of the cell plate/phragmoplast midline in plants might indicate reduced RanGTP at these sites. In analogy to the animal systems, this might cause reduced plus-end growth of the phragmoplast microtubules and/or reduced plus-end motor activity. Such a regulatory function could conceivably fine-tune phragmoplast and vesicle delivery dynamics and thereby contribute to the overall precision of the processes underlying cell plate synthesis and positioning.

Further work will be required to establish the spatial distribution of RanGTP and RanGDP during plant mitosis and its relationship to microtubule biology, and to develop more precise tools to disturb the Ran cycle specifically in mitotic cells. The data presented here connect a spatial organization problem of the plant cell (cell plate positioning) with a well-established spatial regulator of animal mitosis, the Ran cycle.

**Materials and Methods**

**Plant Materials.** Arabidopsis seedlings (Columbia and Wassilewskija ecotype) were grown in soil under standard long-day condition (16 h light and 8 h dark) or on MS (Caisson Laboratories) plates under constant light. Mutant rgl-1 (SALK_058630, with a T-DNA inserted −720 bp downstream of the RanGAP1 start codon) was acquired from the Arabidopsis Biological Resource Center (ABRC, The Ohio State University, Columbus, OH). Mutant rgl2-3...
and transgenic plants expressing RanGAP1-GFP, RanGAP1WPP/AAP-GFP, and signal.salk.edu/tdnaprimers.2.html). The mutant line Homozygous insertion plants were identified by PCR genotyping (http://start codon) was obtained from the Versailles T-DNA lines collection (23).

Inducible RNAi. For Dex-inducible RNAi, the first 334 nt of the RanGAP1 coding region and the last 478 nt of the RanGAP2 coding region were cloned into pENTR3C and pENTR/D-TOPO, respectively. The target fragments were subsequently recombined into destination vector pOpOff2(hyg) (25). Transgenic pok1 pok2

CEFOTAXIM (125 from this transformation that carried a truncated transgene and did not lead to


Immuno/labeling and Confocal Microscopy. Immunolabeling and confocal microscopy were performed as described in ref. 26 and as detailed in SI Materials and Methods.

Antibody Development. Development of the anti-RanGAP1 antibody has been described in ref. 27. For the anti-RanGAP2 antibody, full length RanGAP2 protein was expressed as a His-tag fusion protein using the pDEST17 vector (Invitrogen). After purification of the recombinant protein with a Ni-NTA resin column and excision from a preparative SDS/PAGE gel, a guinea pig antiserum was produced by Cocalico Biologicals (Reamstown, PA).

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Supporting Information

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Cloning of Full-Length POK1 cDNA. RNA was extracted from seedlings (at 6 days after germination) with TRIzol (Invitrogen) according to manufacturer’s specifications. Wild-type cDNA was obtained via reverse transcription of RNA with a Retroscript Kit (Ambion) using oligoDT primers provided with the kit or gene specific primer POK1-SpeR. PCR reactions were performed, using ExTaq (Takara Bio), at annealing temperatures between 55 °C to 60 °C. POK1 full length cDNA was assembled from 3 subclones. The N-terminal half (2428 bp) was amplified with primers POK1-SacII/F and POK1-SpeI-R from cDNA synthesized with POK1-SpeIR and cloned into the SacII and SpeI sites of pSK Bluescript (Stratagene). An EST clone H8C10 (in pSK Bluescript) was obtained from the Arabidopsis Biological Resource Center (ABRC), which contained a 2845 bp C-terminal cDNA fragment of POK1. To bridge the gap between pSK-POK1 N terminus and H8C10, a PCR fragment was amplified from cDNA synthesized with oligoDT18 spanning from the unique POK1 SpeI site to POK1 internal BamHI site at 3660 bp and cloned into pGEM (Promega). The fragment was moved into the vector-internal SpeI site and the POK1 internal BamHI site of H8C10 and the resulting plasmid was designated pSK-POK1-C terminus. Subsequently, the C terminus of POK1 was modified for downstream applications, using the POK1 unique AflII and SacI sites. To remove the SacI site and STOP codon from the C terminus and introduce a KpnI site for cloning, the AIIH and SacI fragment was replaced by an insert amplified with POK1-BamHI/BsmI and POK1-Kpn/SacI-R and digested with AflII and SacI. The N-terminal insert was cloned into the SacII and SpeI sites of the modified pSK-POK1-C terminus to obtain pSK-POK1 N + C. Sequence was confirmed by DNA sequencing using primer extension. Therefore, the POK1 cDNA was flanked by KpnI sites, which were used to move the insert into pENTR3C/EcoRI (the EcoRI fragment in pENTR3C was removed to adjust reading frame for insertion of POK1 cDNA). All primer sequences are available upon request. The POK1 cDNA sequence has been submitted to GenBank (accession no. DQ399529).

Yeast 2-Hybrid Assays. Constructs pAD-POK1C, pBD-AtTAN, pBD-RanGAP1, pBD-RanGAP1C, and pAD-LeWAPCC were described in refs. 5 and 6. For the experiment shown in Fig. S4C, POK1C and full-length POK1 were subjected to LR recombination reaction with the pDEST 22 Gateway vector (Invitrogen) according to the manufacturer’s protocol to create the AD-POK1C and AD-POK1 constructs. All tested plasmid pairs were transformed into the yeast strain PJ694A with plate growth assays following the “Clontech Yeast Protocols Handbook” (PT3024–1). BD-RanGAP1, BD-RanGAP1C and AD-WIP1 were used for the HA blot.

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Fig. S1. Similar to mammalian RanGAP, during metaphase, *Arabidopsis* RanGAP1 (magenta) concentrates around the kinetochores (arrow). A dividing root cell was imaged by immunofluorescence microscopy. HTR12-GFP (green, centromere-specific histone 3; (7)) was used as a marker for centromeres. Note that RanGAP1 also accumulates at the CDS (arrowheads). (Scale bar: 10 μm.)
Fig. S2. Time-lapse (every 90 seconds) images of a dividing root cell expressing RanGAP1-GFP. The series starts from preprophase (judged from the complete nuclear envelope) and ends during cytokinesis (indicated by the two reforming daughter nuclei). RanGAP1-GFP is largely excluded from the nucleus. Note the continuous accumulation of RanGAP1-GFP at the PPB/CDS site (arrowheads) and slight concentration around the kinetochore region (white arrow). The yellow arrow points to RanGAP1-GFP CDS accumulation in the cell beneath. (Scale bar: 10 μm.)
Fig. S3. The WPP domain is necessary and sufficient for RanGAP1 mitotic targeting. (A) RanGAP1\(^{WPP/AAP}\)-GFP appeared mostly cytoplasmic without apparent accumulation at the PPB, kinetochore, phragmoplast midline or CDS. (B) RanGAP1\(^{ΔC}\)-GFP (the RanGAP1 WPP domain fused to GFP) displayed mitotic targeting figures similar to full-length RanGAP1. Labeled compartments in both A and B are PPB (arrowheads in top panel), kinetochore (arrow), spindle midzone (bracket), phragmoplast midline (asterisk) and CDS (arrowheads in second, third and bottom panels). GFP was immunostained, and \(\alpha\)-tubulin (magenta) was costained to indicate the different mitotic stages listed on the left. (Scale bars: 10 \(\mu\)m.)
Fig. S4. (A) RanGAP1 interacts in vivo with the C terminus of POK1 (POK1C) via its WPP domain. Samples were immunoprecipitated with the anti-GFP antibody from *N. benthamiana* leaves coexpressing HA-tagged POK1C (HA-POK1C) with the respective GFP-tagged proteins. RanGAP1ΔC, WPP domain of RanGAP1; RanGAP1WT, full-length RanGAP1 with a 2-amino acid mutation in the WPP motif. Precipitates were probed with anti-GFP antibody (Upper) and anti-HA antibody (Lower). (B) The WPP domain of RanGAP1 (RanGAP1ΔC) interacts with POK1C in a yeast 2-hybrid assay. Combinations of test constructs, listed on the Left and the top were cotransformed into yeast. Auxotrophic markers leucine (-L, for pAD plasmids) and tryptophan (-T, for pBD plasmids) were used to select transformants. Growth on plates without histidine (-H) and adenine (-A) indicates protein–protein interactions. LeWAPCC is a positive control for a WPP domain-binding protein. AtTAN, Arabidopsis TANGLED, is a positive control for POK1C binding. (C) The WPP domain of RanGAP1 (RanGAP1ΔC) interacts with full-length POK1 (POK1) in a yeast-2 hybrid assay. Combinations of test constructs, as listed on the Left and the top were cotransformed into yeast. Auxotrophic markers Leucine (-L, for pBD) and Tryptophan (-T, for pAD) were used to select transformants. Growth on plates lacking Histidine (-H) indicates protein–protein interactions. WIP1 is a positive control for a RanGAP1-binding protein. The lack of binding between full-length RanGAP1 and POK1C or POK1 is similar to findings for other, in planta confirmed RanGAP1–coiled-coil protein interactions [Xu XM, Meulia T, Meier I (2007) Anchorage of plant RanGAP to the nuclear envelope involves novel nuclear-pore-associated proteins. *Curr Biol* 17:1157–1163], and might be due to steric hindrance or other unidentified problems in yeast.
Fig. S5. RanGAP1 mitotic targeting is not affected in the tan-csh mutant. RanGAP1 concentration was readily seen at the PPB (arrowheads in top panel), spindle midzone (bracket), phragmoplast midline (asterisk) and CDS (arrow heads in middle and bottom panel) in dividing tan-csh root cells, revealed by immunofluorescence. α-Tubulin (magenta) was stained to indicate the different mitotic stages listed on the Left. (Scale bars: 10 μm.)
Fig. S6. Identification of mutants of RanGAP1 and RanGAP2. (A) Schematic representation of the gene structure and T-DNA insertions in RanGAP1 and RanGAP2. Dark gray boxes depict untranslated regions and light gray boxes represent ORFs. Black lines indicate introns. Arrowheads indicate the T-DNA insertion sites in \textit{rg1} and \textit{rg2}. (B) Immunoblot analysis with anti-RanGAP1 and anti-RanGAP2 antibodies of total protein extracts from \textit{Arabidopsis} seedlings show that \textit{rg1} and \textit{rg2} are null mutations for RanGAP1 and RanGAP2, respectively. Coomassie Brilliant Blue staining of replica gels is shown below the blots as loading control.
Movie S1. A 3D rotation of a root cell during preprophase showing that RanGAP1-GFP forms a cortex ring (corresponding to the PPB) circulating the nucleus.
Movie S2. A movie showing the complete series of images shown in Fig. S2.