CLASP localizes in two discrete patterns on cortical microtubules and is required for cell morphogenesis and cell division in Arabidopsis

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Accepted 20 November 2007
Journal of Cell Science 120, 4416-4425 Published by The Company of Biologists 2007
doi:10.1242/jcs.024950

Summary
In animals and yeast, CLASP proteins are microtubule plus-end tracking proteins (+TIPs) involved in the regulation of microtubule plus-end dynamics and stabilization. Here we show that mutations in the Arabidopsis CLASP homolog result in various plant growth reductions, cell form defects and reduced mitotic activity. Analysis of Arabidopsis plants that carry a YFP:AtCLASP fusion construct regulated by the AtCLASP native promoter showed similarities to the described localization of the animal CLASP proteins, but also prominent differences including punctate and preferential localization along cortical microtubules. Colocalization studies of YFP:AtCLASP and CFP:EB1b also showed that AtCLASP is enriched at the plus ends of microtubules where it localizes behind the AtEB1b protein. Moreover, AtCLASP overexpression causes abnormal cortical microtubule bundling and array organization. Cortical microtubule arrays have evolved to be prominent in plants, and our findings suggest that plant CLASP proteins may have adopted specific functions in regulating cortical microtubule properties and cell growth.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/24/4416/DC1

Key words: CLASP, EB1, Microtubule, Arabidopsis

Introduction
Microtubule (MT) growth is governed by the incorporation of α/β-tubulin heterodimers at plus ends, whereas MT minus ends are usually protected from dissociation by anchorage to cellular structures. MT plus ends oscillate between growth and shrinkage – a behavior termed dynamic instability. A number of developmental and positional stimuli affect dynamic instability (Kirschner and Mitchison, 1986). Among important regulators of this process are the microtubule (MT) plus-end tracking proteins (+TIPs) which bind to the dynamic plus ends and modulate MT assembly, disassembly, and binding to cellular structures (Akhmanova and Hoogenraad, 2005; Galjart, 2005). The emerging picture is that +TIP proteins, including dynactin, APC, spectrolamins, Lis1, EB1, CLIP170 and CLASP, are functionally linked and may form complexes at MT plus ends (Schuyler and Pellman, 2001; Coquelle et al., 2002; Carvalho et al., 2003; Goodson et al., 2003; Howard and Hyman, 2003; Lansbergen et al., 2004; Akhmanova and Hoogenraad, 2005; Mimori-Kiyosue et al., 2005).

CLASPs (CLIP170-associated proteins) are evolutionary conserved proteins (Akhmanova et al., 2001) and are essential in animal and fungal cells both during mitosis and interphase (Akhmanova et al., 2001; Maiato et al., 2003; Mathe et al., 2003; Galjart, 2005; Wittmann and Waterman-Storer, 2005). Mammalian CLASPs behave differently to many other +TIP proteins because they do not bind to all available MT plus ends, but instead bind plus ends selectively in a spatially defined manner (Akhmanova et al., 2001). In motile epidermal cells, a signal cascade involving glycogen synthase kinase 3β (GSK3β) regulates the association of CLASP with MT lattices in the leading edge lamella or with MT plus ends in the cell body (Wittmann and Waterman-Storer, 2005).

Microtubule-associated proteins (MAPs) in plants have been shown to play essential roles in organizing MT arrays and spatially controlling cell growth (Sedbrook, 2004; Hamada, 2007). Several +TIP proteins have been identified in the fully sequenced genome of Arabidopsis thaliana including EB1, CLASP and LIS1 (Bisgrove et al., 2004; Chuong et al., 2004), whereas other +TIP homologs such as CLIP170, cytoplasmic dynein and dynactin are conspicuously absent. For EB1, it has been shown that two of the three Arabidopsis homologs behave like +TIPs in that GFP-tagged versions bind to the plus ends of MTs (Chan et al., 2003; Mathur et al., 2003; Dixit et al., 2006). When overexpressed, EB1 proteins were found to localize at MT initiation sites in a specific cell line and at endomembranes (Chan et al., 2003; Mathur et al., 2003; Chan et al., 2005). However, when expressed at lower, and presumably more physiological levels, under the control of the endogenous promoter (Chan et al., 2005; Dixit et al., 2006), EB1 protein was not observed in these locations, highlighting
the importance of using appropriate regulatory sequences for localization studies of MAPs.

In addition to the +TIPs already known from animals, plant specific MT plus-end binding proteins have also been identified. For example, the Arabidopsis SPIRAL1 (SPR1) gene was genetically identified as being required for proper growth directionality of various tissues, as the corresponding mutants show twisted organs and abnormal surface-dependent directional growth responses (Nakajima et al., 2004; Sedbrook et al., 2004). SPR1 fusions with GFP revealed localization to MTs in all arrays, with preferential plus-end localization in the cortical array (Sedbrook et al., 2004). Another plant-specific +TIP protein is ATK5, which belongs to the Arabidopsis kinesin-14A superfamily and is thought to have similar functions as the dyneins in animal cells (Ambrose et al., 2005).

In this work, we have analyzed the single-copy gene AtCLASP in Arabidopsis. We show that AtCLASP is required for cell morphogenesis and cell division. We engineered Arabidopsis plants that stably coexpressed YFP:AtCLASP and CFP:AtEB1b and found that AtCLASP has a +TIP behavior and maps adjacent and distal to AtEB1b. To achieve proper expression levels of the YFP:AtCLASP fusion protein, we utilized the native AtCLASP promoter and observed AtCLASP localization on all four MT arrays both in interphase and mitotic cells. Under regulation of its native promoter, AtCLASP complemented clasp mutant phenotypes and showed more prominent localization to cortical MT arrays than to spindle or phragmoplast arrays. Furthermore, in addition to dynamic localization to plus ends it displayed a prominent punctate localization pattern along the MT lattices of cortical MTs, defining a second domain for CLASP localization, which, similarly to plus tip localization of CLASP in animal cells, is apparently selective for a subset of MTs in the cell.

Results
Molecular characterization of the AtCLASP

The fully sequenced Arabidopsis genome revealed only one CLASP gene in BLAST searches – the At2g20190 locus. A comparison of the genomic sequence and a full-length cDNA revealed that AtCLASP has 20 exons and 19 introns (Fig. 1A). The deduced protein of 1439 amino acids shows 19% and 15.6% amino acid sequence identity to the human CLASP1 and CLASP2, respectively, and sequence identities higher than 50% to plant homologs (see dendrogram in supplementary material Fig. S1). From the various domains identified in animal CLASP proteins, plant CLASP homologs show highest similarity in the 167 amino acid long C-terminal Mast-C domain. Within this domain sequence, identity is 25.7% between human and Arabidopsis proteins. In animals, the Mast-C domain mediates CLASP binding to CLIP170 protein, the Golgi and the cell cortex (Galjart, 2005). In addition, several Huntington, elongation factor 3, PR65/A, TOR (HEAT) domains were found scattered over the Arabidopsis CLASP protein. HEAT domains are thought to form helical structures and to mediate protein-protein interactions.

In the Genvestigator database (Zimmermann et al., 2004), AtCLASP is expressed at a low level in all tissues. For a more detailed analysis we used a CLASP::GUS fusion construct containing the regulatory sequences sufficient for full rescue of the mutant phenotype (see below). GUS staining was found in all organs with highest expression in young developing tissues (Fig. 1C).

Identification of Arabidopsis clasp mutants

Three Arabidopsis clasp alleles were identified from the SALK T-DNA collection (Alonso et al., 2003): clasp-1 (SALK_120061), clasp-2 (SALK_083034) and clasp-3 (SALK_049782). The clasp-1 allele carries a T-DNA insertion in exon 13, the clasp-2 allele an insertion in the intron located between exon 11 and 12, and the clasp-3 allele an insertion in the intron located between exons 14 and 15 (Fig. 1A). These positions were verified by amplification and sequencing of border sequences. RT-PCR analysis of the CLASP transcripts in the mutants, using primers located downstream of the T-DNA insertion sites revealed little full-length transcript in clasp-2, and no transcript in clasp-1 and in clasp-3 (Fig. 1B). However, low levels of transcript were detected in all alleles when using primers amplifying sequences upstream of the T-DNA insertions (data not shown). Thus, the question remains as to whether any of these clasp mutant alleles are truly null. We selected the clasp-1 allele for the further experiments.

To verify that the observed phenotypes in these mutants (described below) are due solely to the mutations in the Arabidopsis CLASP gene, we transformed the clasp mutants with a 35S::AtCLASP construct and found complete phenotypic rescue. We also created constructs expressing an N-terminal and a C-terminal fusion of AtCLASP to YFP under the control of a 2.4 kb genomic fragment located upstream to the AtCLASP coding region. Both constructs completely rescued the clasp mutant phenotypes indicating both that the promoter fragment has all essential regulatory regions and that YFP protein fusions were functional.

Fig. 1. Molecular characterization of the Arabidopsis CLASP gene. (A) The Arabidopsis CLASP gene consists of 20 exons and 19 introns. The positions of T-DNA insertions of the three alleles are indicated with arrowheads. (B) RT-PCR analysis of AtCLASP expression in the clasp mutant alleles. Elongation Factor 1a (EF1) was used as a control for RNA extraction and amplification. (C) AtCLASP::GUS expression in the shoot.
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Furthermore, the root cells, and fully elongated cells in the epidermis and cortex were shorter in the mutant (Fig. 3C,D). Roots also exhibited increased lateral root formation (Fig. 3A).

To examine whether cell division defects contribute to the root growth reduction in the clasp mutant, we introduced the pCYCB1;1::GUS marker into the clasp mutant background. The pCYCB1;1 gene is active in the G2-M cell cycle phase and therefore can be used to measure of cell division frequencies (Colon-Carmona et al., 1999). We found that root cells expressing the pCYCB1;1::GUS marker were confined to a zone that was about two times smaller than that found in wild-type root tips (77.6±21.5 μm compared with 133.9±28.0 μm; Student’s t-test, P<0.001; Fig. 3B). Taken together, these data suggest that the AtCLASP gene plays an essential role in both cell expansion and cell division.

AtCLASP is required for cell morphogenesis

To analyze the role of AtCLASP in cell morphogenesis, we focused on two cell types in which the roles of MTs have been well established, namely trichomes and epidermal pavement cells (Fu et al., 2005; Mathur, 2004; Mathur and Hulskamp, 2002). During trichome morphogenesis, MTs are particularly important for the formation of trichome branches. We found that the number of trichome branches in the clasp mutants was reduced (Fig. 4A). Moreover, although in the wild type the first two trichome branches form parallel to the apical basal leaf axis, in the clasp mutants, trichome branch orientations relative to the organ axis were found to be partially randomized. This affect became more pronounced when clasp was crossed to the angustifolia (an) mutant to form clasp an double mutant plants (Fig. 4B). On the an mutant leaves, the average angle at which trichome branches are aligned relative to the leaf longitudinal axis was measured to be 26±19°. By contrast, the average angle of an clasp double mutant two branched trichomes was 50±25° (P<0.001), suggesting that the AtCLASP may be required to sense or to respond to polar information in the leaf epidermis.

Arabidopsis leaf pavement cells form numerous lobes separated by indentations, which gives them a puzzle-piece appearance. Microtubules have been proposed to be important for pavement cell morphogenesis by restricting growth between the lobes (Fu et al., 2005). In clasp mutants, lobing is significantly reduced when compared with the wild type, indicating that AtCLASP is also important for this MT-dependent morphogenesis process (Fig. 4C).

Analysis of AtCLASP function in MT organization

Cell shape and elongation defects in the clasp mutant prompted us to analyze MT organization in hypocotyl cells, which exhibit reduced elongation and bulging relative to the wild type (Fig. 2C-E). In four 4-day-old seedlings of wild-type plants, cells in the upper hypocotyl underwent active elongation and had predominantly transverse MTs (Fig. 5A, upper two cells). Cells further down the hypocotyl were elongating less actively (Refregier et al., 2004) and harbored predominantly oblique MTs (Fig. 5A). Even though these cells were misshapen in the clasp mutants, MT orientation appeared similar to that in the wild type (Fig. 5B). Moreover, no obvious defects were
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observed in the orientation of MTs in etiolated clasp hypocotyl cells (data not shown). Under dark conditions, these cells elongate rapidly and are especially sensitive to MT defects (Kirik et al., 2002a; Kirik et al., 2002b).

To test the effect of AtCLASP overexpression on MT organization, we created transgenic plants expressing a YFP fusion to the AtCLASP cDNA (YFP:AtCLASP) or the C-terminal-truncated form (YFP:ΔC-AtCLASP) under the control of the 35S promoter. The YFP:ΔC-AtCLASP fusion protein lacks 268 amino acids of AtCLASP and it was previously shown that overexpression of the corresponding version of the human CLASP causes MT reorganization and bundling (Aonuma et al., 2005). Both the full-length and the ΔC-AtCLASP constructs labeled spirally arranged MTs as indicated by colocalization with CFP:TUA4 (Fig. 6A,B). CFP:TUA4 labeling was very dense compared with controls, suggesting that these configurations of polymers represent bundles. This MT phenotype was observed in 75% of theOX YFP:ΔC-AtCLASP transgenic lines (n=8) and in about 14% of theOX YFP:AtCLASP lines (n=14). As this spiral arrangement was never observed in the native promoter-driven AtCLASP::YFP (data not shown) and CFP:TUA lines (Fig. 6C), it is likely that this MT bundling and curving phenotype is caused by the overexpression of AtCLASP.

AtCLASP localizes to cortical MTs

The intracellular localization of AtCLASP was studied in Arabidopsis lines stably expressing a YFP:AtCLASP fusion protein under the control of the AtCLASP promoter. This construct rescued the clasp mutant phenotype completely. In these lines, YFP:AtCLASP showed most prominent labeling of the MT lattices compared with the native promoter-driven YFP:AtCLASP expression (Fig. 7C). However, in lines with relatively lower YFP:AtCLASP expression, we observed again discrete spots of YFP:AtCLASP aligned along cortical interphase MTs (Fig. 7C). To confirm that AtCLASP localizes to MTs, we treated plants expressing YFP:AtCLASP with the MT inhibitor oryzalin. As expected, YFP:AtCLASP fluorescence dissipated as MT depolymerized (Fig. 7C).

An inspection of mitotic cells revealed that AtCLASP was associated with all major MT arrays including the preprophase band (PPB), the mitotic spindle and the phragmoplast (Fig. 8). Here again we observed differences in AtCLASP localization depending on the promoter used. We found intense labeling of cortical MTs in the PPB compared with much weaker labeling
of internal MTs in the spindle or phragmoplast when YFP:AtCLASP was expressed under the native promoter (Fig. 8) or in the 35S::YFP:AtCLASP lines with the low expression levels (data not shown). By contrast, all MTs arrays were labeled with similar intensity in 35S::YFP:AtCLASP lines with high expression levels (data not shown). Together, these data show that YFP:AtCLASP is detected on all major MT arrays, but only shows intense accumulation on cortical arrays.

AtCLASP binds to the plus ends of MTs in position adjacent to EB1

Although the most intense localization of YFP:AtCLASP was to immobile spots on cortical MTs, a mobile population of signal was also detected as much more weakly labeled ‘comets’ that resembled the localization of previously observed +TIP proteins. To unambiguously determine whether AtCLASP is a +TIP protein, we generated transgenic plants expressing YFP:AtCLASP together with the +TIP EB1b protein fused to CFP. In this experiment, only MTs that were clearly not overlapping with others were analyzed. As shown in Fig. 9A, the CFP:EB1b exclusively labeled growing MT plus ends. The comet-like pattern of YFP:AtCLASP fluorescence was highest close to the MT tip as identified by CFP:EB1b, and gradually decreased with increasing distance (Fig. 9A and supplementary material Movie 1). Thus, AtCLASP behaves as a +TIP. Close examination of the relative positions of YFP:AtCLASP and CFP:EB1b at the MT plus end showed that AtCLASP was localized in a region close to the MT end but lagging behind AtEB1b. This observation was confirmed by quantifying the fluorescence of both proteins along individual MT plus ends (Fig. 9B). The maximum fluorescence peaks of EB1 and CLASP were clearly separated with an average distance of 0.6±0.16 μm (number of measured MT ends, n=10).

Discussion

The presence of CLASP proteins in diverse phylogenetic groups, including both unicellular and multicellular organisms,
CLASP regulates cortical microtubules indicates that they have been retained over the course of eukaryotic evolution and implies that CLASP proteins have important cellular functions. Although yeast, human and plant CLASP proteins share certain motifs, such as the N-terminal TOG domain and the C-terminal MAST domain, the overall homology level is not high, suggesting that the proteins may have diverged in their functions. Thus it is conceivable that CLASP proteins in different organisms share some overlapping functions but also have acquired specific roles in different lineages. Analysis of the CLASP function among different organisms is likely to make a valuable contribution to understanding both the fundamental principles of cytoskeletal organization and function, and the evolution of specific cytoskeleton functions in different phylogenetic groups.

Function of AtCLASP in cell division, morphogenesis and pattern of cell orientation

CLASP proteins play an essential role during cell division in animal cells, where CLASP proteins are found at spindle poles and MT plus ends within spindles and kinetochores (Maiato et al., 2003; Mimori-Kiyosue et al., 2006). Loss of CLASP function in these cells causes severe defects in spindle formation and chromosome alignment and segregation (Cheeseman et al., 2005; Hannak and Heald, 2006; Mimori-Kiyosue et al., 2006; Pereira et al., 2006). Arabidopsis clasp mutants exhibited mitotic defects, but these phenotypes were relatively mild compared with those described in animal cells; a subtle reduction in the spindle and phragmoplast lengths was observed (Ambrose et al., 2007), as was a reduction in the number of mitotic cells in the root meristem (Ambrose et al., 2007) (this study). These results may reflect loss of an essential role for AtCLASP in the plant spindle, although it is also possible that none of the examined alleles are true null alleles.

Analysis of clasp mutants revealed an essential role for AtCLASP in cell and tissue morphogenesis, with significant alterations in root morphology in particular, and pronounced defects in cell form and cell expansion in a variety cell types including epidermal pavement cells, trichomes, hypocotyl cells and root epidermal cells (Ambrose et al., 2007) (this study). The spectrum of observed phenotypes is consistent with AtCLASP playing an important role in cortical MT array function, which is of central importance in regulating plant cell morphogenesis. Furthermore, our analysis of the orientation of

Fig. 5. MT organization in the upper hypocotyl cells of the clasp mutant. (A) Upper hypocotyl region of a 7-day-old wild-type (Col) seedling. (B) Upper hypocotyl region of a 7-day-old clasp mutant seedling. Microtubules are labeled with CFP:TUA4. Images are montages of projected confocal image stacks. Scale bar: 30 μm.

Fig. 6. Overexpression of AtCLASP and ΔC-AtCLASP induces spiral arrangement of cortical MTs. (A) Spiral arrangement of pavement cell MTs (CFP:TUA4) in a transgenic line with high expression of YFP:AtCLASP. (B) Enhanced spiral MT phenotype and clear MT bundling in pavement cells of a transgenic line overexpressing YFP:ΔC-AtCLASP. (C) Pavement cells expressing CFP:TUA4 only. Images are projections of confocal image stacks. Scale bar: 20 μm.
trichome branching suggests that AtCLASP function is also required for trichomes to either sense or respond appropriately to axial information in leaves.

Cortical array organization
Cortical MTs are hypothesized to guide plant cell morphogenesis by organizing the deposition of cellulose microfibrils in the plant cell wall (Baskin, 2005). Guidance of cellulose deposition by individual cortical MT bundles was demonstrated recently by tracking labeled cellulose synthase complexes in the plasma membrane of live cells (Paredez et al., 2006). Although marked defects in anisotropic cell growth were observed in clasp mutants, the orientation of MTs in the cortical arrays of hypocotyl cells resembled that observed in wild-type cells, being generally oblique to the major axis of cell elongation. Thus, the loss of anisotropic cell expansion in these cells was not obviously attributable to a defect in array orientation. This result was reminiscent of observations of cellulose synthase in cells treated with the MT-destabilizing drug oryzalin, which causes loss of anisotropic cell expansion. Although cellulose synthase complexes were distributed differently on the plasma membrane of cells treated with oryzalin and prominent MT lattice labeling, an oryzalin-treated cell is depicted in the two right-hand images. All images were acquired in hypocotyl cells of 3-day-old seedlings. Scale bars: 5 μm (A,C), 2 μm (B).

Fig. 7. YFP:AtCLASP colocalization with MTs in interphase cells.
(A) Colocalization of YFP:AtCLASP driven from the native promoter and CFP:TUA4. (B) Magnification of a subregion of the cell shown in A. (C) Co-localization of YFP:AtCLASP driven from the 35S promoter at a relatively low level of expression and CFP:TUA4. Note spots of the AtCLASP accumulation on the MTs and prominent MT lattice labeling. An oryzalin-treated cell is depicted in the two right-hand images. All images were acquired in hypocotyl cells of 3-day-old seedlings. Scale bars: 5 μm (A,C), 2 μm (B).

Fig. 8. Localization of AtCLASP to mitotic arrays.
(A) YFP:AtCLASP expressed from the native promoter in dividing root cells, MTs are labeled by expression of CFP:TUA4. On the right is the merged image with YFP:AtCLASP in the red channel and CFP:TUA4 in the green. Note prominent colocalization of YFP:AtCLASP and MTs at the PPB and relatively low signal intensity of YFP:AtCLASP on the spindle MTs. (B) Cells labeled as in A undergoing cytokinesis. Note that phragmoplast MTs are prominently labeled by CFP:TUA4 and almost undetectable by YFP:CLASP. Arrowheads indicate the preprophase band; asterisks depict an anaphase cell with spindle; arrows point to phragmoplast MTs. Scale bars: 10 μm.
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membrane in the absence of an obliquely oriented cortical MT array, their trajectories remained parallel to each other and oblique to the cell axis (Paredez et al., 2006). These results suggest the possibility that there may be aspects of cortical array organization and function in addition to MT and cellulose orientation that are important for regulating plant cell morphogenesis. It is possible that AtCLASP plays a role in one of these functions.

Microtubule stability

Studies of MT dynamic properties in animal cells depleted of CLASP revealed that CLASP regulates transition frequencies, limiting MT depolymerization (Mimori-Kiyosue et al., 2005). In migrating PtK1 epithelial cells it was demonstrated that a regulatory cascade involving GSK3β kinase causes re-localization of CLASP from MT plus ends in the cell body to the polymer lattice in cell lamella (Wittmann and Waterman-Storer, 2005). As MTs behave differently in the two cell body parts, with MT plus ends undergoing rapid growth and shrinkage in the cell body and persistent growth in lamella, it was speculated that CLASP contributes to this switch in dynamic plus-end behavior. Several observations indicate that a general effect on MT stability found in animals is also true for plant AtCLASP: a reduction of MT density and enhanced sensitivity to oryzalin in clasp mutants (Ambrose et al., 2007), and formation of spiral bundles of MTs after overexpression of CLASP (this work).

YFP:AtCLASP localization defines two domains of localization on cortical MTs

We analyzed localization of the YFP:AtCLASP fusion protein expressed under the control of two promoters: the strong viral 35S promoter and the native AtCLASP promoter. Examining stably transformed plants, we found that localization of YFP:AtCLASP is sensitive to the level of expression. In plants expressing high levels of the fusion protein, YFP:AtCLASP localized continuously along the length of MTs and labeled all MT arrays. By contrast, imaging of YFP:AtCLASP expressed from the native promoter revealed two discreet patterns of localization along cortical MTs: decoration of MT plus ends as a dynamic gradient, and a much more intense accumulation of protein to discrete spots distributed at intervals along the lengths of cortical MTs and MT bundles. A very similar combination of +TIP and punctate localization along the polymer wall was observed in 35S::YFP:AtCLASP lines expressing low levels of the fusion protein.

AtCLASP tracks plus ends behind EB1b

YFP:AtCLASP forms a gradient at MT plus ends, allowing AtCLASP to be classified as a +TIP protein. Our fine mapping of AtCLASP with respect to EB1 revealed that these two proteins did not colocalize, rather, YFP:AtCLASP fluorescence intensity peak was found to be about 0.6 μm behind the region of the highest CFP:EB1b intensity. Similar results were reported in migrating epithelial cells (Wittmann and Waterman-Storer, 2005), in which co-localization and photobleaching experiments of EGFP-CLASP2 revealed that CLASP binds immediately behind MT plus ends. These observations suggest that the two proteins track MT plus ends by different mechanisms. CLASP may either recognize a structural feature of the MT lattice that is characteristic of the region just behind the tip (Arnal et al., 2000; Wittmann and Waterman-Storer, 2005), or alternatively, a +TIP-based CLASP gradient may be modified by interactions with other plus +TIPs, EB1 (Chan et al., 2003; Mathur et al., 2003), SPR1 (Sedbrook et al., 2004) and ATK5 (Ambrose et al., 2005) are characterized +TIPs in Arabidopsis. AtCLASP failed to interact with EB1 proteins in the yeast-two-hybrid assay (our preliminary data). Furthermore, in Arabidopsis plants expressing high levels of YFP:AtCLASP such that the entire lattice is labeled, we found that CFP:EB1 remained specifically
localized to MT plus ends. This result is in contrast to the human CLASP proteins, which upon overexpression, induced strong accumulation of EB1 along MTs (Mimori-Kiyosue et al., 2005). Together, these data suggest that, in contrast to its animal and yeast (Grallert et al., 2006) homologues, AtCLASP either does not directly interact with EB1 or its interaction is much weaker. The possible interactions of AtCLASP with other plant +TIP proteins remain to be characterized.

CLASP and MT cell cortex interactions

It is tempting to speculate that the localization of YFP:AtCLASP to punctae along MT walls may be connected to specific properties of cortical MT arrays because these spots were not reported in observations of CLASP in centrosome-organized MT arrays in animal cells. For example, studies in animal cells propose that CLASP mediates the interaction between the plus ends of cytoplasmic MTs radiating from the centrosome to specific cortical sites at the plasma membrane, where it functions as a cortical anchor for plus ends without compromising their dynamic properties (Lansbergen et al., 2006; Mimori-Kiyosue et al., 2005). In HeLa cells, a lipid-binding protein, LL5, restricted contact with the cell cortex.

In plant cells, sites of such interaction may exist not only at MT plus ends but also at other locations along the MT lattice, such as may be reflected by the distribution of AtCLASP spots on these polymers. It is noteworthy that spots of AtCLASP accumulation were not detected on spindle or phragmoplast MTs, which are primarily cytosolic arrays and have much more restricted contact with the cell cortex.

Dynamic imaging of AtCLASP revealed a novel distribution of ATCLASP on cortical MTs and spatial complexity of different MAPs at MT plus ends. The tools we developed here will make new experiments to explore CLASP function possible, such as asking whether MT behavior correlates with the formation and positions of CLASP-rich domains, and what the dynamic relationship of CLASP localization at plus ends might be with other +TIP proteins. Further investigation of the role of AtCLASP in regulating cortical MT dynamics, possible interactions with the cell cortex, and cortical array function will benefit from analysis of individual MT behaviors, such as dynamic properties, association with the cell cortex, and MT-MT interactions in loss- and gain-of-function genetic backgrounds.

Materials and Methods

Plant material

Three clasp alleles used in the study are described in the results section. The angustifolia mutant allele used here was described previously as an-EMI (Folker et al., 2002). Trichome branching was calculated on the second pair of leaves for n=707 (Wild type Columbia), n=632 (clasp-1), n=660 (clasp-2), n=801 (clasp-3). Plants expressing ECFP:TUA1 were described (Walker et al., 2007).

Molecular biology

The AtCLASP cDNA was obtained from the Riken Institute (clone number RFL90-38-C02) (Seki et al., 2002). The full-length cDNA was transferred into pEarleyGate104 (Earley et al., 2006) to generate construct 35S::YFP:AtCLASP or into pEarlyGate101 to generate construct 35S::CLASP::YFP. The 3′ fragment of AtCLASP was amplified with primers 276 (5′-GGGACA-CTTTGTGATACCTGATC-3′) and 299 (5′-GGGGACACCCTTGTGACAGAAATGCTGGTGTTCCAAACCGTTG-TCAGATTAGACC-3′) and subsequent BP and LR reaction with vector pEarleyGate104 to generate construct 35S::YFP:AtCLASP. Tubulin 4 (TUA4) was amplified with primers 326 (5′-GGGACAAGACTTTGATCACAAGGAGGACTGAATGCC-3′) and 327 (5′-GGGGAGGACACTTGGTCCAAACAAC-ACAAAGAGCTGGTGTTATGATGATG-3′) and 391 (5′-GGGGGACGACCAACTAGTTATCAGAAATGCTGGTGTTACCATGTTG-3′) and cloned upstream of Gus, YFP:AtCLASP and AtCLASP:YFP to generate AtCLASP::GUS, AtCLASP::YFP:AtCLASP and AtCLASP:YFP constructs.

For RT-PCR RNA was extracted from leaves with the TRI-Reagent® (Fermentas) according to the manufacturer’s protocol. Detection of the AtCLASP transcript was performed using primers 433 (5′-GAGGGGTTGGTGATGATTGAA-3′) and 434 (5′- CGTACCACGACTTTGATTGG-3′) generating a 580 bp fragment. Amplification of the translation elongation factor EF1α was performed with primers EF1α-U: 5′-ATGC- CCCCAGGATCTGTGATAAGAC-3′ and EF1α-L: 5′-TTGGCGGACCCCTTAGCGTGAATCA-3′ was used as control.

Microscopy and image analysis

Seeds were germinated on 0.5×MS (Murashige and Skoog) agar at 22°C. For drug treatments, seedlings were submersed in solution and incubated in darkness before mounting. Imaging was performed on a spinning disk confocal microscope essentially as described previously (Paredes et al., 2006). Merged images of hypocotyl cells were assembled in Photoshop (Adobe Systems) from 18-24 optical sections. All confocal image processing was performed using Metamorph (Molecular Dynamics, Sunnyvale, CA) and ImageJ (W. Rasband, National Institutes of Health, Bethesda, MD) software. To localize the position of CFP:AtCLASP and YFP:AtCLASP at the growing end of cortical MTs, normalized fluorescence intensity (NFI) was calculated as follows. Images were background-subtracted, a line scan was performed along the axis of the MT end and the mean fluorescence intensity values calculated from a 5-pixel-wide lane. These values were normalized by dividing by the maximum value in each scan (i.e. maximum value becomes 1). The peak values from each scan of EB1 signal were then used to align the scans from a selected population of MT ends and mean values for both EB1 signal and AtCLASP signal at each pixel position were calculated. Finally, the mean YFP:AtCLASP intensities were re-normalized by dividing by the maximum mean value.

For cell-complexity measurements, scanning electron microscopy images of the first leaf pair of the 3-week-old plants were analyzed. For cell-complexity measurements, scanning electron microscopy images of the first leaf pair of the 3-week-old plants were analyzed. The perimeter and the area of 20 different cells were determined by the Diskus Software (http://www.hilgers.com/). A complexity value was calculated by the formula

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4\pi A / P^2
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where A is the area of 20 different cells were determined by the Diskus Software (http://www.hilgers.com/). A complexity value was calculated by the formula

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4\pi A / P^2
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We thank Irene Klinkhammer for excellent technical assistance. We also acknowledge the Signal Program (La Jolla, CA) and the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH) for providing material used for the isolation of clasp-1, clasp-2, and clasp-3 T-DNA insertion lines. This work was supported by NSF grants 0524334 0524355 for collaborative research (V.K., C.P., J.C.S., D.W.E.).
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