RESEARCH PAPER

**Arabidopsis** dyanmin-like protein DRP1A: a null mutant with widespread defects in endocytosis, cellulose synthesis, cytokinesis, and cell expansion

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Abstract

Dynamin-related proteins are large GTPases that deform and cause fission of membranes. The DRP1 family of *Arabidopsis thaliana* has five members of which DRP1A, DRP1C, and DRP1E are widely expressed. Likely functions of DRP1A were identified by studying rsw9, a null mutant of the Columbia ecotype that grows continuously but with altered morphology. Mutant roots and hypocotyls are short and swollen, features plausibly originating in their cellulose-deficient walls. The reduction in cellulose is specific since non-cellulosic polysaccharides in rsw9 have more arabinose, xylose, and galactose than those in wild type. Cell plates in rsw9 roots lack DRP1A but still retain DRP1E. Abnormally placed and often incomplete cell walls are preceded by abnormally curved cell plates. Notwithstanding these division abnormalities, roots and stems add new cells at wild-type rates and organ elongation slows because rsw9 cells do not grow as long as wild-type cells. Absence of DRP1A reduces endocytic uptake of FM4-64 into the cytoplasm of root cells and the hypersensitivity of elongation and radial swelling in rsw9 to the trafficking inhibitor monensin suggests that impaired endocytosis may contribute to the development of shorter fatter roots, probably by reducing cellulose synthesis.

Key words: *Arabidopsis thaliana*, cell wall, cellulose synthesis, cytokinesis, DRP1A, dynamin-related protein, endocytosis, FM4-64, non-cellulosic polysaccharides, radial swelling mutant.

Introduction

Dynamin-related proteins (DRPs) are large GTPases that deform and cause fission of membranes (Praefcke and McMahon, 2004). They are classified into sub-families by sequence and functional domains and have been linked to cell plate formation, endocytosis, exocytosis, protein sorting, and division of mitochondria and chloroplasts. Functional classifications recognize tubulases that draw out membranes into long tubules and pinchases that release vesicles from constricted membranes (Verma and Hong, 2005).

The 16 DRPs of *Arabidopsis* are assigned to sub-families DRP1 to DRP6 (Hong et al., 2003a). In the DRP1 sub-family, DRP1A, DRP1C, and DRP1E are more widely and strongly expressed than DRP1B and DRP1D (Kang et al., 2003a, b). As expected for relatives of phragmoplastin (Gu and Verma, 1996, 1997), DRP1A, DRP1C, and DRP1E accumulate in the *Arabidopsis* cell plate (Lauber et al., 1997; Otegui et al., 2001; Kang et al., 2003a). In tobacco BY-2 cells, *Arabidopsis* GFP-DRP1A and GFP-DRP1C were again located in the cell plate of dividing cells and perhaps with microtubules in interphase cells (Hong et al., 2003b). Further complicating the picture, Jin et al. (2003) found some DRP1C and DRP1E associated with mitochondria and linked them to mitochondrial division while Tang et al. (2006) linked...
mitochondrial DRP1E to salicylic acid signalling and programmed cell death. In addition, Sawa et al. (2005) found that, in the yeast two-hybrid system, DRP1A interacted with VAN3, a regulator of membrane trafficking at the trans-Golgi network. They suggested that DRP1A and VAN3 colocalized in the trans-Golgi network.

Cytokinesis has received the most detailed analysis of the proposed functions for DRP1s. DRP1A forms spiral structures around constricted regions of wide tubules during syncytial cell divisions in Arabidopsis endosperm (Otegui et al., 2001). Ring-like collars and spirals also surround membrane vesicles and tubules in cell plates of apical meristem cells (Seguí-Simarro et al., 2004) and may serve at least two functions. First, they constrict and elongate hourglass-shaped vesicles into the dumb-bells that, with addition of further vesicles, build the tubulovesicular network characterizing cytokinesis stage 2. Secondly, they act as pinchases to release clathrin-coated vesicles and so reduce the plasma membrane area. This allows the tubular network of cytokinesis stage 3 to transform into the planar fenestrated sheet of stage 4. We do not know the exact identities of the protein(s) forming those cell plate collars and spirals nor whether collars and spirals contain the same protein(s). There is some support for the view that DRP1s constrict vesicles and tubules in the cell plate (Otegui et al., 2001; Seguí-Simarro et al., 2004; Verma and Hong, 2005) but whether they act as pinchases is less clear. Endocytosis in animal cells (with the probable exception of macropinocytosis; Johannes and Lamaze, 2002) uses true dynamins as pinchases (Johannes and Lamaze, 2002; Nabi and Le, 2003). Plant DRP2 proteins have all the domains of true dynamins (Hong et al., 2003a), cofractionate with clathrin-coated vesicles, and bind the clathrin adaptor α-adaptin (Lam et al., 2002) leading some to favour them (rather than DRP1s) as likely pinchases in plants (Murphy et al., 2005; Verma and Hong, 2005). Other possible roles for DRP1A during cytokinesis include Golgi vesicle transport (Verma and Hong, 2005).

DRP1A remains at the surface of Arabidopsis cells that have left the root meristem and entered the expansion-only zone (Kang et al., 2003a), suggesting it serves additional functions after cytokinesis is completed. DRP1C occurs in the tip of root hairs and in cells showing diffuse growth where Kang et al. speculate that it is required with DRP1A and DRP1E for endocytosis (Kang et al., 2003b).

Mutant phenotypes have given some insight into the functions of DRP1 proteins. Mutants of the weakly expressed DRP1B and DRP1D lack obvious phenotypes (Kang et al., 2003a) whereas mutations (in the WS background) that disable the widely expressed DRP1A, DRP1C, and DRP1E genes (Kang et al., 2003a) can show strong phenotypes, although sometimes only in double mutants (Kang et al., 2001, 2003a, b). [adl1a and adl1e, the names given by Kang et al. to mutants affecting Arabidopsis dynamin-like proteins 1A and 1E, which are the names used for DRP1A and DRP1E before adoption of the DRP nomenclature of Hong et al. (2003a), are retained in this paper.] Embryos develop slowly in adl1a and seedlings arrest 5 d after sowing but division planes appear normal in both. Exogenous glucose or sucrose rescued arrested adl1a seedlings which then showed only two, very limited morphological abnormalities as they grew to maturity: stigma papillae expanded isotropically rather than anisotropically and trichomes lacked a branch (Kang et al., 2003a). Sawa et al. (2005) subsequently showed that some 10% of adl1a seedlings had vascular discontinuities. Taken together, these observations suggested that rescued adl1a developed with few problems in the absence of DRP1A, presumably relying on DRP1C and/or DRP1E. adl1e showed that no visible phenotype resulted from the absence of DRP1E (Kang et al., 2003a) but adl1a adl1e double mutants were embryonic lethal with disturbed cytokinesis and cell expansion. The convoluted plasma membranes seen in stigma papillae cells of rescued adl1a plants and in embryo cells of adl1a adl1e double mutants could reflect reduced endocytosis increasing the plasma membrane area (Kang et al., 2003a) but could have other causes such as irregular wall deposition. Kang et al. suggested that the limited phenotypes of the single mutants reflect partial redundancy between DRP1A and DRP1E. Mutants have contributed less to understanding the role of DRP1C in vegetative development because pollen lethality precluded obtaining homozygous null mutants (Kang et al., 2003b). Embryo death in adl1a adl1e double mutants indicates that DRP1C cannot cover an absence of DRP1A and DRP1E, at least during embryo development.

Previously described DRP1A mutants have, therefore, provided very few cell types with an obvious phenotype that could be analysed to identify the processes that required DRP1A. This study exploits the widespread phenotype of rsw9 (radial swelling), a DRP1A null in the Columbia background. Many cells in the mutant fail to complete cytokinesis properly or regulate cell division planes but new cells are generated at normal rates although they cannot expand to their normal length or anisotropy (length/width ratio). Mutant cells show reduced endocytosis and we hypothesize that the changes in plasma membrane dynamics inhibit cellulose synthesis, the defect identified as likely to cause the mutant’s short, swollen roots and other visible abnormalities.

Materials and methods

Plant material and growth conditions

Plants were either grown in pots containing seed-raising mix (Debco, Mt Waverley, Vic, Australia) or aseptically in vertical Petri dishes on Hoagland’s solution containing 3% sucrose and 1.2%
ag. Transformants were screened on horizontal plates with Hoagland’s solution containing 3% (w/v) sucrose, 0.75% agar, and 50 µg ml⁻¹ kanamycin. Experimental plants were grown in cabinets under continuous light (100 µmol m⁻² s⁻¹) at either 21 °C or 31 °C. Plants were propagated under higher light (130 µmol m⁻² s⁻¹) at 18 °C to increase seed set.

Map-based cloning and complementation of rsw9

The rsw9 mutant was isolated in the screen described by Baskin et al. (1992). Mapping was essentially as described by Howles et al. (2006) and sequencing candidate genes identified a mutation in At5g42080. Coding and upstream promoter regions of At5g42080 were amplified from BAC MJC20 (Arabidopsis Biological Resource Center, Ohio, USA) using the primers 5'−ATTTGCGGCGCTTTAATCCTCTGTCGGATCTCTGA−3' and 5'−TAAAGGCGGCGACAAGGGGAAAACCTTCA−3' (NolI sites underlined) and cloned into the NolI site of the binary vector pART27 (Gleeve, 1992). The DRP1A cDNA was amplified from clone U21911 (Arabidopsis Biological Resource Center) using the primers 5'−CCGCTCTAGAGTAGAAAACTGTATCTCTCT−3' and 5'−CCTAAGCTCTAATGACCAACAACTCA−3' (XhoI and HindIII sites underlined). The product was cloned into the XhoI and HindIII sites of the expression vector pART7 (Gleeve, 1992) behind the CaMV 35S promoter. This entire cassette was then cloned into the NolI site of pART27. Constructs were verified by sequencing and used to transform Arabidopsis by floral dipping (Clough and Bent, 1998).

RT-PCR

Total RNA (Jacobsen-Lyon et al., 1995) from 1–2 g of 10 d seedlings grown at 20 °C was treated with RQI RNAase-free DNase (Promega, Madison, WI, USA). Primers (Table 1) to amplify 18S rRNA were from Cho and Cosgrove (2000) and primers to amplify DRP1A genes spanned at least one intron. Primer specificity was checked with BLAST and by sequencing PCR products. RT-PCR used the Superscript One-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA) with Platinum Taq, 1 µg of RNA, and followed the manufacturer’s instructions. The PCR cycle was: 20 min at 45 °C; 2 min at 94 °C; (15 s at 94 °C; 30 s at 55 °C; 30 s at 72 °C)×n cycles (see Table 1 for values of n); 7 min at 72 °C. For semi-quantitative RT-PCR, the OD₂₆₀ in each sample was adjusted to generate 18S rRNA bands of similar intensity. To ensure amplification stayed within the log phase, at least five reactions were set up for each gene and tubes were removed from the thermocycler at varying times to determine the optimal number of cycles. Product amounts were compared visually or through Photoshop (version 7, Adobe).

Immunological techniques

Anti-DRP1A raised against residues 498–512 (Kang et al., 2001), a kind gift from Dr S Bednarek (University of Wisconsin), was diluted 1:250. Anti-DRP1A raised against residues 85–253 was kindly provided by Dr W Lukowitz (Cold Spring Harbor). It recognizes sequences in DRP1A and DRP1E but not DRP1C (Kang et al., 2001, 2003b) and was diluted 1:500. Rabbit polyclonal anti-maize actin (courtesy of Dr C Staiger, Purdue University) was used as a loading control. For immunoblots, 7 d seedlings grown at 21 °C were ground in SDS sample buffer containing Roche (Castle Hill, NSW, Australia) protease inhibitor cocktail (100 µg tissue in 500 µl buffer). Proteins in 20 µl aliquots were separated by SDS-PAGE on 10% acrylamide gels for anti-DRP1A or 4–15% gradient gels (Bio-Rad, Hercules, CA, USA) for anti-DRP1. Proteins were blotted onto Hybrid-C Extra (Amersham Biosciences, Little Chalfont, Bucks, UK), blocked with milk powder, and incubated successively with primary antibody, anti-rabbit biotinylated Ig (Amersham; 1:300 dilution), and biotinylated streptavidin horseradish peroxidase complex (Amersham; 1:500 dilution), and developed using 4-chloro-1-naphthol (Sigma, St Louis, MO, USA) in methanol with 3% hydrogen peroxide.

Immunofluorescence was modified from Collings and Wasteneys (2005). Five day seedlings were fixed (40 min) in PEM solution (50 mM PIPES pH 7.0, 2 mM MgSO₄, 2 mM EGTA) containing 1.0% (v/v) DMSO, 0.1% (v/v) Triton X-100, and 4.0% (v/v) formaldehyde. After extraction in PEM containing 1.0% Triton X-100 (1 h) and washing, cell walls were digested (10 min) in 1% (w/v) cellulysin Y6 and 0.1% (w/v) pectolyase Y23 (MP Biomedicals, Irvine, CA, USA) in PEM containing 1.0% (w/v) bovine serum albumin (BSA), and 0.4 M mannitol, washed in PEM (2×10 min), and dehydrated in methanol (−20 °C, 20 min). After rehydration (5 min) in phosphate-buffered saline (PBS; 131 mM NaCl, 5.1 mM Na₂HPO₄, 1.56 mM KH₂PO₄, pH 7.2), material was blocked (10 min) in incubation buffer (IB; PBS containing 1.0% BSA and 0.1% Tween-20). Roots were then incubated in primary antibodies overnight at 4 °C, with these either being tubulin antibodies [monoclonal anti-α-tubulin clone B512, (Sigma) diluted 1:1000 in IB] or tubulin antibodies mixed with either anti-DRP1 or anti-DRP1A, diluted in IB. After washing (PBS, 3×20 min), roots were incubated with secondary antibodies (2 h) [either 1:100 FITC-conjugated sheep anti-rabbit IgG (Silenus Laboratories, Melbourne, Australia) or a mixture of 1:100 FITC-conjugated sheep anti-rabbit IgG (Silenus) concurrently with 1:200 Cy-5-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA)], washed in PBS (3×10 min), and mounted in AF1 antifade agent (Citifluor, London, UK). Roots were viewed with a confocal laser scanning microscope (model SP2; Leica, Wetzlar, Germany) using a ×40 NA 1.2 oil immersion lens, concurrent excitation at 488 and 633 nm, and with emission collected from 500–530 nm and 650–700 nm.

FM4-64 assay of endocytosis

The endocytosis assay (Ueda et al., 2001; Grebe et al., 2003) used FM4-64 (Molecular Probes, Eugene, OR, USA). Five or six day seedlings (20 °C) were incubated (30 min, 0 °C) in distilled water containing 0.5% (v/v) DMSO and 10 µM FM4-64 diluted from a 10 mM stock solution in DMSO. Seedlings were briefly rinsed in

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Table 1. Primers and cycle number to amplify transcripts for DRP1 and 18S rRNA

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primers (5'−3')</th>
<th>Product (bp)</th>
<th>Cycle number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRP1A</td>
<td>1-F: ATCTTGTCATCTCAGATGCA</td>
<td>184</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>2-F: TGGTACACCCACACACTCCTA</td>
<td>221</td>
<td>20</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>1-R: ATCTGCGTGGGAACGGTTGA</td>
<td>400</td>
<td>13</td>
</tr>
</tbody>
</table>
ice-cold distilled water containing 0.5% DMSO and mounted on slides in this solution. Roots were imaged (excitation 488 nm, emission 600–800 nm) at 5 min intervals at room temperature by confocal microscopy with a water-immersion lens (×63, NA 1.3). In some experiments, dye incubation and wash solutions were modified to contain 3% sucrose and/or Hoagland’s solution. All images were collected as single focal planes with 8-fold line averaging using identical collection and processing conditions to allow comparisons of different time points.

**Growth experiments and inhibitor hypersensitivity assays**

Inhibitors were stored at −20 °C as stock solutions in DMSO: 3.2 mM monensin, 10 mM brefeldin A, 100 mM amantadine, 33 mM wortmannin, 10 mM 2,6-dichlorobenzonitrile, 50 mM triiodobenzoic acid (all from Sigma), 50 mM naphthylphthalamic acid (Chem Service, West Chester, PA, USA), 2 mM latrunculin B (ICN, Costa Mesa, CA, USA), and 20 mM oryzalin (Lilly Research Laboratories, Greenfield, IN, USA). Inhibitors were added to liquid agar containing Hoagland’s solution and 3% sucrose, and adjusted to provide a uniform 0.2% DMSO. Seedlings grown for 5 d or 6 d on inhibitor-free plates were transferred to inhibitor plates and incubated for 48 h at 20 °C. Methods for measuring root elongation and radial expansion were modified from Collings et al. (2006).

Root diameters were measured from micrographs (MZ FLIII dissecting microscope with DC200 camera; Leica), while root elongation over a 24 h period was measured from digital scans of the plates made 24 h and 48 h after transfer to inhibitors. To facilitate comparison between wild-type and the more slowly growing rsw9 seedlings, root elongation rates were expressed as relative elongation rates, i.e. as a percentage of the elongation rate of the same genotype growing without inhibitors.

**Other methods**

Cell walls stained with 10 μM propidium iodide (Molecular Probes) in 0.5% DMSO were observed in living roots by confocal microscopy (488 nm excitation, 600–800 nm emission). Longitudinal optical sections (collected in a z-series at 0.5 μm intervals) gave the lengths of recently matured cells in trichoblast files and reconstructed cross-sections gave cell widths. These measurements provided cell anisotropy (length/width ratio). Cell flux (number of cells added per day to each longitudinal cell file) was calculated as the elongation rate of the preceding 24 h of average length of recently matured cells. Root elongation was deduced from scanned images of agar plates and root diameter from micrographs of living material. For measurements of stem epidermal cells, plants were grown in pots at 20 °C and the primary stem cut back soon after elongation began (Burn et al., 2002). Stem regrowth at 31 °C was followed with approximately daily measurements of stem height. Plots of height against time showed an extended period where elongation rate was approximately constant. Epidermal peels were taken from regions on fully grown stems that would have reached maturity during the period when growth rate was constant. Stem diameter was from photographs. Micrographs provided cell lengths, widths, and anisotropy, and cell flux was determined as before.

Monosaccharide composition of cell walls was determined for the non-cellulosic polysaccharides, respectively. and -soluble fractions were analysed to quantify monosaccharides in cellulose and non-cellulosic polysaccharides, respectively.

Expression of the five Arabidopsis DRP1 genes (DRP1A, At5g42080; DRP1B, At3g61760; DRP1C, At1g14830; DRP1D, At2g44590; DRP1E, At3g60190) was examined using the gene chip data available at Genevestigator (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004) and using the massively parallel signature sequencing (MPSS) global expression data (http://mpss.udel.edu/at; Meyers et al., 2004). Data in Fig. 2 come from samples 21 (cotyledons), 22 (hypocotyl), 23 (radicle), 34 (stem), 41 (immature leaves), and 42 (mature leaves) as defined at Genevestigator.

**Results**

**Mutant isolation and molecular analysis**

rsw9 was identified in the screen for root radial swelling mutants that show a stronger phenotype at 31 °C than at 18 °C (Baskin et al., 1992). The mutation was mapped to ∼100 kb of chromosome 5 lying between markers PHYC and ciw9. Sequencing candidate genes among the 28 loci in the interval showed that At5g42080 encoding DRP1A contained a single base pair change from G to A in an AG dinucleotide at the 3′ border of intron 8 (Fig. 1A). To prove that the mutation caused the phenotype, rsw9 was transformed with a genomic fragment including the coding and upstream promoter region of At5g42080 and with the full-length DRP1A cDNA expressed behind the CaMV 35S promoter. Both constructs gave complemented seedlings showing longer, thinner roots than rsw9.

*Arabidopsis* 3′ splice sites have a conserved AG (Brown, 1996) so it was expected that mutation to AA would impair removal of intron 8 in rsw9. RT-PCR using primers 1F and 1R (Table 1) amplified a product from rsw9 RNA that was slightly shorter than the major product from wild-type RNA (Fig. 1B) and sequencing showed that 10 extra nucleotides had been spliced out with intron 8. This was consistent with the splicing machinery in rsw9 removing part of the following exon by using as splice site the next AG that was 10 nucleotides downstream of the wild-type splice site (Fig. 1A). rsw9 RNA also gave small amounts of a larger product from which intron 7 but not intron 8 had been spliced out. This suggested that the splicing machinery occasionally failed to recognize the next AG motif as a 3′ splice site for intron 8.

Conceptual translation of the mRNA from which the additional 10 bp had been removed showed that the frame shift created a premature stop codon after residue 252. This might lead to a truncated protein product (DRP1A1−252) but should also favour mRNA destruction by the nonsense-mediated degradation pathway (Hori and Watanabe, 2005). Semi-quantitative RT-PCR using primers amplifying at the 3′ end of the mRNA (2-F and 2-R in Table 1) showed that rsw9 indeed had less DRP1A mRNA than wild type (data not shown).

**Expression of DRP1 genes**

The transcript levels for the five DRP1 genes were examined using gene chip data available at Genevestigator. Tissue samples were chosen from seedlings (cotyledon, hypocotyl, and radicle), leaves (immature and mature), and stems since they are relevant to the rsw9
phenotype described below. DRP1A was the most strongly expressed gene in all these tissues, whereas the relative levels of DRP1C and DRP1E could differ between tissues (Fig. 2). DRP1B and DRP1D were very weakly expressed in these samples (but were strongly expressed in stamen and pollen samples; data not shown). The gene chips used RNA from various ecotypes, confirming that the widespread expression of DRP1A, C, and E in Kang et al. (2003b) applies beyond the Ws ecotype that they used. MPSS offers a less extensive selection of tissue samples but the data there point to a broadly similar picture (not shown).

rsw9 lacks immunologically detectable DRP1A and DRP1A1–252

Two antibodies (Kang et al., 2001) (Fig. 3A) were used to determine whether rsw9 contained either full-length DRP1A or DRP1A1–252. Anti-DRP1 recognized DRP1A and DRP1E (but not DRP1C) through a shared sequence that DRP1A1–252 would retain, while anti-DRP1A recognized a sequence present only in DRP1A and not DRP1A1–252. After immunoblotting, anti-DRP1A stained a single band in wild-type extracts that rsw9 extracts lacked (Fig. 3B), whereas anti-DRP1 stained a doublet (DRP1A and DRP1E) in wild type, the lower band of which rsw9 lacked (Fig. 3C). No immunostained band had the mobility expected for a 27 kDa protein such as DRP1A1–252 (Fig. 3B, C). It is concluded that rsw9 is a null mutant lacking detectable amounts of DRP1A or DRP1A1–252.

Abnormal phragmoplasts and division planes in cells lacking DRP1A but retaining DRP1E

Whole roots were double labelled with anti-tubulin and either anti-DRP1 or anti-DRP1A. Anti-tubulin clearly showed the difference between phragmoplasts in wild type, which were straight and transversely oriented (Fig. 4A, C), and those in rsw9 which were sometimes...
abnormally oriented or curved (Fig. 4B, D). The organization of phragmoplast microtubule arrays was quantified in epidermal and cortical cells of 12 \textit{rsw9} and 9 wild-type roots. Some 39\% of \textit{rsw9} phragmoplasts (20 from 51) were aberrant, whereas only 7\% (2 out of 30) of wild-type phragmoplasts were aberrant (slightly skewed from a normal transverse cell division plane). Of the 20 abnormal phragmoplasts in \textit{rsw9}, 6 were incomplete and 14 were skewed. Therefore, \textit{rsw9} has a clear cell division phenotype but it is not as severe as found, for example, in the \textit{mor1} microtubule disruption mutant where 50\% of phragmoplasts are incomplete (Kawamura \textit{et al.}, 2006).

Anti-DRP1A labelled cell plates within those wild-type phragmoplasts and recently formed transverse walls (Fig. 4C) but, as expected, did not label any structures in \textit{rsw9} (Fig. 4D). Anti-DRP1 strongly stained cell plates and newly formed transverse walls in wild type (Fig. 4A) where it would be binding DRP1A and DRP1E. There was much weaker staining in \textit{rsw9} (compare Fig. 4B with Fig. 4A), where anti-DRP1 would only bind DRP1E. This is consistent with DRP1A accounting for substantial antibody binding in root tip cells and DRP1E for relatively little. It was not possible to recognize differences between anti-DRP1 and anti-DRP1A labelling that...
would have suggested that DRP1A and DRP1E had different locations in the cell. Labelling of transverse walls and cell plates by anti-DRP1 and anti-DRP1A is consistent with previous observations using roots of wild type and the adl1a mutant (Kang et al., 2003a). Observations of living cells (mitotic and post-mitotic) containing a DRP1A–GFP fusion protein show it also occurs close to the plasma membrane underlying longitudinal walls. This association is lost after fixation for immunofluorescence (Kang et al., 2003a) to give a more punctate pattern of staining similar to that present in wild-type roots after fixation and staining with anti-DRP1A in the present study (Fig. 4C).

Cell division abnormalities in rsw9 were investigated further by staining walls with propidium iodide and observing intact roots by confocal microscopy. Optical sections showed that the reliably transverse and longitudinal walls of wild-type roots (Fig. 5A) were abnormally aligned and sometimes incomplete in epidermal, cortical, and stelar cells of rsw9 (Fig. 5B). Focal sections forming a z-series clearly show incomplete cell walls and cell wall stubs (Fig. 5C), a pattern revealed in three-dimensional reconstructions of the cells (Fig. 5D). Tubulin immunolabelling also revealed the presence of incomplete cell walls and multinucleate cells within the root tip of rsw9 (Fig. 5E), a pattern never observed in wild-type plants. The presence of interphase cortical arrays in these cells shows that cell plate growth has finished and that the incomplete cell walls are not still expanding.

**Morphology of the mutant**

By contrast to mutants found in the same screen that appear almost normal at 20 °C (e.g. rsw1, Williamson et al., 2001; rsw3, Burn et al., 2002), rsw9 showed a strong phenotype at 20 °C (Fig. 6A, B) that increased in severity at 31 °C. The mutant’s splicing defect is complete at 20 °C so this stronger phenotype probably reflects extra deficiencies exposed by the demands of rapid growth at 31 °C rather than the existence of a genuinely temperature-sensitive protein. Although somewhat atypical in the extent of the phenotype seen at 20 °C, the major morphological features of the phenotype are shared by most rsw mutants. Seedlings of rsw9 have severely stunted and swollen roots after growth at 20 °C in either light (Fig. 6A, B) or dark (Fig. 6D, E). Root swelling was also seen in median optical sections obtained by confocal microscopy (compare left-hand panels in Fig. 5A, B) and was quantified (125 µm versus 175 µm approximately) through the zero concentration points in the inhibitor experiments (Fig. 9B, D, F) that will be described below. Hypocotyls were also shorter and fatter after growth at 20 °C in either light (Fig. 6A, B) or dark (Fig. 6D, E). Initial hypocotyl growth is often almost horizontal and the subsequent growth direction of dark-grown hypocotyls and roots seems less precise than it is in wild type (Fig. 6B, E). This widespread phenotype was identified in the presence of sucrose, a component of normal agar medium. Elongation rates of rsw9 roots responded to withdrawal of sucrose, to changes in its concentration, or to its replacement by glucose, but there was no parallel to the complete arrest which Kang et al. (2001, 2003a) reported when adl1a was grown without sucrose or glucose (data not shown).

Plants of rsw9 grown without sugar supplements on potting mix also showed a strong phenotype. Leaves emerged more slowly and formed smaller rosettes (Fig. 6C), although trichomes were not obviously abnormal as was reported for adl1a. Bolts of rsw9 grew more slowly and were shorter than in wild type. In the experiments to determine cell size and cell flux described below, rsw9 stems grew at 25 mm d−1 during the phase where they showed a constant growth rate, whereas wild-type stems grew at 34 mm d−1. Elongation lasted slightly longer in rsw9, reducing the differences in final heights (383 mm and 419 mm). Flowers of rsw9 resembled flowers of adl1a in that stigma papillae failed to elongate (Kang et al., 2003a) and seed set was low. Seeds that did form in rsw9 were not shrivelled as found for adl1a seed (Kang et al., 2001).  

**Reduced cell expansion rather than reduced cell production underlies the shorter roots and stems**

The abnormal cell divisions in rsw9 might slow the rate at which it can add new cells to longitudinal cell files, a parameter termed cell flux in kinematic analyses. However, cells retain DRP1A after leaving the meristem so cell expansion might be affected by its absence. After measuring root and shoot elongation rates and final cell sizes, the relationship

\[
\text{rate of organ elongation} = \text{cell flux} \times \text{final cell size}
\]

was used to determine cell flux (the number of cells each file added in 24 h). No statistically significant changes in cell flux in the epidermis of either roots (trichoblast files) or stems were found (Table 2). rsw9 had shorter cells in both organs, suggesting that lack of DRP1A reduced cell expansion rather than cell flux. Cell anisotropy (final length:final width) fell in both organs but cell width increased only in roots. The lower anisotropy in stems resulted solely from reduced cell lengths with neither cell width nor stem diameter increasing (Table 2).

**Changed wall composition**

It was noted before that rsw9 shares with many other rsw mutants the phenotype of shorter and fatter roots and dark-grown hypocotyl, smaller rosettes, and shorter but not fatter stems. Among the ~40 rsw lines identified in the present screen, it is estimated that the phenotype in
approximately half of them (representing nine loci) results from reduced cellulose production. To see if rsw9 belonged to this category, its walls were analysed using the trifluoroacetic acid method. Walls of rsw9 seedlings did indeed have less cellulose than wild type (Fig. 7A), suggesting that reduced cellulose does cause much of the visible phenotype of rsw9. By contrast to the reduction in cellulose, however, rsw9 incorporated more galactose,
arabinose, and xylose into non-cellulosic polysaccharides (Fig. 7B). If root and shoot cell walls had different compositions, the changes seen in analysing whole seedlings could result from changes in the root:shoot balance in the mutant. The dry weights of the very stunted mutant root and the wild-type root were very similar so that the roots contribute a very similar percentage to total seedling dry weight in the two genotypes (data not shown). It is concluded that the reductions in cellulose and increases in some non-cellulosics reflect changed wall composition rather than changed tissue contribution.

Reduced endocytosis

The root phenotype of rsw9 provided the opportunity to see whether endocytosis was reduced as suggested from static images of stigma papillae and embryo cells (Kang et al., 2003a). FM4-64 labels plasma membranes of living cells and reaches the cytoplasm only by endocytosis (Ueda et al., 2001; Bolte et al., 2004). Intact seedlings labelled at low temperature with FM4-64 in distilled water (following Grebe et al., 2003) were returned to room temperature and the roots observed by confocal microscopy. Wild-type cells in the distal elongation zone showed FM4-64 at punctate cytoplasmic sites within 5 min of transfer to room temperature and the number of fluorescent spots increased over 40 min (Fig. 8A). Dye uptake was also seen in the root cap (Fig. 8C) and in cells in the elongation zone (Fig. 8D). rsw9 cells, by contrast, showed little uptake even after 45 min (Fig. 8B, E, F). Adding Hoagland’s salts and/or sucrose to labelling and uptake solutions did not affect uptake into wild type (Fig. 8G–J) but increased uptake into rsw9, particularly when both Hoagland’s salts and sucrose were supplied (Fig. 8L–N). The effects of sucrose probably result from changed osmotic potential, rather than from sucrose metabolism since 88 mM mannitol (a similar osmotic potential to 3% sucrose) gives a comparable stimulation in FM4-64 uptake in rsw9 (data not shown).

Hypersensitivity to trafficking inhibitors

Vesicle trafficking inhibitors (Baskin and Bivens, 1995), like the loss of DRP1A in rsw9, promote radial swelling and reduce elongation in Arabidopsis roots. This led to the consideration whether the reduced endocytosis in rsw9 contributes to the mutant having shorter and fatter roots. This hypothesis was tested by examining whether rsw9 was hypersensitive to endocytosis inhibitors, i.e. whether inhibitor concentrations that are minimally effective in wild type, slow growth and/or cause radial swelling in rsw9. Mutant hypersensitivity can reflect an increased binding constant for the inhibitor but more commonly it reflects mutation and inhibitor acting on the same pathway and hypersensitivity appears particularly under conditions where mutant and inhibitor effects are individually sub-threshold or at least non-saturating. The accepted explanation is that mutation and inhibitor together create an above-threshold response while individually each is sub-threshold (or at least non-saturating) for an effect. Arabidopsis radial swelling mutants exemplify this origin for hypersensitivity; mor1, defective in a microtubule-associated protein, is hypersensitive to microtubule disruption.
with oryzalin (Collings et al., 2006) but not to the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile, while rsw1, defective in a CESA glycosyltransferase, is hypersensitive to 2,6-dichlorobenzonitrile but not oryzalin (DA Collings, unpublished data).

Various compounds reported to inhibit endocytosis and trafficking were tested: brefeldin A which blocks exocytosis from the Golgi and recycling from endosomes (Nebenführ et al., 2002); monensin A, a putative inhibitor of both exocytosis and endocytosis in plants that may raise vesicle pH (Mollenhauer et al., 1990; Roszak and Rambour, 1997; Signoret et al., 2004); wortmannin which inhibited endocytosis in tobacco and sycamore culture cells (Emans et al., 2002; Etxeberria et al., 2005); amantadine which inhibited the uptake of bacterial elicitors into tobacco culture cells (Gross et al., 2005); latrunculin B, a microfilament inhibitor suggested to block the endocytotic pathway (Grebe et al., 2003; Šamaj et al., 2004); naphthylphthalamic acid and triiodobenzoic acid, auxin transport inhibitors suggested to inhibit endocytosis (Geldner et al., 2001).

Higher concentrations of all inhibitors reduced elongation of wild-type roots but only brefeldin, monensin, latrunculin, and naphthylphthalamic acid caused radial swelling at similar concentrations. Of these, only monensin produced convincing hypersensitivity: 0.33 μM reduced root elongation by 67% in rsw9 whereas even 1.0 μM monensin inhibited elongation in wild type by only 28% (Fig. 9A). Furthermore, only rsw9 showed significant root swelling in 0.33 μM monensin (Fig. 9B). Differences with brefeldin and latrunculin were not convincing. Both inhibited elongation slightly more in rsw9 than in wild type (Fig. 9C, E) but there were no major differences in the response of root diameter to these compounds (Fig. 9D, F).

**Discussion**

It is argued that many important morphological and microscopic features in rsw9 can be linked to reduced cellulose synthesis and it is hypothesized that reduced endocytosis might be the underlying defect that reduces cellulose synthesis.
Low cellulose walls probably cause the visible phenotype

Cell walls in rsw9 have less cellulose than wild-type walls and rsw9 plants resemble plants of eight other cellulose-deficient rsw mutants (rsw1, rsw2, rsw3, rsw5, rsw10 to rsw13): roots (and usually dark-grown hypocotyls) are shorter and fatter, rosettes smaller, and stems are shorter (Lane et al., 2001; Williamson et al., 2001; Burn et al., 2002; Howles et al., 2006; Wang et al., 2006; PA Howles, LK Gebbie, RE Williamson, unpublished results for rsw11 to rsw13). It can therefore be concluded with some confidence that the low cellulose walls documented for rsw9 cause those morphological features in rsw9. Reduced cellulose plausibly changed the shape of stigma papillae cells in sugar-rescued adl1a plants (Kang et al., 2003a) and Kang et al. themselves noted similarities between embryos of the adl1a adl1e double mutant and embryos of cyt1, a cellulose-deficient, cytokinesis-defective mutant (Nickle and Meinke, 1998; Lukowitz et al., 2001).

At least some, perhaps all, cellulose-deficient rsw mutants share further changes to wall composition with rsw9, changes that point to lack of DRP1A inhibiting cellulose production without inhibiting production of
non-cellulosic polysaccharides. The cellulose-deficient walls of rsw9 have more xylose, galactose, and arabinose than wild type when analysed by the trifluoroacetic acid method. Four other cellulose-deficient rsw mutants have been analysed by that method (Howles et al., 2006 for rsw10; RE Williamson, CH Hocart, AH Cork, unpublished data for rsw11, rsw12, and rsw13). All four had more arabinose, three had more xylose, and three had more galactose (although increases were statistically significant in only one of the four mutants). This strengthens the proposition (His et al., 2001; Pagant et al., 2002; Mouille et al., 2003) that reducing cellulose synthesis often increases production of non-cellulosics. In rsw9, increased non-cellulosics could reflect reduced endocytosis, a process that may remove non-cellulosics from the wall (Balusˇka et al., 2002; Sˇamaj et al., 2004). That endocytosis effect cannot, however, explain why non-cellulosics increase in other mutants where the defects are unrelated to endocytosis (e.g. defective ribose 5-phosphate isomerase in rsw10; Howles et al., 2006). The increased non-cellulosics in all five of these rsw mutants also implies that their mutations are specifically inhibiting cellulose synthesis rather than simply causing some general inhibition of wall deposition. This specificity holds even when the mutated protein seems well removed from cellulose synthesis per se (ribose 5-phosphate isomerase, Howles et al., 2006; DRP1A, this study) as well as when it may be more closely involved (His et al., 2001; Pagant et al., 2002). Raised non-cellulosics in rsw9 also suggests that there is no block to vesicle trafficking from the trans-Golgi network to the wall; other proteins must sustain any function DRP1A may normally provide with VAN3 at the trans-Golgi network (Sawa et al., 2005).

Aberrant cell division planes and impaired cell expansion

The present immunofluorescence observations place DRP1A at the cell plate (see also Lauber et al., 1997; Otegui et al., 2001; Kang et al., 2003a; Hong et al., 2003b), presumably reflecting an actual location adjacent to the plasma membrane surrounding the plate (Seguí-Simarro et al., 2004). DRP1E, the sole target of anti-DRP1 in rsw9, has a similar location when viewed at this resolution, a result echoing the finding of Kang et al. (2003a) for adl1a. In the rsw9 root but not in the adl1a root, growing phragmoplasts/cell plates can be curved, division planes disturbed, and some walls remain incomplete. Previous studies suggested that DRP1A might deform membranes, transport Golgi vesicles, or release endocytotic vesicles during cell plate growth (Verma and Hong, 2005), and any of these deficiencies could prevent completion of cytokinesis in rsw9. However, reduced cellulose synthesis itself could also contribute. Cellulose

Fig. 9. rsw9 is hypersensitive to monensin but not to other inhibitors of vesicle trafficking. Response of roots to varying concentrations of monensin (A, B), brefeldin A (C, D), and latrunculin B (E, F). Root elongation rate (A, C, E) was derived from length measurements made 24 h and 48 h after seedlings were transferred to inhibitor plates and root diameters (B, D, F) were measured at 48 h. Relative elongation rates were derived by expressing the rate as a percentage of that for seedlings of the same genotype not exposed to inhibitors. (A, B) Monensin at 0.33 μM has limited effects on wild type but strongly inhibits elongation (A) and promotes radial swelling (B) of rsw9. (C, D) Brefeldin A effects on rsw9 are not drastically greater than its effects on wild type for either elongation (C) or radial swelling (D). (E, F) Latrunculin inhibits elongation of rsw9 slightly more strongly than it inhibits wild type (E) but it promotes radial swelling to a comparable extent (F). Data are means with SEM from three or more replicate experiments with n >18 roots for all data points.
is deposited in cell plates late in cytokinesis (Samuels et al., 1995) after the proposed DRP1-dependent release of endocytic vesicles transforms the stage 3 tubular network into the stage 4 fenestrated sheet (Seguí-Simarro et al., 2004). Problems in this transformation may restrict subsequent cellulose deposition and contribute to incomplete cell divisions since walls also remain incomplete when cellulose synthesis is inhibited by chemicals (Buron and Garcia-Herdugo, 1983; Venverloo et al., 1984) or by some mutations (Nickle and Meinke, 1998; Lane et al., 2001; Lukowitz et al., 2001). How lack of DRP1A alters division planes is unclear but soybean phragmoplastin (a DRP1) perturbs cell division planes in tobacco when mutated to inhibit GTPase activity (Hong et al., 2003b; Wyrzykowska and Fleming, 2003) or overexpressed (Gu and Verma, 1997; Geisler-Lee et al., 2002).

Kinematic analysis shows that these abnormal cell divisions do not reduce the rate at which root and stem epidermis adds new cells. Reduced cell lengths account for most of the decline in organ elongation. Changed final cell dimensions must also reduce the length and increase the diameter of hypocotyls since that organ generates no cellulose synthase directly, although we note suggestions that they may bind callose synthase (Hong et al., 2001). Endocytosis recycles many important plasma membrane components (Murphy et al., 2005) such as the KOR endocellulase (Robert et al., 2005) that cellulose synthesis requires (Lane et al., 2001) and the CESAs of rosette terminal complexes (Paradez et al., 2006). As a result, reduced endocytosis in rsw9 has potential routes to change plasma membrane properties in ways that reduce cellulose synthase activity.

Monensin, like several other trafficking inhibitors, reduces elongation and promotes radial swelling in Arabidopsis roots (Baskin and Bivens, 1995), features of the rsw9 phenotype that are linked to low cellulose walls. These growth responses in rsw9 are hypersensitive to monensin. This points to the genetic defect (lack of DRP1A) and exposure to monensin causing similar growth changes by acting on pathways having a common step. That step may be endocytosis, given that both monensin (Roszak and Rambour, 1997) and lack of DRP1A (this study) inhibit it. Reduced endocytosis may therefore be an early step in the chain of events that leads, via low cellulose walls, to altered growth anisotropy in rsw9.

**Reduced endocytosis**

Root cap, meristematic, and expanding cells of the rsw9 root take up less FM4-64 than their wild-type counterparts. This probably reflects reduced endocytosis at the plasma membrane but the possibility cannot be excluded that the defect is downstream, for example, in fusion with endosomes. The reduction is dramatic for roots bathed in distilled water but less pronounced for roots exposed to sucrose, mannitol, and/or Hoagland’s salts. This identifies an important process that is inhibited during the cell expansion that kinematic analyses show is the dominant effect in reducing organ elongation.

**Events leading through low cellulose walls to the visible phenotype**

We suggested that low cellulose walls explain many visual features of rsw9. DRP1s are not known to bind cellulose synthase directly, although we note suggestions that they may bind callose synthase (Hong et al., 2001). Endocytosis recycles many important plasma membrane components (Murphy et al., 2005) such as the KOR endocellulase (Robert et al., 2005) that cellulose synthesis requires (Lane et al., 2001) and the CESAs of rosette terminal complexes (Paradez et al., 2006). As a result, reduced endocytosis in rsw9 has potential routes to change plasma membrane properties in ways that reduce cellulose synthase activity.

A more extensive mutant phenotype in rsw9 than in adl1a

The phenotype of rsw9 differs from that of null alleles of DRP1A that are in the Ws background (adl1a of Kang et al., 2001, 2003a; Sawa et al., 2005); rsw9 continues growth but with a strong, widespread phenotype, whereas adl1a arrests at day 5 with little visible phenotype or, after rescue with sugars, grows with only stigma papillae and trichomes being abnormal. Growth of rsw9 seedlings responds to changing concentrations of sugars in the medium (as do other genotypes) but rsw9 shows no parallels to the arrest/rescue behaviour that characterizes the response of adl1a to sucrose and glucose. Although much less extensive than the morphological changes in rsw9, loss of anisotropic growth in stigma papillae cells and faulty branching of trichomes in adl1a could plausibly reflect a cellulose deficiency in those cells, a phenomenon that seems to affect many cell types in rsw9. Embryo cells of the adl1a adl1e double mutant (no DRP1A or DRP1E) show further parallels with rsw9. Cell divisions were misaligned and incomplete, highly reminiscent of those seen in seedlings of rsw9. Moreover, Kang et al. (2003a) described arrested embryo cells as ‘blown’ after they failed to expand anisotropically and noted other features reminiscent of cellulose-deficient, embryo-lethal mutants. It therefore seems that the major differences between rsw9 and adl1a are not in the defects they show (which always involve loss of cell anisotropy and cytokinesis defects) but rather that rsw9 shows widespread defects in cell anisotropy and cytokinesis, whereas adl1a either arrests completely or, after rescue, grows with only very few abnormally shaped cells. Removing DRP1E as well as DRP1A in the adl1a adl1e double mutant causes cell shape and cytokinesis defects that are embryo lethal but again they resemble the defects seen widely in rsw9 after germination. Preliminary findings suggest that rsw9 may be typical of DRP1A mutants in the Columbia background. Seedlings of the
Salk line 069077, a T-DNA insert in the Columbia background, resembled rsw9 seedlings in having stunted and swollen roots.

Kang et al. (2001, 2003a) argue persuasively that DRP1A and DRP1E provide redundancy, and differences in the extent of that redundancy could underlie rsw9/adl1a differences. For example, the widespread rsw9 phenotype requires DRP1E to sustain growth but with reduced cellulose production, whereas DRP1E must be unable to sustain growth at all in adl1a when it arrests without a sugar supplement. The near normal morphology of sugar-rescued adl1a implies that DRP1E then supports essentially wild-type cellulose production, except probably in misshapen stigma papillae cells and trichomes. In thinking of redundancy between DRP1A and DRP1E, it should be remembered, however, that the role of the widely expressed DRP1C is not clearly understood. A major obstacle remains that homozygous DRP1C mutants have not been obtained because lack of DRP1C is lethal to male reproductive development (Kang et al., 2003b). Embryo lethality in adl1a adl1e clearly shows that DRP1C cannot support embryo development without DRP1A and DRP1E but DRP1C could contribute to an unknown extent to the growth of single mutants such as rsw9.

In summary, the strong but non-lethal phenotype of rsw9, a null mutant of DRP1A in the Columbia background, was used to show that lack of DRP1A disrupts cytokinesis. However, reduced cell expansion contributes more towards the reduced elongation seen in roots and stems of rsw9. Impaired cellulose synthesis plausibly causes (or, in the case of cytokinesis, contributes to) these changes and to increased radial swelling in root and hypocotyls. It was confirmed here that lack of DRP1A inhibits endocytosis in interphase cells as Kang et al. (2003a) hypothesized from static images of stigma papillae cells. Hypersensitivity of rsw9 to monensin strengthens the case that reduced endocytosis is on the chain of events that leads, via reduced cellulose synthesis, to shorter and fatter roots and hypocotyls.

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