Tethering Factors Required for Cytokinesis in Arabidopsis[1][W]

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At the end of the cell cycle, the nascent cross wall is laid down within a transient membrane compartment referred to as the cell plate. Tethering factors, which act by capturing vesicles and holding them in the vicinity of their target membranes, are likely to play an important role in the first stages of cell plate assembly. Factors required for cell plate biogenesis, however, remain to be identified. In this study, we used a reverse genetic screen to isolate tethering factors required for cytokinesis in Arabidopsis (*Arabidopsis thaliana*). We focused on the TRAPPI and TRAPPII (for transport protein particle) tethering complexes, which are thought to be required for the flow of traffic through the Golgi and for trans-Golgi network function, as well as on the GARP complex, thought to be required for the tethering of endocytotic vesicles to the trans-Golgi network. We found weak cytokinesis defects in some TRAPPI mutants and strong cytokinesis defects in all the TRAPPII lines we surveyed. Indeed, four insertion lines at the TRAPPII locus *AtTRS120* had canonical cytokinesis-defective seedling-lethal phenotypes, including cell wall stubs and incomplete cross walls. Confocal and electron microscopy showed that in *trs120* mutants, vesicles accumulated at the equator of dividing cells yet failed to assemble into a cell plate. This shows that *AtTRS120* is required for cell plate biogenesis. In contrast to the TRAPP complexes, we found no conclusive evidence for cytokinesis defects in seven GARP insertion lines. We discuss the implications of these findings for the origin and identity of cell plate membranes.

Cytokinesis is the division of the cytoplasm following nuclear division. This process differs considerably in plant and animal cells, due predominantly to the presence of the plant cell wall. In animal cells, a contractile ring pinches a dividing cell in two. In plants, by contrast, cross walls are deposited from the middle out within a transient membrane compartment, the cell plate, which is assembled during the anaphase-to-telophase transition. In addition to extensive membrane trafficking and cross wall deposition, plant cytokinesis requires the assembly of two plant-specific cytoskeletal structures, the preprophase band (PPB) and the phragmoplast (Assaad, 2001a). The PPB is a cortical ring of microtubules and actin filaments that marks the division site (Van Damme, 2009). The phragmoplast is a large array consisting of both microtubules and actin filaments, which delivers vesicles to the cell equator and guides the expanding cell plate to the division sites formerly marked by the PPB (Samuels et al., 1995; Seguí-Simarro et al., 2004; Van Damme, 2009).

A genetic dissection of plant cytokinesis has identified a relatively large proportion of trafficking genes (Jürgens, 2005). The cellular processes underlying vesicle traffic are largely conserved in all eukaryotic cells and can be broken down into six steps: budding, transport, tethering, docking, fusion, and recycling (Mellman and Warren, 2000; Assaad, 2001b; Assaad, 2009). With the exception of factors required for tethering, players potentially required for each of these six steps have been identified as being involved in plant cytokinesis. Budding of vesicles from donor membranes requires the action of ARF GTPases. These are encoded by a large family with 45 members in Arabidopsis (*Arabidopsis thaliana*), of which at least one (*TITAN5*) has been shown to play a role in mitosis (McElver et al., 2000). A number of motor proteins and microtubule-associated proteins (for review, see Jürgens, 2005) have been identified that could play a role in the transport of cell plate vesicles to the equator of a dividing cell. Membrane fusion events are mediated by the assembly of a four-helix bundle in which one helix is derived from an R (or v) SNARE, one from a Q (or t) SNARE, and two additional helices are provided by a protein referred to as SNAP25 (Mellman and Warren, 2000; Assaad, 2009). To date, two Q-SNAREs, KNOLLE and NPSN11, as well as a SNAP25 homolog (SNAP33) have been shown to play a role in cytokinesis (Lukowitz et al., 1996; Heese et al., 2001; Zheng et al., 2002), but no R-SNARE has been described. A Sec1/Munc18 protein, KEULE, as well as two Rab GTPases have been implicated in plant cytokinesis, and these could act to regulate fusion events at the cell plate in space and in time as well as to ensure the specificity and fidelity of membrane traffic (Assaad et al., 2001; Chow et al., 2008). Dynamin-related proteins are thought

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to constrict and shape the thin tubular membrane networks that characterize the initial stages of cell plate formation (Seguí-Simarro et al., 2004; Fujimoto et al., 2008). Finally, ESCRT (for endosomal sorting complexes required for transport) proteins involved in endosomal sorting and protein degradation have been shown to be required for cytokinesis (Spitzer et al., 2006, 2009).

Notable for their absence from the above list are tethering factors. Tethering is a process that brings and holds vesicles in close proximity to their target membranes. There are two classes of tethering factors. The first class consists of long coiled-coil proteins, and the second class consists of large multisubunit complexes (Grosshans et al., 2006; Cai et al., 2007). Seven multisubunit tethering complexes have been conserved among all eukaryotes (Koumandou et al., 2007). Each of these tethering complexes resides on a specific cellular compartment and mediates a specific series of membrane fusion events in the secretory pathway. Thus, based on the yeast literature and as depicted in Figure 1, the TRAPPI (for transport protein particle) complex would mediate endoplasmic reticulum (ER)-to-Golgi, COG intra-Golgi, and TRAPPII exit from the Golgi as well as trans-Golgi network (TGN) functions. From the trans-Golgi, secretion to the plasma membrane would be mediated by the Exocyst complex, whereas vesicles destined for the prevacuolar compartment and the vacuole would be tethered by the HOPS (or VPS) complex. The tethering of early or late endosomes to the TGN would be mediated by the GARP complex. Finally, retrograde traffic from the Golgi to the ER would be mediated by the Dsl1 complex on the ER membrane (Koumandou et al., 2007). Studies on the GARP, HOPS, VPS, and Exocyst complexes in plants (see below) support this general model (Fig. 1).

Among the plant tethering complexes, the HOPS/VPS, GARP, and Exocyst complexes have been the most extensively studied. In Arabidopsis, the HOPS/VPS complex resides on the vacuole and prevacuolar compartment and is required for vacuolar biogenesis (Rojo et al., 2001, 2003). The Exocyst is encoded by a number of single and expanded gene families in Arabidopsis and has been shown to play a role in pollen and root hair tip growth and in hypocotyl elongation (Cole et al., 2005; Synek et al., 2006; Hála et al., 2008; Chong et al., 2010). In addition, the Exocyst has recently been shown to be required in the stigma for the acceptance of compatible pollen in both *Brassica* and Arabidopsis (Samuel et al., 2009). Some Arabidopsis GARP mutants have also been implicated in pollen tip growth (Lobstein et al., 2004; Guermonprez et al., 2008) as well as in resistance to heat and osmotic stress (Lee et al., 2006). The TRAPPI and -II complexes, however, have not been studied in plants to date. The TRAPPI complex consists of seven subunits, and the TRAPPII complex consists of the TRAPPI complex and three additional subunits in yeast (Kim et al., 2006; Sacher et al., 2008). All seven TRAPPI and two TRAPPII subunits are conserved in Arabidopsis (Table I).
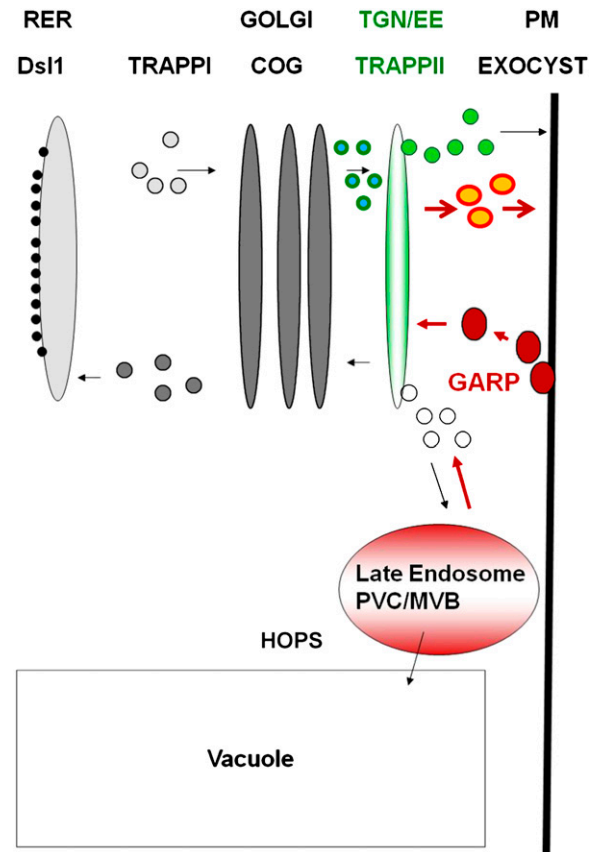

Figure 1. Conserved tethering complexes and the plant secretory pathway. Based on the yeast literature and as adapted from Koumandou et al. (2007), the TRAPPI complex would mediate ER-to-Golgi traffic and the COG complex would mediate intra-Golgi traffic; the TRAPPII complex would mediate exit from the Golgi as well as TGN functions. The TRAPPII complex is thought to reside on the TGN and to tether Golgi-derived as well as endocytotic vesicles in yeast (Cai et al., 2005). The GARP complex is also thought to reside on the TGN, where it tethers early (large, red) and late (small, white) endocytotic vesicles (Conibear et al., 2003; Quenneville et al., 2006). From the trans-Golgi, secretion to the plasma membrane would be mediated by the Exocyst complex, whereas vesicles destined for the prevacuolar compartment and the vacuole would be tethered by the HOPS (or VPS) complex. Finally, retrograde traffic from the Golgi to the ER would be mediated by the Dsl1 complex on the ER membrane. The TGN has been shown to function as an early endosome in plants (Dettmer et al., 2006; Lam et al., 2007). It thereby functions as a sorting station, being the first point of entry for vesicles coming from the plasma membrane (endocytotic vesicles; red) and delivering secretory vesicles (light green) and recycling endosomes (orange) to the plasma membrane as well as clathrin-coated vesicles to the vacuole. The cell plate is thought to be a specialized TGN compartment (Dettmer et al., 2006; Chow et al., 2008). As such, it could tether both Golgi-derived (small, blue-green) as well as endocytotic (red) vesicles. It follows that the TRAPPII complex may play a critical role in cytokinesis. We also predicted that if endocytosis contributes significant membranes to the cell plate, GARP mutants might be impaired in cytokinesis as well. MVB, Multivesicular body; PM, plasma membrane; RER, rough endoplasmic reticulum. See text for a more extensive description and for further references.

Table I. *TRAPP and GARP subunits and their Arabidopsis homologs*

The amino acid conservation between the Arabidopsis proteins and their yeast and mammalian homologs is given in the last four columns. Alignment data were obtained from pairwise, global alignments of the protein sequences by EMBOSS (Rice et al., 2000 [Blossom62; gap open, 10.0; gap extend, 0.5]). Our phylogenetic analyses are consistent with the published data of Koumandou et al. (2007) and of Cox et al. (2007). % Sim., Percent similar; % Ident., percent identical. Dashes indicate that the yeast gene has no homolog in mammals or in Arabidopsis, or that the column header does not apply.

Yeast Subunit	Mammalian Subunit	TRAPP Complex	Arabidopsis Homolog	GenBank Accession No.	*Saccharomyces cerevisiae*		*Homo sapiens*	
					% Sim.	% Ident.	% Sim.	% Ident.
Bet5	C1	I and II	At1g51160	841539	39.5	23.1	53.9	37.1
Trs20	C2	I and II	At2g20930	816627	49.2	31.6	69.3	49.3
Bet3	C3	I and II	At5g54750	835565	67.0	42.8	70.4	50.0
Trs23	C4	I and II	At5g02280	831680	38.5	24.3	46.8	33.2
Trs31	C5	I and II	At5g58030	835915	37.0	26.3	65.0	45.7
Trs33	C6	I and II	At3g05000	819661	32.2	20.3	58.2	42.4
Trs85	KIAA1012	I and II	At5g16280	831488	17.8	9.3	40.2	23.7
Trs65	–	II	–	–	–	–	–	–
Trs120	C9	II	At5g11040	830971	33.2	18.6	36.2	20.0
Trs130	C10	II	At5g54440	835532	34.6	17.3	31.6	19.1
Vps52	Vps52/SACM2L	–	At1g71270	843471	38.0	19.9	56.3	33.6
Vps53	Vps53	–	At1g50500	841472	37.8	18.8	51.3	30.9
Vps54	Vps54	–	At4g19490	827690	31.4	17.2	41.5	23.8

Several questions pertaining to the membrane dynamics of plant cytokinesis are still open. First, the origin of the cell plate is currently debated. Some reports suggest that Golgi-derived vesicles build the cell plate (Reichardt et al., 2007; Chow et al., 2008), others that extensive endocytosis contributes cell wall components as well as enzymes required for polysaccharide biosynthesis to emerging cross walls (Dhonukshe et al., 2006). The question that follows is whether the cell plate arises via the homotypic fusion of like vesicles or whether it arises via heterotypic fusion between different populations of vesicles. Second, little is known about the biogenesis of the cell plate. As tethering factors promote the first contact between donor and acceptor membranes and define specific membrane compartments and trafficking steps, identifying which tethering factors are required for cytokinesis could help answer some of the questions outlined above.

Table II. *Insertion lines used in this study*

n = refers to the total number of individuals analyzed. Asterisks indicate that homozygous mutant individuals were recovered.

Locus	Polymorphism	Position in Gene	Accession No.	Allele	Cytokinesis Defective[a]	Transmission[b]
TRAPPI						
Bet3	GABI_318C08	Fourth intron	N430464	*bet3-1*	0% (n = 200)	7.6% (n = 66)
Bet5	SALK_099482	Second intron	N599482	*bet5-1*	1% (n = 192)	15.2% (n = 79)
	SAIL_634_G07	Second intron	N827313	*bet5-2*	1% (n = 98)	67.2%* (n = 64)
Trs31	FLAG_488E06	Third intron	EWSTV26T3	*trs31-1*	1% (n = 211)	13.6% (n = 59)
Trs33	SALK_109244	Third exon	N609244	*trs33-1*	5.7% (n = 614)	55.6% (n = 36)
	SALK_109724	Third intron	N609724	*trs33-2*	2.9% (n = 104)	15.9% (n = 69)
TRAPPII						
Trs120	SALK_125246	First intron	N625246	*trs120-1*	6.3% (n = 221)	11.8% (n = 34)[c]
	SALK_021904	First intron	N521904	*trs120-2*	8.4% (n = 455)	7.7% (n = 78)
	SALK_111574	Seventh exon	N611574	*trs120-3*	11% (n = 349)	13.1% (n = 107)
	SAIL_1285_D07	Seventh intron	N879232	*trs120-4*	11% (n = 617)	23.8% (n = 105)
GARP						
Vps52	SALK_055433	16th exon	N555433	*vps52-2*[d]	0% (n = 169)	50% (n = 56)
Vps53	SAIL_87_D06	Fifth intron	N870946	*vps53-2*	0% (n = 540)	27.5% (n = 69)
	GABI_400C01	Seventh exon	N344145	*vps53-3*	0% (n = 620)	82% (n = 62)
	SAIL_117_D11	13th intron	N871277	*vps53-4*	0% (n = 221)	50.8% (n = 59)
	GABI_463D10	23rd exon	N344181	*vps53-5*	0% (n = 600)	87.8%* (n = 49)
Vps54	SALK_036485	First exon	N536485	*vps54-1*[d]	0% (n = 196)	25.5% (n = 55)
	SALK_062261	Eighth exon	N562261	*vps54-2*[d]	0% (n = 340)	39.1% (n = 69)

[a]Cytokinesis-defective mutant seedlings on the plate in the progeny of a selfed hemizygous line (%). In the absence of defects in transmission of the mutant alleles, 25% would be expected. [b]Hemizygous or homozygous segregants in the progeny of a hemizygous line grown on soil (%). In the absence of defects in transmission of the T-DNA insertion, and in case of lethality, 67% would be expected. All numbers are based on PCR analysis of genomic DNA isolated from soil-grown individuals (Supplemental Fig. S1), except as noted in subsequent footnotes. [c]For *trs120-1*, we scored cytokinesis-defective mutants in the progeny of individual lines. [d]These alleles have been described by Guermonprez et al. (2008).

In this study, we used a reverse genetic approach to identify tethering factors required for cytokinesis in Arabidopsis. We focused on the TRAPPI, TRAPPII, and GARP tethering complexes because they mediate the flow of traffic through the Golgi apparatus and from the cell surface. Both the TRAPPII and GARP complexes have been shown to reside on the TGN in yeast (Conibear et al., 2003; Cai et al., 2005; Quenneville et al., 2006). Whereas the TRAPPII complex is required for both exocytosis and endocytosis, the GARP complex has only been implicated in endocytosis (Conibear et al., 2003; Cai et al., 2005; Quenneville et al., 2006). The TGN has been shown to function as an early endosome in plants (Dettmer et al., 2006; Lam et al., 2007; Wang et al., 2010). It thereby functions as a sorting station, being the first point of entry for vesicles coming from the plasma membrane and delivering secretory vesicles and recycling endosomes to the plasma membrane (Fig. 1) as well as clathrin-coated vesicles to the vacuole. The cell plate is thought to be a TGN-derived compartment (Dettmer et al., 2006; Chow et al., 2008). As such, it could tether both Golgi-derived and endocytotic vesicles (Fig. 1). If the cell plate is assembled from both Golgi-derived and endocytotic vesicles, we predicted that the TRAPP and GARP complexes might play equally important roles in its biogenesis.

RESULTS

Screening Insertion Mutants in Arabidopsis Tethering Factors for Cytokinesis Defects

All the insertion lines surveyed in this study are listed in Table II. Insertion lines were available for four TRAPPI subunits: Bet3, Bet5, Trs31, and Trs33. The TRAPPII complex consists of the TRAPPI complex and three additional subunits in yeast, of which two (Trs120 and Trs130) are encoded by unique genes in Arabidopsis. Four insertion lines were surveyed for Trs120. The GARP complex consists of four subunits in yeast and animal cells. Of these, only three, Vps52, Vps53, and Vps54, are encoded by unique genes in the Arabidopsis genome. Insertion lines were obtained for all three loci. T-DNA flanking sequences were amplified by PCR on genomic DNA isolated from individual soil-grown plants (for an example, see Supplemental Fig. S1). We were able to confirm the insertion sites by PCR analysis for all the lines listed in Table II.

We plated all the lines and surveyed seedlings for cytokinesis-defective phenotypes. The strongest cytokinesis-defective mutants described to date in the plant literature are typically seedling lethal (Söllner et al., 2002, and refs. therein). Therefore, we specifically screened for the canonical seedling-lethal cytokinesis-
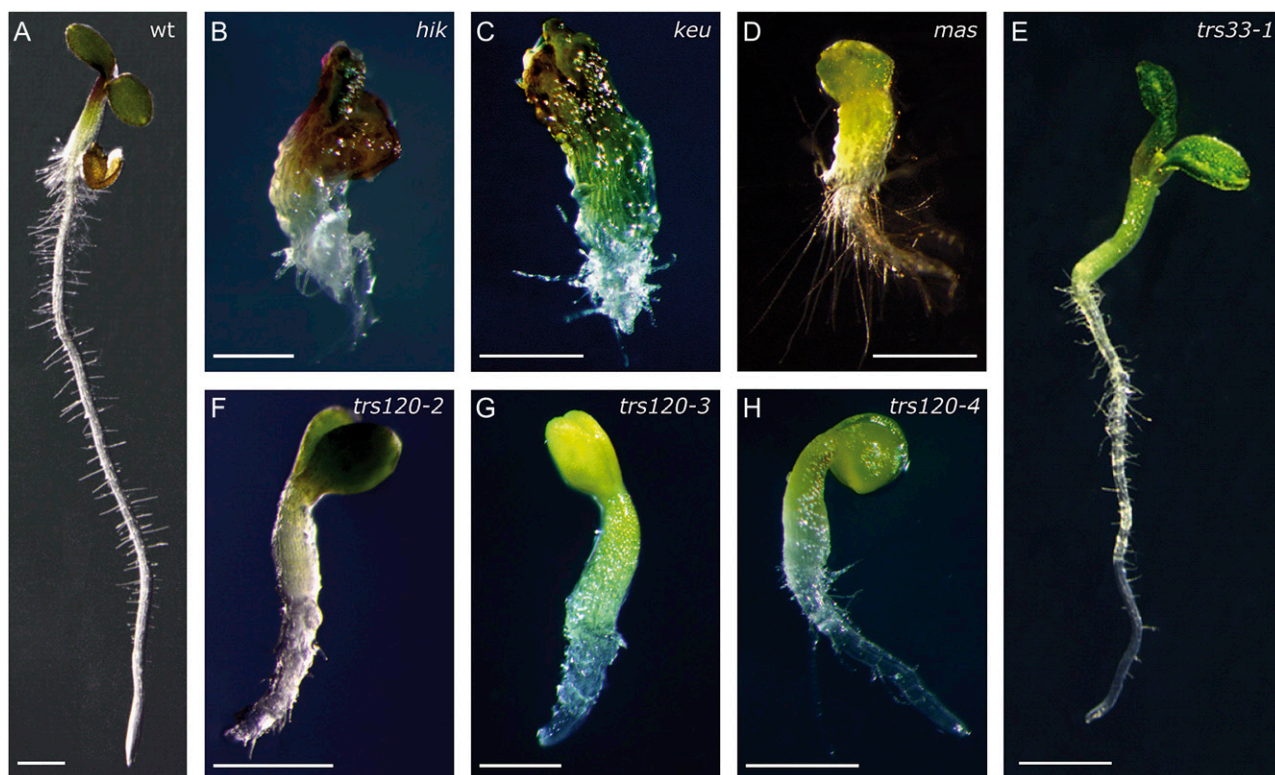

Figure 2. Seedling phenotypes of TRAPPI and TRAPPII mutants and their comparison with canonical cytokinesis-defective mutants. Five-day-old seedlings were grown on Murashige and Skoog Suc plates. A, The wild type. B, *hinkel*. C, *keule*. D, *massue* (*mas-5*). E, *trs33-1*. F, *trs120-2*. G, *trs120-3*. H, *trs120-4*. Note the reduced root and apical meristem growth and bloated appearance of the mutants. Bars = 1 mm.

defective phenotype, which includes a reduced growth of the shoot and root meristems, a rough surface layer with enlarged cells, and a somewhat bloated general appearance (Söllner et al., 2002). We found no evidence of cytokinesis defects in the progeny of seven GARP insertion lines, even though transmission rates were reduced in five of these lines (Table II; reduced transmission has also been reported for GARP mutants by Guermonprez et al. [2008]). In TRAPPI lines, we occasionally observed an attenuated version of the canonical cytokinesis-defective phenotype (Fig. 2E; Table II). The TRAPPII lines segregated 6% to 11% cytokinesis-defective seedlings (Fig. 2, F–H; Table II), whereby seedling morphogenesis was not as severely disrupted in TRAPPII mutants as it was for *hinkel* and *keule* (Fig. 2, compare B and C with F–H), which have some of the more extreme phenotypes (Assaad et al., 1996; Söllner et al., 2002; Strompen et al., 2002). Histological sections confirmed that TRAPPI and -II mutant seedlings were cytokinesis defective, as inferred from the presence of cell wall stubs and floating walls in the mutants (Fig. 3). As the canonical cytokinesis-defective mutant phenotype occurred more reproducibly and reliably in TRAPPII than in TRAPPI lines, we focused on the TRAPPII complex. An insertion mutant in the TRAPPII gene Trs130 was found to be allelic to *club-1* (Söllner et al., 2002); therefore, we named it *trs130/club-2*. *club* mutants, which were identified as being cytokinesis defective in a forward genetic screen, are described elsewhere (Jaber et al., 2010). In this study, we characterize the Arabidopsis Trs120 ortholog (*AtTRS120*).

Characterization of *AtTRS120* Insertion Lines

All four insertion lines in *AtTRS120* were verified by sequencing (Table III; Fig. 4) and shown to be transcriptionally null by reverse transcription (RT)-PCR (Fig. 4; Supplemental Fig. S2). The *AtTRS120* gene spans 5,636 bp and encodes a protein of 1,186 amino acids (Fig. 4). The Trs120p signature spans the entire length of the protein, with five well-conserved domains (designated by black boxes in Fig. 4) ranging from 20 to 165 amino acids in length and characterized by high-quality sequence similarities and by the presence of highly conserved amino acids in the alignment (Cox et al., 2007). In budding yeast, Trs120 is an essential gene, and truncations upstream of the functional break point (designated by an arrow in Fig. 4) are lethal, while truncations downstream of the arrow yield temperature-sensitive phenotypes (Cox et al., 2007). All four insertions in *AtTRS120*, depicted in Figure 4, are upstream of the yeast break point, consistent with their seedling-lethal phenotype.

To assess T-DNA transmission in the insertion lines, DNA was isolated from viable plants grown on soil, and the presence or absence of the T-DNA was monitored via PCR. For a seedling-lethal mutation, one would expect 67% hemizygous and 33% wild-type plants in the progeny of a selfed hemizygous
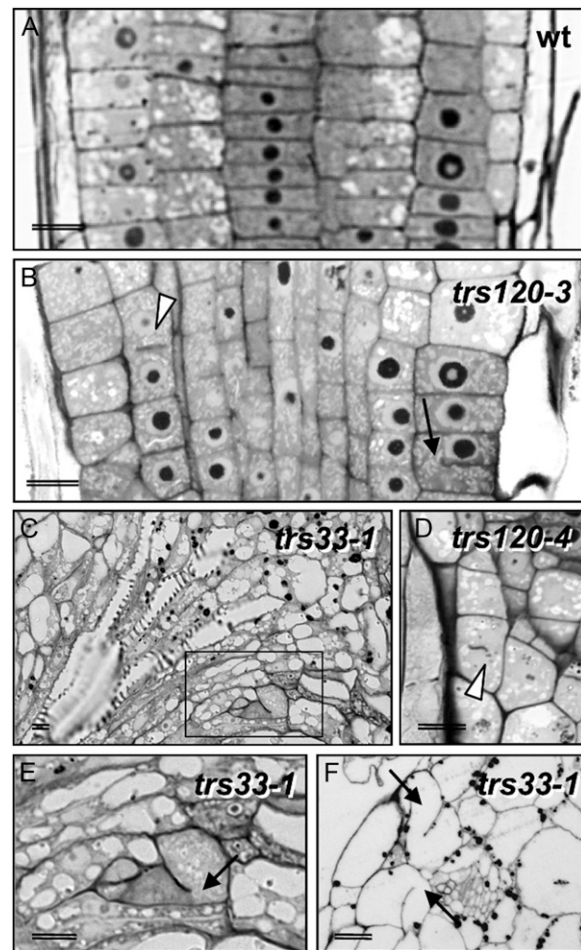

Figure 3. Toluidene blue-stained histological sections of *trs120* and *trs33* seedlings. A, The wild type. B, *trs120-3*. C, E, and F, *trs33-1*. D, *trs120-4*. E corresponds to the boxed area in C. Black arrows (B, E, and F) point to selected cell wall stubs, and the arrowheads in B and D point to "floating" walls.

line. For the four *AtTRS120* lines, which segregated the highest frequency of cytokinesis-defective seedlings (Table II), we observed only between 7.7% and 23.8% hemizygous segregants among the progeny of selfed hemizygous lines (Table II). Thus, these insertion lines showed reduced transmission of the mutant allele. To test whether the T-DNA segregated with the phenotype, we selfed hemizygous plants and analyzed their progeny. In 290 segregants we looked at (for three alleles: *trs120-2*, *trs120-3*, and *trs120-4*), lines harboring a T-DNA insertion (as shown in Supplemental Fig. S1) segregated cytokinesis-defective mutants, but wild-type segregants did not. Thus, the data (Table II) show an absolute segregation of the T-DNA insertion with the cytokinesis-defective phenotype. In a total of 324 plants, we did not recover a single individual that was homozygous for the T-DNA insertion, indicating that null mutations in *AtTRS120* are lethal.

Table III. *Four AtTRS120 alleles*

Allele	Polymorphism	Position in Gene	Position on the Open Reading Frame[a]	Plate Phenotype
trs120-1	SALK_125246	First intron	517	Canonical cytokinesis defective
trs120-2	SALK_021904	First intron	542–562	Canonical cytokinesis defective
trs120-3	SALK_111574	Seventh exon	2,626	Canonical cytokinesis defective
trs120-4	SAIL_1285_DO7	Seventh intron	2,832	Canonical cytokinesis defective

[a]Nucleotide position on the full-length genomic sequence, including introns, starting from the start (ATG) codon. The polymorphisms are depicted graphically in Figure 4.

AtTRS120 Is Required for Cell Plate Assembly

To determine the role of *AtTRS120* in cytokinesis, we used the KNOLLE polyclonal rabbit antibody (Lauber et al., 1997) to label cell plate vesicles and membranes. In addition, a 4′,6-diamino-2-phenylindole (DAPI) stain of the nucleus and a microtubule monoclonal antibody stain were used to monitor cell cycle progression. Confocal analysis of a total of 139 wild-type and 296 *trs120* dividing cells showed that *trs120* mutants are impaired in cell plate assembly. The comparison between the wild type and mutants is depicted in Figure 5 and described in detail for each cell cycle stage below. Although we looked at root tips of three alleles, *trs120-2*, *trs120-3*, and *trs120-4*, and ascertained that they shared the same phenotypes, for the sake of consistency we depict only the *trs120-4* allele in Figure 5. A quantitative analysis was carried out for two alleles, *trs120-3* and *trs120-4* (Table IV).

Metaphase

At this stage, chromosomes are aligned along the equatorial plane of the cell (Fig. 5, A and E) and are surrounded by the spindle apparatus (Fig. 5, B and F). In both wild-type and *trs120* mutant cells, KNOLLE-positive vesicles were distributed throughout the cell (Fig. 5, C and G). We did not notice any difference in the abundance or appearance of vesicles between the mutant and wild type at this stage.

Late Anaphase

At the end of anaphase, once the daughter chromosomes have been brought to opposite poles of the cell (Fig. 5, I and M), the spindle apparatus disintegrates and phragmoplast microtubules can be seen to form two bands at the cell equator (Fig. 5, J and N). As of this stage, the wild type and mutant differ. Indeed, 87.8% of wild-type cells but only 11.9% to 12.5% of mutant cells had a fully assembled cell plate (Fig. 5K) at this stage. In the mutant cells, the cell plate was entirely missing, abnormally thin, or patchily assembled (Table IV). The failure to assemble a cell plate correlated with the presence of loose vesicles that could be seen at the equator of *trs120* cells (Fig. 5O; Table IV).
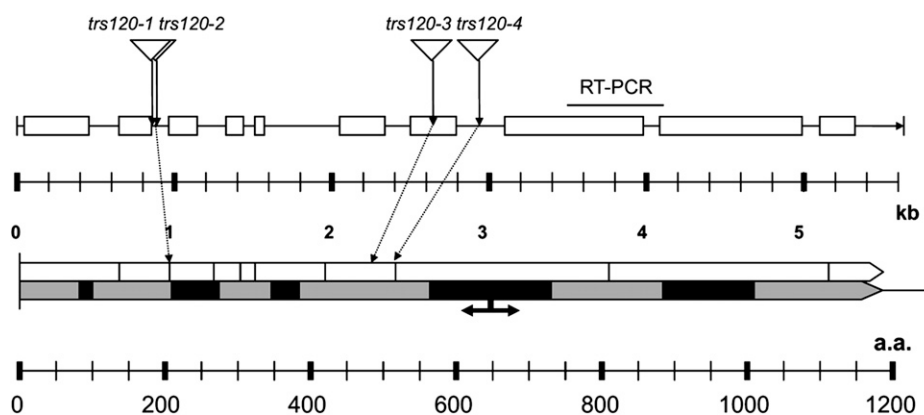

Figure 4. Four insertion lines in *AtTRS120*, a putative TRAPPII tethering factor. The top line represents the *AtTRS120* gene. A single open reading frame is depicted by a line, and the exons are drawn as boxes. Four alleles, depicted with triangles, show a cytokinesis-defective phenotype (Table III). The scale is for nucleotides in kb. A line above the gene designates the interval spanned by RT-PCR primers, with which no transcripts were detected downstream of the four insertions. The bottom line represents the AtTRS120 protein. The Trs120p signature (designated by a gray arrow) spans the entire length of the protein, with five well-conserved domains (designated by black rectangles) characterized by the presence of highly conserved amino acids in the alignment (Cox et al., 2007). The scale is for amino acid (a.a.) residues. The black arrow represents a functional break point in yeast, with mutations upstream of the break point being lethal and mutations downstream being temperature sensitive (Cox et al., 2007). Note that all four insertions are upstream of the functional break point.

Figure 5. Cell plate assembly during anaphase in wild-type and *trs120-4* root tips. Columns are as follows: DAPI/nucleus (blue, first column); microtubules (red, second column); KNOLLE protein (green, third column); and merge (fourth column). The cell cycle stage is indicated on the left, and the genotype (wild-type [wt], A–D, I–L, and Q–T; *trs120-4*, E–H, M–P, and U–X) is indicated in the first column. In all cases, representative cells are shown (see Table IV). Note that cell plate formation, as judged with the KNOLLE antibody, is complete by late anaphase in the wild type (K). In *trs120* mutants, by contrast, the cell plate has failed to assemble throughout cytokinesis (late anaphase [O] and telophase [W]). See text for a detailed description. Bars = 5 μm.
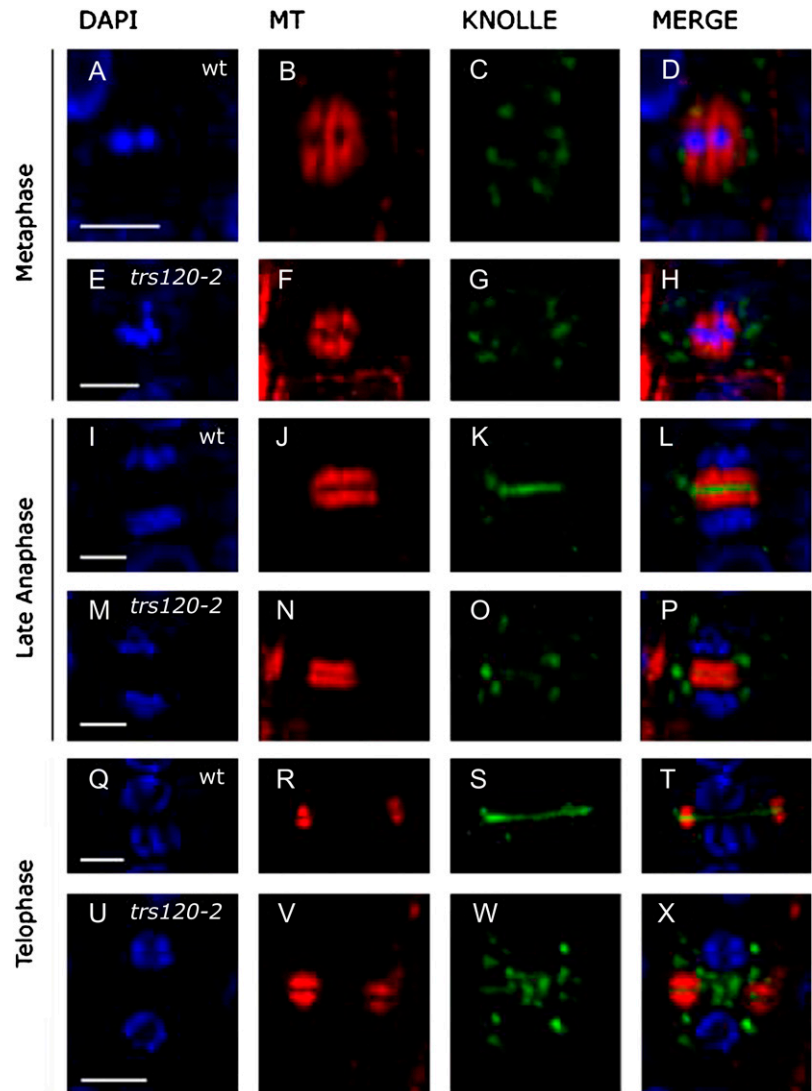
Telophase

Telophase cells were identified based on a DAPI stain showing an ovoid nucleus in which the DNA is decondensed and the nucleoli are visible (Fig. 5, Q and U), together with the presence of a phragmoplast in which the microtubules had depolymerized from the middle and repolymerized at the leading edges (Fig. 5, R and V). A total of 91.6% of wild-type telophase cells (Fig. 5S) but only 5.9% to 14.6% of *trs120* mutant cells had formed a complete cell plate by this stage (Table IV). A cloud of loose vesicles could be seen at the equator in more than 90% of the mutant cells (Fig. 5W), but we scored vesicles as being abundant in only 1.4% of wild-type cells (Table IV).

Conclusion

In wild-type cells, cell plate formation was complete by late anaphase. In contrast, in *trs120* mutants, cell plate assembly was impaired and untethered or un-fused vesicles were abundant at the cell equator throughout cytokinesis.

Electron microscopy on root tips confirmed the impaired cell plate assembly we observed with confocal analysis. Figure 6C, depicts a *trs120-4* cell in which large vesicles (arrows in C and closeup in F) are aligned along the equator of a cell but have failed to form a contiguous cell plate. The nuclei in this cell, however, have decondensed chromosomes and a nuclear envelope (Fig. 6F), which indicates that the cell is well past the anaphase stage and therefore should have a more mature cell plate or a cross wall, as shown in the wild-type cells depicted in Figure 6, A and B. Similarly, Figure 6, D and E, show a patchily assembled incomplete cell plate in a *trs120-3* mutant, resulting in a short stub at one end of the cell and two small walls that, in the focal plane of this sample, appear to be floating (arrowheads). Collectively, the confocal and electron micrographs point to a requirement of AtTRS120 for cell plate biogenesis.

Table IV. *Confocal analysis*

The extent of cell plate assembly correlates negatively with vesicle abundance. Some of the more pronounced differences between the mutant and the wild type are highlighted in boldface.

Phase	Cell Plate Assembly				Vesicle Abundance				Total Cells
	Cell Plate Absent	Patchily Assembled	Intermediate or Thin	Complete	None	Few	Intermediate	Abundant	
	%				%				
Late anaphase									
Wild type	6.1	2.0	4.1	**87.8**	30.6	20.4	38.8	10.2	*n* = 49
trs120-3	33.3	28.6	26.2	**11.9**	4.8	21.4	59.5	14.3	*n* = 42
trs120-4	25.0	50.0	12.5	**12.5**	0.0	12.5	54.2	33.3	*n* = 24
Telophase									
Wild type	**0.0**	1.4	7.0	**91.6**	50.7	22.5	25.4	**1.4**	*n* = 71
trs120-3	**37.2**	45.1	11.8	**5.9**	2.0	7.8	58.8	**31.4**	*n* = 51
trs120-4	**50.9**	29.1	5.5	**14.5**	3.6	5.5	50.9	**40.0**	*n* = 55

DISCUSSION

TRAPPII Mutants Have a Strong Cytokinesis-Defective Phenotype

In this study, we specifically screened insertion lines for strong cytokinesis defects. The strongest cytokinesis-defective mutants described to date in the plant literature are typically seedling lethal (Söllner et al., 2002). Embryo lethality has been reported only in rare cases, most notably in a double mutant between *keule* and *knolle* (Waizenegger et al., 2000). Weak cytokinesis-defective mutants are viable but small and compromised, with seedling phenotypes that are uninformative in the absence of extensive histological analyses (Heese et al., 2001). Canonical seedling-lethal cytokinesis defects were most frequently observed in TRAPPII mutants. This correlated with an impaired biogenesis of the cell plate, a transient compartment thought to be derived from the TGN.

In yeast, the TRAPPII complex has been shown to reside on the TGN, where it mediates both exocytosis and endocytosis (Cai et al., 2005). The KNOLLE syntaxin, which is cell cycle regulated at the transcriptional level, remains the best marker for the cell plate. KNOLLE-positive vesicles appear at the onset of the cell cycle, and KNOLLE antibodies label the cell plate throughout cytokinesis (Lauber et al., 1997). The cell plate is also labeled by Rab GTPases Rab-A2 and Rab-A3, which colocalize with KNOLLE throughout mitosis (Chow et al., 2008). Rab-A2/A3 reside on an early endosome (EE)/TGN compartment, implicated in both endocytosis and exocytosis (Chow et al., 2008). The Rab-A2/A3 compartment only partially overlaps with the TGN marker VHA-a1, which is excluded from the cell plate (Dettmer et al., 2006; Chow et al., 2008). The partial colocalization of the VHA-a1 and Rab-A2/3 markers and their difference at the cell plate reflect the existence of subdomains or subcompartments within the TGN.

As the major sorting station within the plant cell (Dettmer et al., 2006; Lam et al., 2007), the TGN contributes membranes in the form of secretory or clathrin-coated vesicles to the plasma membrane, to the late endosome/prevacuolar compartment (PVC), and (in retrograde traffic) to the Golgi. Yet neither the PVC nor the Golgi colocalizes with Rab-A2/A3 gene fusions, which also predominantly label punctate mobile structures (Chow et al., 2008). The observation that Rab-A2/A3 labels the cell plate throughout cytokinesis suggests that the cell plate has a Rab-A2/A3 EE/TGN identity. Chow et al. (2008) propose that the Rab-A2/A3 TGN/EE compartment is the precursor to the cell plate and that the cell plate may even arise substantially by KNOLLE-mediated homotypic fusion of Rab-A2/A3-like membranes. Our observations are entirely compatible with this view. We show that KNOLLE-positive compartments fail to assemble into a cell plate in the absence of a tethering factor thought, by orthology, to reside on the TGN.

Tethering factors comprise an important addition to existing markers, such as SNAREs and Rab GTPases, that have been shown to colocalize with other markers thought to best define a given cellular compartment (Robinson et al., 2008; Spitzer and Otegui, 2008; Richter et al., 2009). This is because tethers define the target membrane as well as the nature of the vesicles that fuse with this target membrane. As such, they not only define membrane compartments but also specific trafficking steps. We postulate that TRAPPII mutants have strong cytokinesis defects because they impact a crucial early phase in cytokinesis, namely cell plate biogenesis. Tethering factors have been implicated in the biogenesis of other compartments, such as the vacuole. Indeed, a very striking, embryo-lethal phenotype has been reported for the HOPS or VPS tethering complex. Electron micrographs of Arabidopsis VPS mutants show a severe defect in cell elongation due to the absence of a vacuole, and the mutants were accordingly named *vacuoless1* (Rojo et al., 2001). The HOPS complex is thought to tether prevacuolar compartments to each other, as the initial step in vacuolar biogenesis (Rojo et al., 2003). Similarly, and based on our confocal and electron microscopy, we postulate that the TRAPPII complex tethers Golgi-derived and

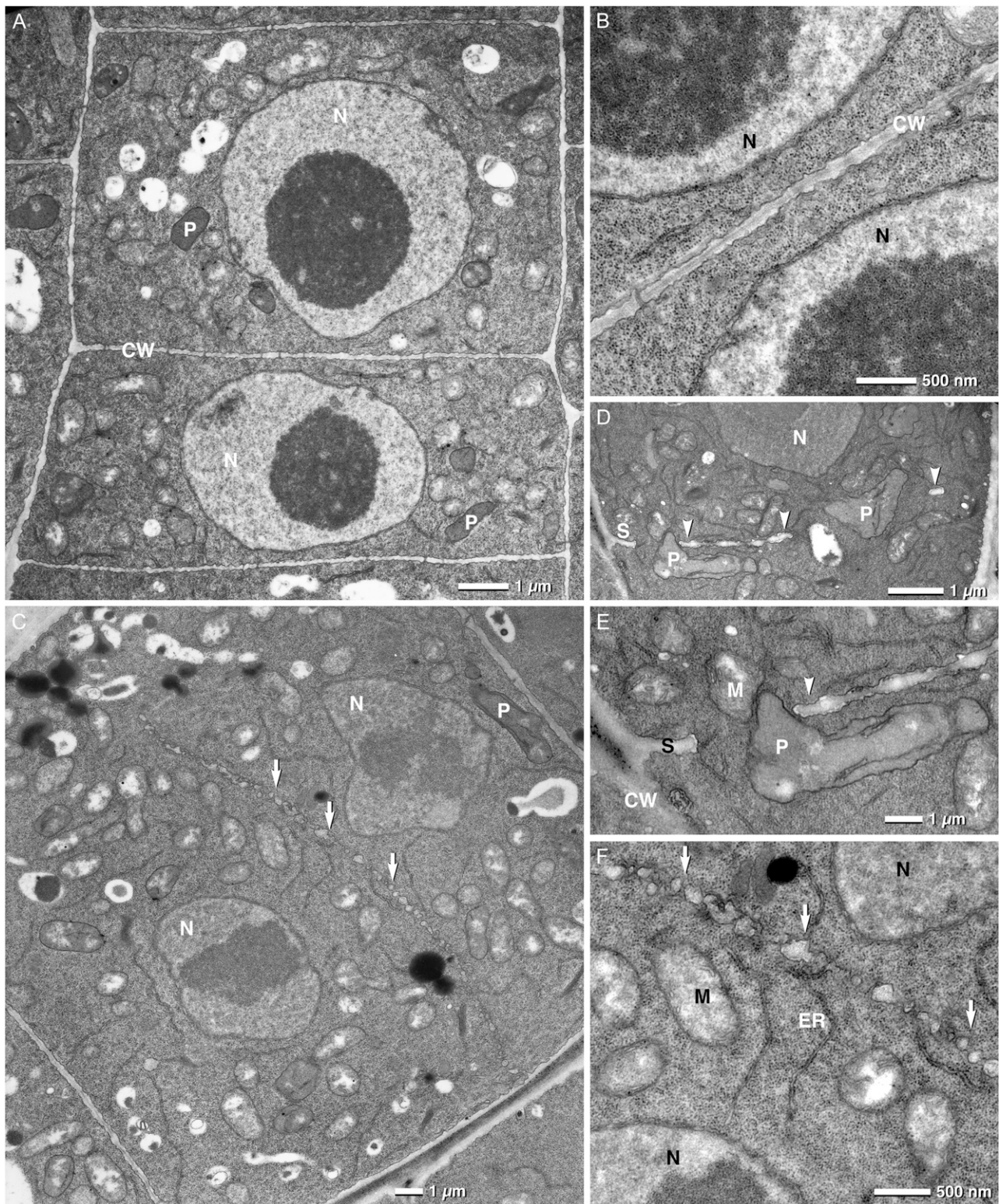**Figure 6.** Incomplete cell plate assembly in *trs120* mutants. Electron micrographs of 5-d-old root tips are shown. A and B, The wild type. Note the regular shape of the cells and the complete cross wall. C and F, *trs120-4*. D and E, *trs120-3*. F is an enlargement of C, and E is an enlargement of D. CW, Cell wall; M, mitochondria; N, nucleus; P, proplastid; S, cell wall stub; V, vacuole. In C and F, white arrows point to vesicles or compartments that have failed to assemble into a contiguous cell plate or cross wall. In fact, the cell plate appears to be in an initial assembly stage characteristic of anaphase. The nuclei, however, are at telophase or interphase, as judged by the presence of decondensed chromosomes and a nuclear envelope (G). Thus, the nuclear

possibly endocytotic vesicles at the cell equator as an initial step in cell plate biogenesis. While there are 26 different Rab-A's in Arabidopsis (Vernoud et al., 2003), the TRAPPII *AtTRS120* and *AtTRS130* proteins are encoded by single genes. These would thus be expected to fulfill all TRAPPII-mediated TGN functions. In this context, cell plate biogenesis might be considered to be a specialized TGN function in mitotic cells.

The Role of Endosomal Trafficking in Plant Cytokinesis

Cross wall deposition requires extensive recycling, reorganization, and remodeling, presumably via distinct and specialized forms of endosomal trafficking. KNOLLE marks the juvenile phase of cross wall deposition, as it appears at the onset of mitosis and disappears once cytokinesis is complete (Lauber et al., 1997; Thiele et al., 2009). As a membrane compartment, the cell plate is initially distinct from, yet after cytokinesis contiguous with, the plasma membrane. Similarly, cross walls are considerably remodeled shortly after they are deposited, with callose being the dominant luminal component of the cell plate yet absent from intact cross walls (Samuels et al., 1995; Thiele et al., 2009). Thus, cell plate or cross wall maturation requires the active removal of certain juvenile traits such as KNOLLE or callose. Proteins targeted for degradation are sorted into luminal vesicles of multivesicular bodies by the ESCRT machinery (Winter and Hauser, 2006; Otegui and Spitzer, 2008). Consistently, ESCRT mutants of Arabidopsis have been reported to have cytokinesis defects (Spitzer et al., 2006, 2009).

The role of GARP proteins is distinct from the role of ESCRT proteins, in that GARP proteins are required not for the degradation but for the recycling of proteins back to the TGN. One potential role for GARP proteins in this context would be to recycle membranes and cell plate materials from the middle of the cell plate to its leading edges as the cell plate expands and joins the parental walls. In a recent study, endocytosis has been suggested to be required for the spatial restriction of the KNOLLE syntaxin to the cell plate (Boutté et al., 2009). Indeed, an impairment of endocytosis via drugs or mutation resulted in the lateral diffusion of KNOLLE after the cell plate had fused with the parental plasma membrane (Boutté et al., 2009). If the role of GARP proteins is restricted to limiting lateral diffusion at late stages of cytokinesis, then one might predict a weaker, more subtle phenotype than the seedling lethality we screened for. It is to be noted, however, that the TRAPPII complex could in principle fulfill all potential endocytotic functions at the cell plate, such that our inability to observe cytokinesis defects in GARP lines does not necessarily exclude a role for endocytosis in cell plate biogenesis.

However, there are three lines of evidence that contest the model, put forth by Dhonukshe et al. (2006), that endocytosis contributes substantially to the cell plate. First, the observation that RabF2b, RabF2a, and GNOM, which are markers for the late endosome (PVC) in plants, were found to colocalize with KNOLLE at the cell plate (Dhonukshe et al., 2006) has been contested (Chow et al., 2008). Second, a pharmacological inhibition of endocytosis did not impact cytokinesis (Reichardt et al., 2007). Third, mutations in retromer proteins that impact endosomal recycling (Niemes et al., 2010) have not been reported to have an effect on cytokinesis (Jaillais et al., 2007; Kleine-Vehn et al., 2008; Yamazaki et al., 2008).

The GARP and TRAPPII Tethering Complexes and the TGN

Both the GARP and TRAPPII tethering complexes have been shown to reside on the TGN in yeast, but their distinct functions at the TGN in space and in time remain to be elucidated. The TGN is emerging as the predominant sorting station in the plant secretory pathway and as a hub in the flow of information to and from the cell surface. Electron tomography has shown that the TGN cannot be perceived as a single compartment but as a continuum of early, late, and mature compartments that release their vesicles by means of cisternal fragmentation (Staehelin and Kang, 2008). TGN cisternae can be seen to generate budding secretory vesicles targeted to the cell surface as well as clathrin-coated vesicles targeted to the vacuole. Interestingly, the ratio of secretory to clathrin-coated vesicles can vary from 5:1 to 1:3 within a single cell (Staehelin and Kang, 2008). This is an example of the plasticity of TGN compartments and of their ability to rapidly adapt to changes in the nature of the cargo molecules present. While electron tomography and live imaging (Nebenführ and Staehelin, 2001; Yang et al., 2005; Moreau et al., 2007; Staehelin and Kang, 2008) have greatly contributed to our understanding of the plant secretory pathway, little is known at a molecular level about how the TGN is subcompartmentalized to fulfill its diverse functions.

The cell plate is widely accepted to be a TGN-derived compartment (Dettmer et al., 2006; Chow et al., 2008) whose assembly can readily be monitored in space and in time. In this study, we show that the TRAPPII *AtTRS120* locus is required for cell plate biogenesis. By contrast, we found no evidence for an involvement of the GARP tethering complexes in cytokinesis in our analysis, but we are not able to exclude this possibility. There are two possible interpretations for these findings. The first is that endocytosis is not required for cell

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Figure 6. (*Continued.*)
cycle is complete but the cell plate has failed to assemble. This is in contrast to the wild type shown in B. D and E show an incomplete, patchy cell plate or cross wall. Arrowheads point to walls that appear to be floating, at least in the focal plane of the micrograph. Note that in E, a protoplastid (P) can be seen inside a large gap in the cross wall.

plate biogenesis. A second interpretation is that the TRAPPII complex tethers both Golgi-derived and endocytotic vesicles at the cell plate. Regardless of which interpretation turns out to be correct, an interesting point is that the GARP and TRAPPII complexes differ with respect to their role in the biogenesis of a TGN-derived compartment. This may open up new avenues for research into the different facets of the TGN, a plastic, complex compartment of pivotal importance for the regulation of differential growth in plants.

MATERIALS AND METHODS

Lines

Due to the lethality of the majority of Arabidopsis (*Arabidopsis thaliana*) TRAPP and GARP mutants, and more specifically of all *trs120* mutants, lines were propagated as hemizygotes. Insertion mutants were selected via the www.arabidopsis.org (Swarbreck et al., 2008) or www.signal.salk.edu (Alonso et al., 2003) Web sites and were obtained from the Nottingham Arabidopsis Stock Centre or the Arabidopsis Biological Resource Center (http://Arabidopsis.info; Scholl et al., 2000) as well as from the Institut National de la Recherche Agronomique (http//www-ijpb.versailles.inra.fr; Samson et al., 2002) and GABI (http://www.gabi-kat.de; Rosso et al., 2003). The insertion lines are in Columbia, and this ecotype was subsequently used as a wild-type control. The *hinkel* allele used was G235 (F.F. Assaad, unpublished data), the *keule* allele was MM125 (Assaad et al., 2001), and the *massue* allele was *mas-5* (Thiele et al., 2009). Plants were grown in the greenhouse throughout the year under controlled temperature conditions and with supplemental light or under controlled growth chamber conditions (16-h/8-h photoperiod at 250 μmol m^{-2} s^{-1}). For an analysis of embryo lethality in seed pods, we used healthy plants grown in the greenhouse during the fall or in controlled growth chambers. Seedlings were surface sterilized, stratified at 4°C for 2 d, and plated on Murashige and Skoog medium supplemented with 1% Suc and B5 vitamins (Sigma; http://www.sigmaaldrich.com) as described previously (Assaad et al., 1996). The root tips of 5-d-old plate-grown seedlings were used for light, confocal, and electron microscopy.

Characterization of Insertion Lines

DNA was isolated from rosette leaves or inflorescences of soil-grown plants. Cetyl-trimethyl-ammonium bromide minipreps were prepared as described by Assaad et al. (2001), and RT-PCR was carried out as described in Supplemental Materials and Methods S1. A full description of the PCR and RT-PCR primers we designed for the analysis of the insertion lines can be found in Supplemental Materials and Methods S1 or in Supplemental Table S1.

Antibody Stains and Confocal Microscopy

Antibody stains were carried out on root tips as described (Völker et al., 2001). The KNOLLE rabbit polyclonal antiserum was generated as described (Lauber et al., 1997) and used at a 1:2,000 dilution. Monoclonal anti-tubulin antibody (Sigma) was used at a dilution of 1:2,500. Goat anti-rabbit monoclonal antibody was coupled to Alexa-m488 (Molecular Probes; http://www.invitrogen.com), and goat anti-mouse secondary antibody was coupled to Cy3 (Dianova; http://www.dianova.de). Nuclei were stained with 1 mg mL^{-1} DAPI (Sigma). Slides were analyzed with a Fluoview 1000 confocal laser scanning microscope (Olympus; http://www.olympus.de). A 40× water-immersion 0.9 numerical aperture objective (Olympus) was used, and scanning was carried out with 4- to 6-fold electronic magnification. Images were acquired and processed with the Fluoview 1000 acquisition software (Olympus) and subsequently processed with Adobe Photoshop. Images were assembled with Adobe Illustrator (http://www.abode.com).

Light and Electron Microscopy

Seedlings for light and electron microscopy were fixed and prepared as described by Schumann et al. (2007), and infiltration was as described by Assaad et al. (1996). Five-day-old seedlings were used for light and electron micrographs. The light microscope was an Olympus BX61 microscope equipped with an F-view II digital CCD camera (Olympus). Transmission electron micrographs were taken with an EM 912 electron microscope (Zeiss; http://www.zeiss.com) equipped with an integrated OMEGA energy filter operated at 80 kV in the zero loss mode. For scanning electron microscopy of fresh material, samples were placed onto stubs and examined immediately in low vacuum with a Zeiss (LEO) VP 438 scanning electron microscope operated at 15 kV. Electron micrographs were digitally recorded from the backscattered electron signal.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers listed in Table I.

Supplemental Data

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

Supplemental Figure S1. PCR analysis of the *trs120-3* and *trs120-4* T-DNA insertion.

Supplemental Figure S2. RT-PCR analysis of the four *trs120* alleles.

Supplemental Table S1. PCR primers for an analysis of polymorphisms.

Supplemental Materials and Methods S1. PCR conditions.

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