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Rapid Paper

Actin Control Over Microtubules Suggested by *DISTORTED2* Encoding the *Arabidopsis* ARPC2 Subunit Homolog

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In *Arabidopsis*, based on the randomly misshapen phenotype of leaf epidermal trichomes, eight genes have been grouped into a '*DISTORTED*' class. Three of the *DIS* genes, *WURM*, *DISTORTED1* and *CROOKED* have been cloned recently and encode the ARP2, ARP3 and ARPC5 subunits respectively, of a conserved actin modulating ARP2/3 complex. Here we identify a fourth gene, *DISTORTED2* as the *Arabidopsis* homolog of the ARPC2 subunit of the ARP2/3 complex. Like other mutants in the complex *dis2* trichomes also display supernumerary, randomly localized cortical actin patches. In addition *dis2* trichomes possess abnormally clustered endoplasmic microtubules near sites of actin aggregation. Since microtubules are strongly implicated in the establishment and maintenance of growth directionality in higher plants our observations of aberrant microtubule clustering in *dis2* trichomes suggests a convincing explanation for the randomly distorted trichome phenotype in *dis* mutants. In addition, the close proximity of microtubule clusters to the arbitrarily dispersed cortical actin patches in the *dis* mutants provides fresh insights into cytoskeletal interactions leading us to suggest that in higher plants microtubule arrangements directed towards the establishment and maintenance of polar growth-directionality are guided by cortical actin behavior and organization.

Keywords: Actin — *Arabidopsis* — ARP2/3 complex — cytoskeleton — *DISTORTED2* — Microtubules.

Abbreviations: ARP2/3 complex, actin-related protein 2/3 complex; MS medium, Murashige and Skoog medium (1962).

Introduction

The unicellular, stellate, 3–5 branched leaf epidermal trichome in *Arabidopsis* is an accepted model cell-type for studies on plant cell morphogenesis. Mutations in eight *Arabidopsis* genes *ALIEN*, *CROOKED*, *DISTORTED1*, *DISTORTED2*, *GNARLED*, *KLUNKER, SPIRRIG* and *WURM* display randomly distorted trichomes (Fig. 1, Hülskamp et al. 1994). The recent molecular characterization of *CROOKED*, *DISTORTED1* and *WURM* has identified a highly conserved actin-modulating ARP2/3 complex in higher plants (Mathur et al. 2003a, Mathur et al. 2003b, Le et al. 2003, Li et al. 2003). Cell biological studies in the respective mutants have suggested a strong role for the actin cytoskeleton in generating and maintaining the regular trichome cell morphology. Despite the detailed molecular and cell biological analysis of the *distorted* mutants a convincing explanation for the most important defect, namely, shape distortion (Fig. 1), has not emerged so far. The trichome distortions are believed to result from arbitrary alterations in localized expansion (Szymanski et al. 1999, Mathur et al. 1999). Intriguingly, changes in growth directionality are usually linked to alterations in the microtubule rather than the actin cytoskeleton (Bibikova et al. 1999, Mathur and Hülskamp 2002, Smith 2003). Though intracellular co-localization and interactions between microtubules and actin microfilaments have been described in tobacco BY-2 cells (Hasezawa et al. 1998, Collings et al. 1998), in cultured *Zinnia* cells undergoing tracheary element differentiation (Kobayashi et al. 1988), in elongating pollen tubes (Lancelle et al. 1991), in epidermal cells of azuki bean epicotyls (Takesue and Shibaoka 1998), and in root cells in maize (Blancaflor 2000), similar studies have not been carried out for trichomes exhibiting distorted shapes. Thus it is unclear whether microtubules play any role in creating the randomly distorted trichome phenotype of *Arabidopsis 'dis'* mutants.

Here we identify *DISTORTED2* as the gene encoding the fourth subunit (ARPC2) of the seven-subunit ARP2/3 complex (Welch 1999, Machesky and Gould 1999) and present cell biological evidence that suggests that defects in the actin cytoskeleton impinge upon the microtubule cytoskeleton to create random alterations in trichome shape.

Results

Molecular characterization of DISTORTED2

Following the earlier identification of *CRK*, *DIS1* and *WRM* as different subunits of the ARP2/3 complex (Mathur et

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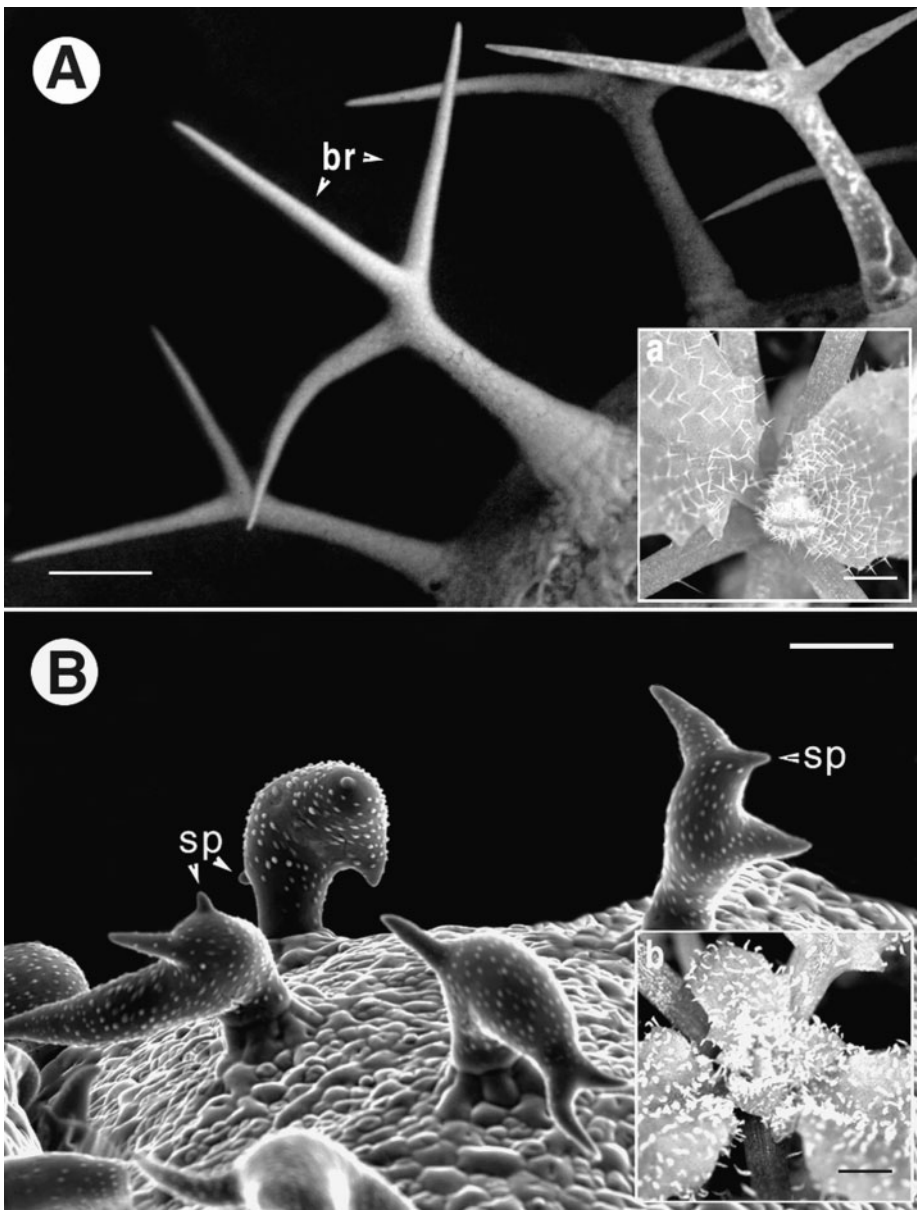

Fig. 1 Comparative morphology of leaf trichomes in WT *Arabidopsis* (ecotype Landsberg erecta) and *distorted 2–1* mutant. WT trichomes (A) are erect, with a well-extended stalk and branches (br). 'a' provides a general view of trichomes on WT leaves. (B) Mutant trichomes are short, randomly distorted and display spike-like (sp) unextended branches. 'b' provides a general view of trichomes on mutant leaves. Note that *dis2-1* trichomes (B) are swollen as compared to WT (A) trichomes. Bars in A, B = 50 μm; a, b = 250 μm.

al. 2003a, Mathur et al. 2003b, Le et al. 2003, Li et al. 2003) cloning the *DIS2* gene utilized a candidate gene approach. The *Arabidopsis* homologs for the ARPC2 and ARPC3 subunits of the ARP2/3 complex lie on chromosome-I (Mathur et al. 2003a) in the general region described for *DIS2* (Feenstra 1978). cDNAs for the At1g60430 (ARPC3; human p21 homolog) and the At1g30825 (ARPC2; human p35 homolog) were PCR amplified and cloned under a trichome cell-specific *GLABRA2* promoter (Szymanski et al. 1998) and the ubiquitously expressed cauliflower mosaic virus (CaMV) 35S promoter in a pCAMBIA 1300 binary vector (accession number AF234296). Twenty-five independent hygromycin-resistant mutant transgenic lines were obtained for pGL2-At1g60430-cDNA and pGL2-At1g30825 each and were screened for rescue of the distorted trichome cell into the wild-type (WT) shape. All 25 plants carrying the At1g30825 transgene displayed WT-trichomes indicating that overexpression of this gene in trichomes could complement the effects of the mutation. The T2 progeny of 18 pGL2-At1g30825 cDNA transgenic lines showed a 3 : 1 Mendelian segregation for the WT : mutant trichome phenotype indicating that these lines had a single insertion. No rescue of the trichome phenotype was observed with the At1g60430 cDNA. Similar results were obtained using the 35S CaMV promoter driving the two genes. In addition a genomic rescue was achieved by bombarding 1-μm gold particles coated with BAC-DNA (t17h7 containing the At1g30825 gene) and pCaMV35S-mGFP into 8- to 10-day-old *dis2* seedlings. WT-like trichomes expressing GFP were

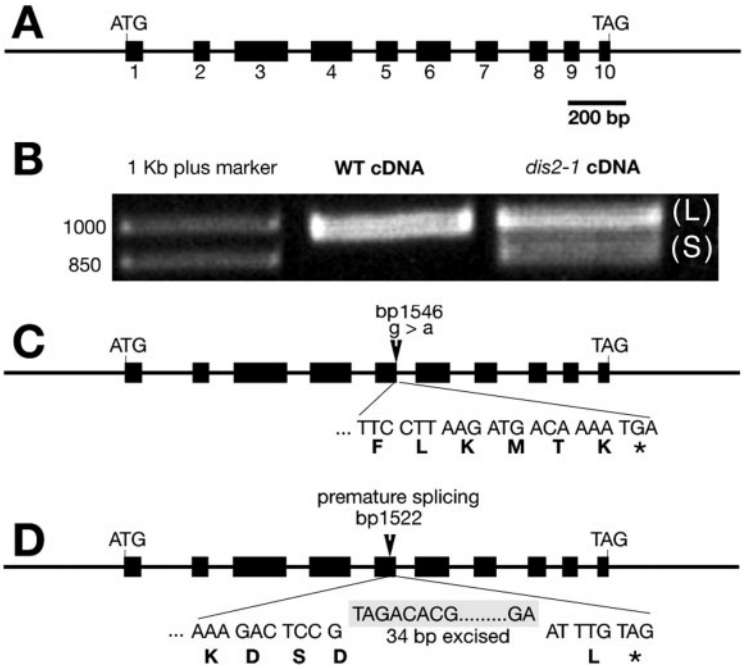

Fig. 2 Molecular characteristics of the *DISTORTED2* gene and the *dis2-1*mutant. (A, B) The *DIS2* (At1g30825) gene contains 10 exons and produces a 957-bp transcript (B). The mutant produces two transcripts; a longer one (L) of 1,032 bp and a shorter one (S) of 923 bp. (C, D) A change (g to a) at position 1,546 relative to the start codon destroys the 'gt' splice donor site at the end of exon 5. This results in either a longer transcript (2B-L) as a fresh ATG is created or generates a premature splice site that is recognized at 1,522 bp (2D) and leads to the excision of 34 bp from the transcript. Both situations introduce premature stop sites.

observed between non-GFP-expressing distorted trichomes and confirmed that the BAC containing genomic *DIS2* fragment rescues the trichome phenotype.

The At1g30825 gene consists of 10 exons (Fig. 2A) with conserved 'ag/gt' nucleotides at the exon-intron splice junctions and produces a 957-bp WT (Landsberg ecotype) transcript (Fig. 2B, WT cDNA). Several alleles have been described for *dis2* mutant but are no longer available in public stocks. However, the sequencing of the At1g30825 gene from *dis2-1* genomic DNA revealed the alteration of a 'g' to 'a' at position 1546 in the genomic clone (according to the MIPS data-base annotation; Fig. 2C). The mutation abolishes the 'gt' splice donor site at the end of exon 5 and results in two different transcript sizes (Fig. 2 B, *dis2-1* cDNA); one that is longer than the WT due to an extension of exon-5 by 75 bp and a second that is shorter than WT by 34 bp. In the first case the replacement of the 'g' at the splice donor site by an 'a' creates a fresh ATG codon (Fig. 2C). The second, smaller transcript, appears to result from premature splicing that takes place 32 bp upstream of the mutation, at position 1522 where the GT nucleotide combination in exon 5 is apparently recognized as a new donor site (Fig. 2D). Both sequence alterations create new stop codons in the mutant open reading frame (Fig. 2C, D).

Though the ARPC2 subunit of the ARP2/3 complex is conserved between different organisms the overall level of sequence identity is fairly low (Fig. 3). A close plant homolog (68% identity/84% similarity) is found in rice (*Oryza sativa*, cultivar japonica) gi-20161526 encoding a 428 amino acid protein. Amongst non-plants its highest sequence similarity of 54% is with the *Dictostelium* ARPC2 ortholog (Fig. 3). The identical residues are, however, totally conserved between the different orthologs. Based on our mutant sequence analysis two potential mutant peptides of 180 or 191 amino acid residues only can be created as compared to the 318 amino acid WT protein (Fig. 3).

The *dis2-1* mutant displays alterations in hypocotyl, cotyledon and root hair cells similar to those observed in *wurm*, *distorted1* and *crooked* mutants. As observed for other ARP2/3 complex mutants (Mathur et al. 2003a, Mathur et al. 2003b), hypocotyl and petiole cells of *dis2-1* when challenged into rapid growth also de-link from each other and undergo non-coordinated growth. Given these similar phenotypes of all the ARP2/3 mutants, including *dis2-1*, here we have concentrated on trying to figure out the cause behind the random distortion of the growing trichome cell. Rescue of the *dis2-1* trichome phenotype (Fig. 1) to WT structure followed by identification of the mutation in the gene confirmed the cloning of *DIS2* and by virtue of it being a subunit of the actin-modulating ARP2/3 complex established its link to the actin cytoskeleton. Our focus now shifted to a cell biological analysis of the mutant.

dis2-1 mutant displays an aberrant actin cytoskeleton

A detailed description for the aberrant F-actin cytoskeleton in *dis2-1* mutant trichomes has been published (Schwab et al. 2003) and matches that described for other mutants in subunits of the ARP2/3 complex (Mathur et al. 2003a, Mathur et al. 2003b). Briefly, actin cytoskeleton defects accrue during trichome cell expansion and manifest maximally in extending trichome branches. The F-actin instead of being structured into fine, longitudinally extended cables (as in WT trichomes, Fig. 4A, B) is typically organized into thick, cross-linked, short bundles (Fig. 4C, D). This aberrant F-actin organization

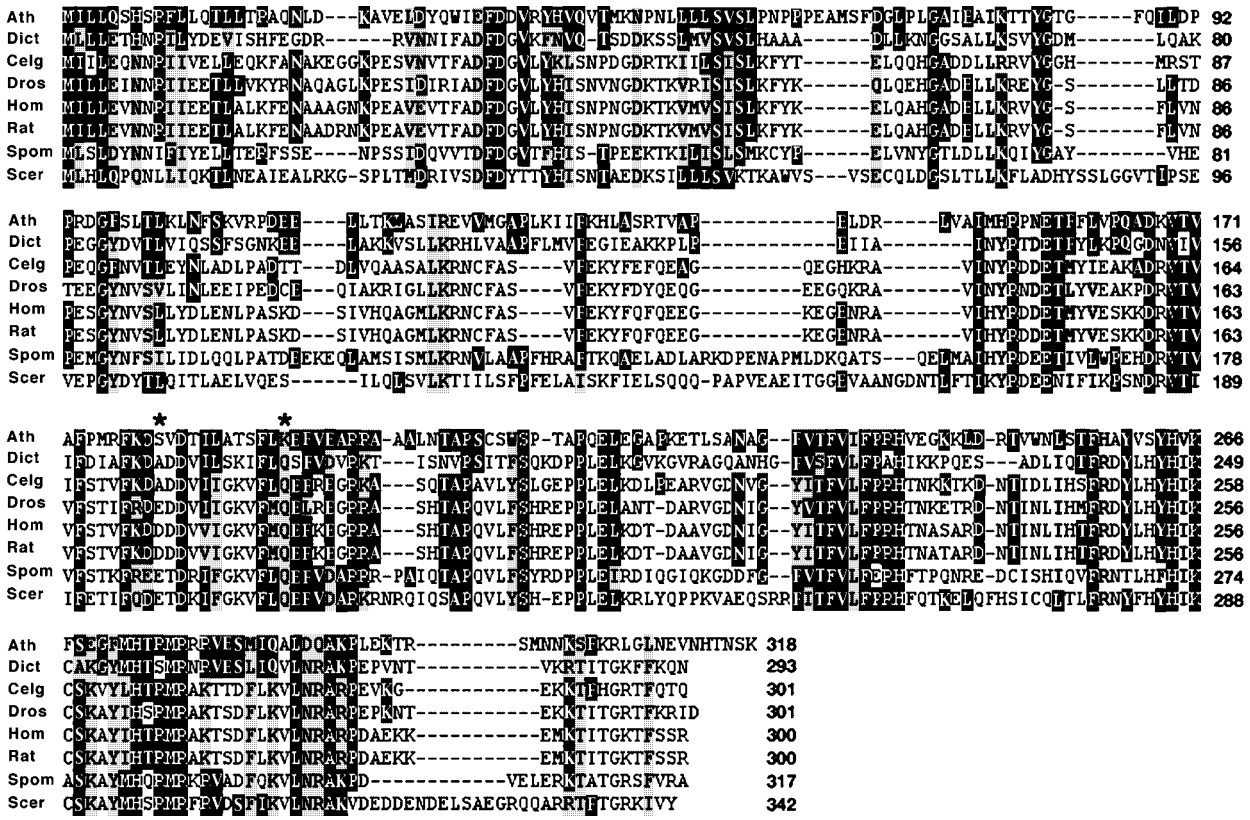

Fig. 3 The predicted amino-acid sequence for *DISTORTED2* aligned against protein sequences of the ARPC2 subunit of ARP2/3 complex from other organisms. (Ath, *A. thaliana* gi-28950897-At1g30825; Dict, *D. discoideum*: gi-10719881; % identity 33/% similarity 54; Celg, *C. elegans* gi-25144430;28/48; Dros, *D. melanogaster* gi-27923962;26/48; Hom, *H. sapiens* AF006085;26/46; Rat, *Rattus* sps gi-27684953; 26/46; Spom, *S. pombe* gi-3121760 26/46; Scer, *S. cervevisiae* gi-1730675; 28/46). Black areas denote identical amino acids while grey-shaded residues are similar. Asterisks placed above the *A. thaliana* sequence indicate the predicted protein truncation sites in the mutant.

increases the number of dense actin patches at the cell cortex (Fig. 4 C, d), and creates regional pockets of dense and fine actin (Fig. 4D, d). Though dense actin-patches and fine F-actin regions are observed in WT trichomes too at the junction and tips of branches (Mathur et al. 2003b), the number of dense actin patches and their location is characteristically randomized in *dis* trichomes. As with other ARP2/3 complex mutants (Mathur et al. 2003a, Mathur et al. 2003b) we also assessed the state of vacuoles, motility of Golgi-bodies and peroxisomes and found these parameters to be similarly altered locally (data not shown). However, these data did not directly reveal the cause for random changes in growth directionality and led us to look at the microtubule cytoskeleton in the mutant.

The microtubule cytoskeleton is aberrant in dis2-1 trichomes

Earlier observations on trichomes from actin drug-treated plants (Mathur and Chua 2000) and different *dis* mutants (Schwab et al. 2003) have suggested that cortical microtubules maintain their flexibility and usual arrays (WT array Fig. 4E) despite major alterations in the cell morphology. Consistent with these earlier observations, we found that cortical microtubules followed the general contours of the distorted cell in *dis2-1* trichomes (Fig. 4F) and concluded that cortical microtubules simply mold themselves to the altered shape. However, in the earlier study by Schwab et al. (2003) the focus had been on cortical microtubules in mature trichomes and specific attention had not been paid to sub-cortical (endoplasmic) microtubules located 7–15 μm below the outermost optically sectioned layer. Since endoplasmic microtubules (EMTs) have been shown to play a major role in reorienting the growth in *Arabidopsis* trichomes (Mathur and Chua 2000) here we focused on their organization in short, still expanding *dis2-1* trichomes using the *GFP-MAP4* fusion construct (Marc et al. 1998).

In WT expanding trichome cells EMTs are prominent at the tubular stage where they preferentially localize to the growing tip. As the cell bifurcates EMTs can be observed at the region of bifurcation and the newly defined tips of branches. With subsequent trichome-stalk and branch expansion EMTs are pushed against the expanding cell wall by an enlarging central vacuole and it became increasingly difficult to distinguish them from cortical microtubule arrays. EMT clusters usually persist at the bifurcation point and the sub-apical region in the tips of trichome branches even in mature WT trichomes (Fig.

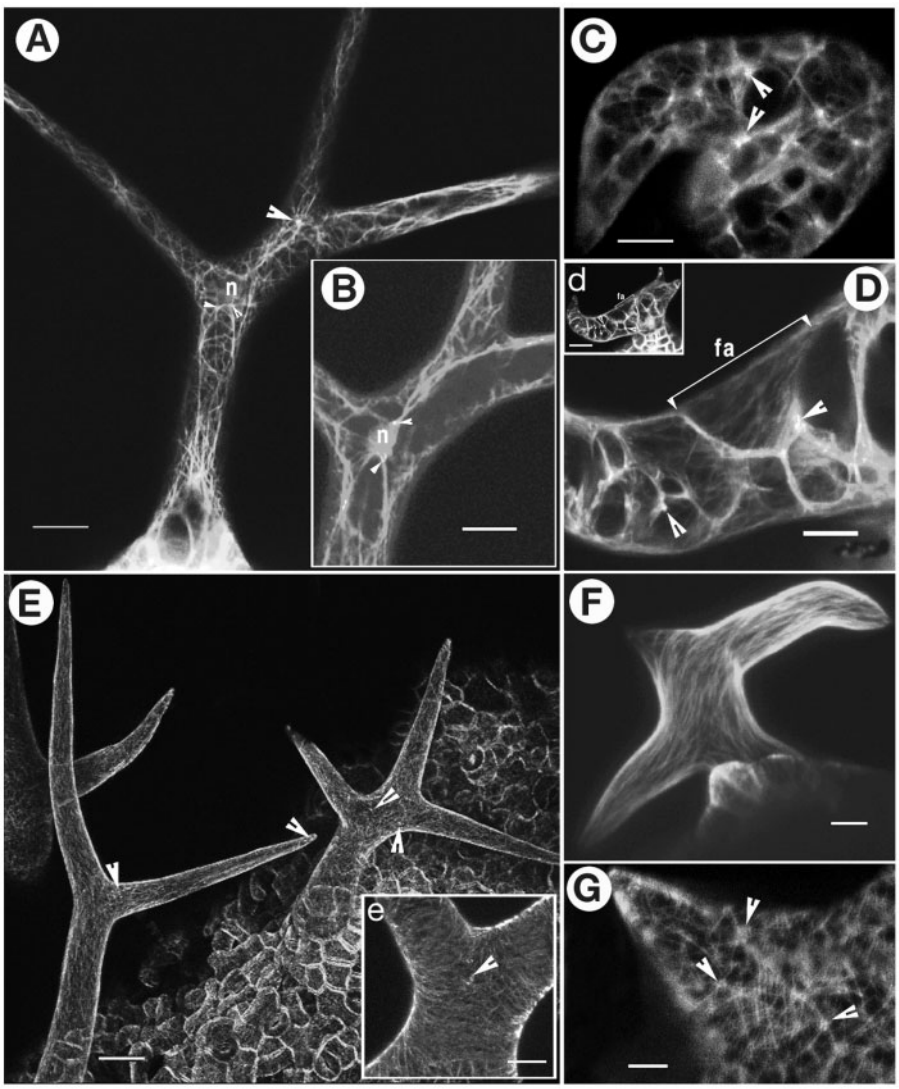

Fig. 4 Actin and microtubule organization in *Arabidopsis* WT (Landsberg erecta) and mutant trichomes. (A) Cortical actin organization in an expanding WT trichome displaying characteristic longitudinally stretched F- (filamentous) actin stands and few actin patches (arrow-heads). 'n' marks the nucleus. (B) A single optical section of trichome in 'A' shows actin-patches (arrowheads) in continuity with F-actin strands connecting to the nucleus 'n'. (C) Cortical actin organization in a *dis2-1* trichome at a comparable stage to 'A' displaying more bundled actin filaments and pronounced, randomly placed actin patches (arrowheads). 'n' denotes the nucleus. (D) Portion of *dis2* trichome (d) showing regional difference in actin organization. Dense actin patches (arrowheads) are interspersed between regions with fine F-actin (fa). (E) The microtubule cytoskeleton in still expanding WT trichomes. A few dense endoplasmic microtubular foci are observed near branch points and the tips of branches (arrowheads). (e) Shows a magnified view of the branching region in 'E' where microtubule aggregates appear as small bright dots (arrowheads). (F) A stack of five optical sections (1-μm apart) shows that the cortical microtubule organization in a *dis2-1* trichome follows the general contours of the distorted cell. (G) Optical sections taken between 8 and 16 μm below the outermost optically section-able region of the trichome cell shown in 'F' shows distinct endoplasmic microtubule aggregates (arrowheads). Compare with WT (e). Bars in A, d, E = 20 μm; B, C, D, F = 10 μm; e, G = 5 μm.

4E, e, Mathur and Chua 2000). By contrast expanding mutant trichomes display numerous randomly localized EMT clusters varying in diameter from 3 to 5 μm (Fig. 4G, 5). These large inter-connected clusters persist in mature trichomes giving the microtubule cytoskeleton in *dis2-1* trichomes a general patchy appearance in strong contrast to that seen in WT trichomes (compare Fig. 4e with 4G). Similar microtubule aggregates were observed in distorted trichomes of the *crooked* and *wurm* mutants, encoding for the ARPC5 and ARP2 subunits, respectively, of the ARP2/3 complex (data not shown).

EMTs have been shown to be more labile than cortical microtubules and depolymerize readily upon treatment with very low concentrations of microtubule depolymerizing drugs (Sieberer et al. 2002). Table 1 shows that as compared to the WT, microtubule clusters in *dis2-1* trichomes take a longer time to break down upon drug induced de-polymerization suggesting that they comprise of more stabilized microtubules.

Actin and microtubule patches in dis2 trichomes concur spatially

The distribution pattern of EMT aggregates in *dis2-1* trichomes was strongly reminiscent of the cortical actin cytoskeleton observed in these trichomes and led us to investigate whether a positional correlation existed between the two elements. Using transient expression of CFP-mTalin (labels F-actin in cyan color) and YFP-MAP4 (labels microtubules in yellow color) we found that the pattern of EMT clusters was similar to that of actin aggregates in the mutant trichomes (Fig. 5A–C). Both actin patches (Fig. 5B) and EMTs (Fig. 5A), displayed varying degree of vertical as well as lateral movement and though they both appeared in the same intracellular locality (Fig. 5C), the degree of their actual co-localization within a particular patch could not be quantitatively estimated due to the transient nature of the assay with differing levels of gene expression. A more global and detailed analysis aimed at establishing the degree of transient co-localization between the two

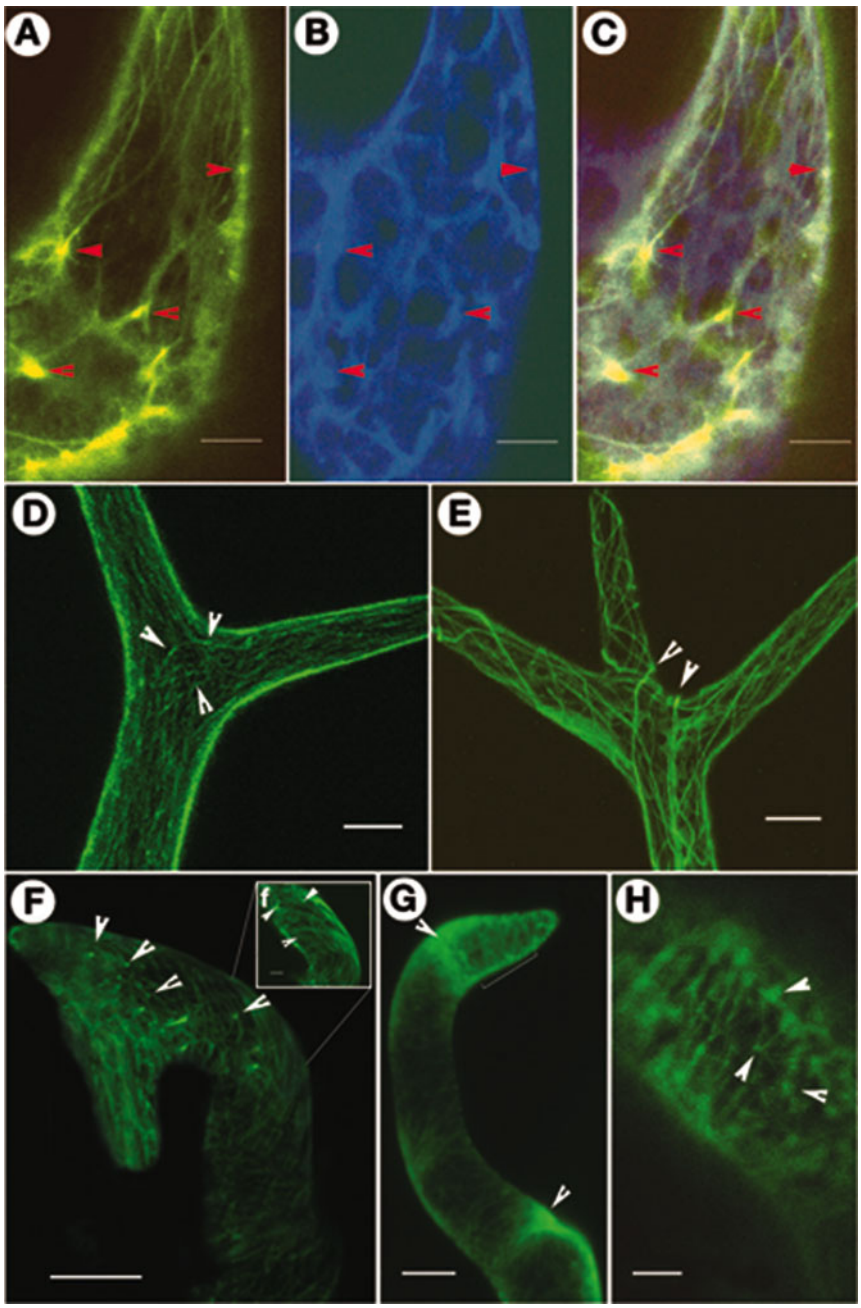

Fig. 5 A–C show a compressed stack of eight optical sections (1 μm apart) shows portion of a mutant trichome transiently co-expressing YFP-MAP4 and CFP-mTalin constructs that allow simultaneous visualization of microtubules (in yellow) and F-actin (in Cyan). Panels D–G show the effect of actin-polymerization inhibitor latrunculin-B on microtubule cytoskeleton in trichomes of GFP-MAP4 plants. (A) Dense microtubule aggregates (red arrowheads) and elongated microtubules connecting them are visualized in the YFP-channel (excitation at 488 nm/emission collected 424–440 nm). (B) The same cell as in 'A' visualized for CFP-labeled F-actin organization displays dense, interconnected actin bundles (excitation at 458 nm/emission collected 470–480 nm). (C) A merged image of A, B shows positional proximity of YFP-labeled microtubule foci with CFP-labeled actin patches (red arrowheads). (D) An untreated WT (GFP-MAP4-transgenic) trichome maintained in MS liquid medium for 2 h exhibits characteristic small microtubule aggregates in the branch region (arrowheads). (E) A trichome from GFP-MAP4 transgenic plant 2 h after treatment with 100 nM lat-B displays widespread bundling of microtubules. (F) A stack of 12 confocal sections shows numerous microtubule aggregates in the branch regions (arrowheads) in an expanding trichome treated with lat-B for 2 h. 'f' shows a magnified view of the bracketed region to show oblique cortical microtubule arrays which follow the curve of the trichome cell as well as the denser endoplasmic microtubules (arrowhead). (G) An already extended trichome branch exposed to 100 nM lat-B for 12 h exhibits areas of increased fluorescence (arrowheads) indicative of microtubule aggregation. (H) A magnified view of endoplasmic microtubules in the region indicated by a white-line in 'G' shows bundled and aggregated microtubules (arrowheads). Size bars in A, B, C, f, H = 10 μm; D, E = 50 μm; F = 80 μm; G = 20 μm.

elements in the *X*, *Y*, *Z* axes over time is in progress in transgenic plants stably expressing the two markers and shall be reported separately. The present observations on the increased number and localization of EMTs in the mutant trichomes strongly suggest that EMTs cluster exist in the same intracellular regions as the aberrant cortical actin patches. Comparable dense cytoskeletal patches are not observed in WT but EMT presence has been reported near trichome branch points (Mathur and Chua 2000), which are also areas of dense actin aggregation (Mathur et al. 2003b). Though the mechanism for this coincidental localization is presently unclear, further evidence supporting these observations comes from actin drug-based experiments.

Actin-polymerization inhibitors reorganize endoplasmic microtubules

Treatment of WT seedlings with either 1 μM of cytochalasin-D (cyto-D) or latrunculin-B (lat-b) for 6–12 h is sufficient to completely inhibit actin polymerization and readily induces distortions in WT trichomes within 24 h (Mathur et al. 1999). Alternatively low concentrations (10–100 nM) of lat-B have been shown to induce several changes in actin organization

Table 1 Characteristics of endoplasmic microtubule organization in the 6–12 μm region from the outermost surface in expanding [a] WT (Landsberg erecta) and *distorted 2–1* mutant trichomes

	WT	*distorted 2–1*
Number of foci	7±3	45±18
Position of cluster	Branch point/Branch tip	Random distribution
Size of cluster (*x*-axis)	2–5 μm	5–12 μm
Drug stability of cluster [b]	10–15 min	35–>45 min

All observations are based on 25 individual trichomes each from WT and mutant.

[a] Trichomes without papillate surface decorations on their branches were considered immature and still expanding.

[b] Leaves each of WT (GFP-MAP4 transgenic plants) and *dis2-1* carrying the GFP-MAP4 transgene) were dipped in 2. 5 μM Oryzalin and visualized at 5-min intervals for a total of 45 min.

including an initial bundling of F-actin filaments prior to their final disappearance due to non-polymerization (Blancaflor 2000, Mathur et al. 2003a). We assessed the effects of 100 nM lat-B treatment on microtubule cytoskeleton between 6 and 12 h (Fig. 5D–H). *Arabidopsis* plants carrying the GFP-MAP4 transgene (Mathur and Chua 2000), were used for this purpose and alterations in EMT organization became apparent in lat-B-treated cells within 2 h as microtubules became more bundled (compare Fig. 5D–E, F, f). Since microtubule bundling could have been caused by general lat-B-induced toxicity in the cell, continued cytoplasmic streaming was taken as an indicator of non-toxicity and cell health. Trichome cells that did not exhibit cytoplasmic streaming were not considered for observation. The 2–6 h duration under lat-B treatment coincides with that observed for increased F-actin bundling prior to cessation of active actin polymerization in trichome cells (Mathur et al. 2003a). Maximum EMT bundling in expanding trichomes was noticed in the branch-bifurcation region of the trichome (Fig. 5F), which is also a known actin-rich region (Mathur et al. 2003b). Trichomes treated with lat-B for 12 h or more (up to 24 h) showed the cytoplasm segregated into small pockets. These cytoplasm-dense regions exhibited a stronger fluorescence than surrounding regions (Fig. 5G). While cortical microtubules were clearly organized in oblique arrays in lat-B-treated cells (Fig. 5F, f), EMTs in such cells were arranged into random aggregates nearly indistinguishable from those observed in *dis2-1* trichomes (Fig. 5H compared to Fig. 4G). Untreated trichome cells did not exhibit aggregation of endoplasmic microtubules. Since lat-B is a well-known and potent inhibitor of actin polymerization we concluded that changes in actin polymerization dynamics could profoundly affect endoplasmic microtubule organization and behavior.

Discussion

Three genes belonging to the *DISTORTED* class have already been identified as subunits of the ARP2/3 complex (Mathur et al. 2003a, Mathur et al. 2003b, Le et al. 2003, Li et al. 2003). The results presented here identify *DISTORTED2* as the fourth subunit of the ARP2/3 complex and thus serve to re-emphasize the importance of the complex in modulating the actin cytoskeleton during trichome cell expansion. Despite providing a clear molecular basis for the changes observed in the actin cytoskeleton in the three previously characterized ARP2/3 mutants the observations did not provide sufficient explanation for the distorted trichome phenotype, which apparently results from random localized changes in growth directionality. As changes in growth directionality are usually associated with alterations in the microtubule cytoskeleton (Bibikova et al. 1999, Mathur and Chua 2000, Mathur and Hülskamp 2002, Ketelaar et al. 2003) our finding that the microtubule cytoskeleton is also aberrant in the *dis2-1* mutant assumes major significance and allows us to discuss the causes for polar growth directionality fixation in plant cell in a new light.

Localized growth is an internal response to an external cue

The growth of a plant cell in a particular direction reflects decisions made in the cell's interior. The deposition of external cell-wall building components and the typical alignment of cellulose microfibrils are thus manifestations of internal decisions and should not be considered as external impositions that can determine growth directionality in a developing cell. This is why, despite well-documented observations that microtubules are involved in establishing and maintaining growth directionality in plant cells (Bibikova et al. 1999, Mathur and Chua 2000, Mathur and Hülskamp 2002), an earlier study (Schwab et al. 2003), that reported normal configurations of cortical microtubule arrays in distorted trichomes, could not conceive of a convincing role for microtubule involvement in cell shape distortion. However, as shown here, the internal EMT organization is considerably altered in *dis2-1* mutants even though the cortical microtubule array appears normal (Fig. 4, 5). EMTs have been clearly implicated in polarized growth and are known to be very labile and active in living cells (Bibikova et al. 1999, Mathur and Chua 2000, Sieberer et al. 2002, Ketelaar et al. 2003). How EMTs are directed towards specific cortical locations to perform their direction-finding and polarity fixation functions during polarized growth of plant cells is unclear.

Contextual studies in other organisms have clearly shown that cytoplasmic microtubules search for cortically located cues defined by the actin cytoskeleton (Hayles and Nurse 2001, Rodriguez et al. 2003). Since the molecular lesions in the four distorted mutants described hitherto relate to ARP2/3 complex-mediated cortical actin-cytoskeleton organization our observations suggest that a similar actin cue-based mechanism may be operating in higher plant cells too.

ARP2/3 complex modulation of actin loosens the cortical cytoskeletal mesh

Before we can discuss the possible recruitment of microtubules by actin-cues we need to understand the intracellular basis for such an action. Studies in different cells have shown that the earliest indication of a change in growth directionality in a cell is the appearance of a small bulge on the cell surface (Baluska et al. 2000, Mathur and Chua 2000). This bulge appears to arise from a localized weakening of the cell cortex (Baluska et al. 2000). Such a weakening and bulge formation has been experimentally invoked through drug-induced increase in actin cytoskeleton instability (Ketelaar et al. 2003). A similar requirement for dynamic cortical actin has also been reported for polarization and rhizoid-pole establishment in fucoid zygotes (Alessa and Kropf 1999, Hable et al. 2003). A link between fine F-actin formation and cell morphology is also suggested through observations of atypically swollen cells in transgenic plants overexpressing a constitutively active form of AtROP2 (Fu et al. 2002). In vivo, using the *crooked* phenotype as a discussion point we had proposed that loosening of the actin mesh that delineates a cellular site for localized growth is accomplished by ARP2/3 complex-mediated increase in actin-polymerizing activity (Mathur et al. 2003b). Further, depending upon the F-actin mesh size the cortical F-actin may act as a barrier for the movement of vesicles as well as for the targeted deposition of growth materials by them. However, the actin mesh-based model did not elaborate how a weakening of the actin mesh, once initiated, could be channeled to produce localized expansion. Nor did it explain how the directionality of localized expansion could be maintained in a growing cell. Our observations on *dis2-1* now allow us to expand further on this theme.

Reinforcement of the cortical mesh and fixation of growth-directionality requires microtubules

It is plausible that the ARP2/3 complex-mediated increase in actin-polymerization dynamics can continue indefinitely as long as upstream regulatory signals are active. Conceivably, a broadened weakening of the cell cortex would spread the growth process over a larger area by allowing non-targeted delivery of growth material by vesicles. This would lead to global expansion of a cell. However, if cortical weakening could be limited to a small region of the cell and not allowed to extend uncontrollably the process of growth would become targeted to that small area only. Since the actin cytoskeleton, implicated in the weakening process may be unable to restrict its own activity, another fibrous, rapidly polymerizing cytoskeletal component may be required at the weakened site. Ideally, the same molecules that regulate the actin cytoskeleton should not regulate this protein. Moreover, in addition to providing structural support the second cytoskeletal element may also bring along membrane vesicles for immediate replenishment of the weakened site. We propose that endoplasmic microtubules carry out these vital functions by responding to the weakening of the cortical actin cytoskeleton and establishing a 'reinforcing patch' around the site. A recent observation involving the microtubule plus-end-binding protein AtEB1b (Mathur et al. 2003c), suggests that growing microtubule ends can pull along and reconfigure endo-membranes and may thus provide membrane replenishment too.

Strong support for the microtubule involvement in restricting growth to a region comes from the fact that loss of microtubule activity invariably leads to non-polarized, isotropically expanding cells (Mathur and Chua 2000, Mathur and Hülskamp 2002). Moreover, observations on tip growing root hairs and pollen tubes have revealed that microtubules are conspicuously absent from the apolar bulge that is produced in cells prior to their embarking on polarized growth (Baluska et al. 2000, Carol and Dolan 2002). Their subsequent appearance in the bulged domain usually signifies the fixation of growth directionality and leads to true polarized growth (Baluska et al. 2000, Carol and Dolan 2002). Polar growth fixation obviously requires microtubule presence at a cortical site that has become weakened due to alterations in actin organization.

A cell biological explanation for the distorted trichome phenotype

Based on the molecular lesions the primary cytoskeletal defect in *crk*, *dis1*, *dis2*, and *wrm* trichomes is in their cortical actin organization. Whereas WT trichomes exhibit maximum actin aggregation near trichome branch points and the tips of branches (Mathur et al. 2003b), extensive actin aggregates occur in random locations in the mutant trichomes. The same regions where actin aggregation is seen in WT trichomes are also areas where aster like, more stable microtubule formations have been described in WT trichomes (Mathur and Chua 2000). Moreover, a transient stabilization of endoplasmic microtubules is sufficient to alter growth directionality in expanding trichomes. The coincident localization of actin patches and microtubule foci in cellular domains where growth directionality appears to change suggests an intimate spatio-temporal cooperation and interdependence between the two cytoskeletons. Studies in other plant cell types using immunocytochemical methods have also observed localized proximity between actin and microtubule elements (Collings and Allen 2000 and references therein). The random localization of cortical actin patches in the hitherto described *distorted* mutants appears to be reflected in placement of endoplasmic microtubules and suggests that at these sites of coincident localization the cell

may exercise alterations in growth directionality. The increase in number of such coincident spots as compared to WT trichomes strongly suggests that such localized changes in growth may occur many times during mutant trichome development. The cumulative result of these numerous changes in growth directionality is a randomly distorted trichome cell.

Conclusions

The cloning of *DIS2* identifies a new functional component of the ARP2/3 complex in higher plants. More importantly our results suggest that ARP2/3 complex-mediated cortical actin organization plays a very important role in growth site selection and defines cortical regions for recruiting endoplasmic microtubules. Subsequent fixation of polarity and continued growth in a particular direction is accomplished only when both actin and microtubule cytoskeletons are in concurrence. The precise mechanics governing this actin–microtubule interaction as well as the global veracity of our observations are presently unclear and shall be the subject of further studies.

Materials and Methods

Molecular methods and plant transformation

For identification of the *DIS2* gene PCR primers JM217F/JM218R were used for cloning a ca. 900-bp genomic fragment of At1g60430 WT gene from Landsberg erecta genomic DNA. For the Atg1g30825 gene the primers used were JMF406/JM407R (genomic clone ca. 1,900 bp) and JM408F/JM409R (cDNA clone ca. 960 bp). Standard molecular protocols were according to Sambrook and Russell (2001). The respective genomic and cDNA fragments were sub-cloned into a pGEM-T-easy vector (Promega), sequenced on an ABI-prism sequencer and finally cloned into a pCAMBIA 1300 binary vector (accession number AF234296) under the control of CaMV p35S, and a trichome specific *GLABRA2* promoter (Szymanski et al. 1998) for plant transformation. Different transgenes were introduced into the *dis2-1* mutant (Landsberg erecta background; (Feenstra 1978) by genetic crossing or *Agrobacterium tumefaciens* (strain GV3101)-mediated floral dip transformation (Clough and Bent 1998). For achieving a genomic rescue through transient expression the BAC DNA (t17h7 containing the At1g30825 gene) was mixed with a green fluorescent protein (mGFP5), precipitated around 1 μm diameter gold particles (BioRad, Hercules, CA, U.S.A.) following the manufacturer's directions, loaded onto carrier membranes and shot into 7- to 10-day-old seedlings at 1,100 psi Helium pressure under a vacuum of 25 inches of Hg, using a PDS-1000/Helium driven Biolistic delivery apparatus (BioRad). Following complementation of the trichome cell phenotype JMF406/JM407R primers for the Atg1g30825 gene were used on *dis2-1* mutant DNA to extract the genomic and cDNA clones and subjected to sequence analysis.

Further details of primers and PCR conditions are available on request. DNA and protein sequence homology searches were carried out using the respective BLAST programs (Altschul et al. 1997). Amino-acid alignments were carried out using the ClustalW multiple sequence alignment algorithms (http://www.ch.embnet.org/).

Cell biology methods and microscopy

The GFP-mTalin and GFP-MAP4 constructs have already been described (Kost et al. 1998, Marc et al. 1998) and were introduced in *dis2* mutants by crossing with WT plants carrying the construct. CFP-mTalin and YFP-MAP4 were constructed by replacing the GFP in respective constructs with CFP/YFP. These constructs were combined in equal amounts and expressed transiently using gold-particle bombardment as described above. Phenotypic and cell-biological characterization was carried out in-vitro on MS (Murashige and Skoog 1962) medium plates (MS salts supplemented with 3% sucrose, 1% Phytagar; 23°C temperature, 16 h daylight/8 h dark) and on soil grown plants (ambient light/dark conditions). Seven-day-old intact seedlings with well-exposed trichomes on the first pair of leaves were treated with 100 nM of the actin inhibitor lat-B (Molecular Probes) by dipping the seedlings in MS medium supplemented with the drug. For subsequent treatment up to 48 h the lat-B-dipped seedlings were transferred onto a 1 cm wide moist filter paper wick that dipped into the MS–lat B medium in a 5 cm plastic Petri dish kept upright.

Scanning electron microscopy of *dis2-1* trichomes was carried out as described (Mathur et al. 1999). Light and fluorescence microscopy was carried out on a LEICA-DMRE microscope equipped with a high resolution KY-F70 3-CCD JVC camera and a frame grabbing DISKUS software (DISKUS, Technisches Büro, Königswinter). For confocal laser scanning microscopy WT and *dis2-1* plants carrying GFP-mTalin and GFP-MAP4 transgenes were grown on MS medium plates as described (Mathur et al. 1999), mounted in water and observed using a 40× water-immersion lens. The descriptions provided here were obtained on 35 (each observation) randomly selected WT and transgenic plants. A spectro-photometric confocal laser scanning microscope (Leica TCS-SP2 AOBS) was used for visualizing EGFP (excitation maximum 490/emission maximum 510 nm) as described (Mathur et al. 2003a). Images were sized, processed for brightness/contrast and CMYK alterations using the Adobe Photoshop 6.0 software.

Note added in proof

Since the submission of this article the cloning of DIS2 has been independently reported by El-Assal et al. *Plant J.* 38: 526–538.

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