Arabidopsis Casein Kinase 1-Like 6 Contains a Microtubule-Binding Domain and Affects the Organization of Cortical Microtubules[1][W][OA]

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Members of the casein kinase 1 (CK1) family are evolutionarily conserved eukaryotic protein kinases that are involved in various cellular, physiological, and developmental processes in yeast and metazoans, but the biological roles of CK1 members in plants are not well understood. Here, we report that an Arabidopsis (*Arabidopsis thaliana*) CK1 member named casein kinase 1-like 6 (CKL6) associates with cortical microtubules in vivo and phosphorylates tubulins in vitro. The unique C-terminal domain of CKL6 was shown to contain the signal that allows localization of CKL6 to the cortical microtubules. This domain on its own was sufficient to associate with microtubules in vivo and to bind tubulins in vitro. CKL6 was able to phosphorylate soluble tubulins as well as microtubule polymers, and its endogenous activity was found to associate with a tubulin-enriched subcellular fraction. Two major in vitro phosphorylation sites were mapped to serine-413 and serine-420 of tubulin β. Ectopic expression of wild-type CKL6 or a kinase-inactive mutant form induced alterations in cortical microtubule organization and anisotropic cell expansion. Collectively, these results demonstrate that CKL6 is a protein kinase containing a novel tubulin-binding domain and plays a role in anisotropic cell growth and shape formation in Arabidopsis through the regulation of microtubule organization, possibly through the phosphorylation of tubulins.

The casein kinase 1 (CK1) family is an evolutionarily conserved eukaryotic Ser/Thr protein kinase family composed of a highly similar catalytic domain and a variable domain mostly located at the C terminus (Gross and Anderson, 1998; Vielhaber and Virshup, 2001). In yeast, CK1 homologs are known to function in vesicular trafficking (Wang et al., 1996; Panek et al., 1997, 2000; Feng and Davis, 2000; Babu et al., 2002, 2004; Sun et al., 2004), cell morphogenesis (Robinson et al., 1993), cell cycle progression (Wang et al., 1996; Robinson et al., 1999; Petronczki et al., 2006), and DNA repair (DeMaggio et al., 1992). The CK1 family in metazoans has also been shown to regulate a number of cellular, physiological, and developmental processes (Polakis, 2002; Knippschild et al., 2005; Nusse, 2005; Heeg-Truesdell and LaBonne, 2006; Price, 2006). It is notable that CK1 can recognize and phosphorylate several components of a signaling pathway, regulating the signal relays from the cell surface to the cytoskeleton or nucleus at multiple levels. Members of CK1 in yeast and animals are partitioned to various subcellular domains, including the cytoplasm, nucleus, vesicles, cytoskeleton, and plasma membrane. Spatial and temporal subcellular compartmentalization is one of the key mechanisms for controlling the specificity of multifunctional CK1 family members (Gietzen and Virshup, 1999; Babu et al., 2002; Swiatek et al., 2004).

In a previous study (Lee et al., 2005), we reported the biochemical isolation of a tobacco (*Nicotiana tabacum*) member of CK1 named plasmodesma-associated protein kinase (PAPK). It was the specific phosphorylation activity toward the tobacco mosaic virus (TMV) movement protein (MP) that allowed for the isolation of the tobacco PAPK. TMV MP, a viral protein that plays an important role in spreading viral infection in plants through the cell-to-cell trafficking activity, was shown to undergo phosphorylation in planta (Waigmann et al., 2000) and to interact with host systems, including microtubules (Kragler et al., 2003; Brandner et al., 2008). The Arabidopsis (*Arabidopsis thaliana*) genome encodes 14 casein kinase 1-like (CKL) isoforms from 13 genes that are distinctively localized to the cytoplasm, nucleus, endoplasmic reticulum, or uncharacterized punctate structures (Lee et al., 2005). Based on a similar in vitro substrate preference and a partial colocalization with TMV MP, an Arabidopsis CKL member, CKL6, was proposed as a functional homolog

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of tobacco PAPK and was called Arabidopsis PAPK1. In the interest of simplicity and clarity in referring to CKL gene family members, the name CKL6 instead of PAPK1 will be used here.

Among animal CK1 members, CK1δ is bioinformatically predicted to be a homolog of CKL6. It is notable that this isoform is implicated in increasing microtubule instability in mammals (Behrend et al., 2000; Li et al., 2004). CK1δ is thought to play a role in regulating the interaction between the microtubules and membrane, perhaps in microtubule-dependent vesicle transport. It was shown to localize to punctate particles that label interphase microtubules and post-Golgi structures and to phosphorylate tubulins and some microtubule-associated proteins (MAPs) in vitro (Behrend et al., 2000). These studies suggest the possibility that CK1δ has unique structural and functional properties that allow for the recognition and regulation of the microtubule cytoskeleton. The exact mechanism underlying vesicle and microtubule association and the biological function of tubulin phosphorylation by CK1δ has yet to be established. However, it is conceivable that this kinase may play a role in the regulation of interphase microtubule dynamics or array organization through the phosphorylation of tubulins and/or MAPs. Direct modulation of tubulin by phosphorylation was shown to be one of the mechanisms underlying the control of microtubule dynamics during cytokinesis (Fourest-Lieuvin et al., 2006). Phosphorylation of tubulin β by cyclin-dependent kinase Cdk1 inhibits the incorporation of the phosphorylated tubulin β into the microtubule polymers. Dynamic microtubule assembly also plays a critical role in plants by regulating various cellular processes, such as intracellular trafficking of macromolecules and organelles, cytokinesis, cell expansion, and morphogenesis (Cyr and Palevitz, 1995; Shaw et al., 2003; Lloyd and Chan, 2004; Sedbrook, 2004; Smith and Oppenheimer, 2005; Ehrhardt and Shaw, 2006). However, tubulin modifications in plants have not been extensively explored.

In this study, we tested the possibility that CKL6 may play a function in microtubule regulation in plants and showed that CKL6 is a protein kinase that can regulate the organization of the cortical microtubules in Arabidopsis. CKL6 was found to localize to the cortical microtubules in addition to the association with punctate structures. Microtubule association of CKL6 was shown to be conferred by the interaction of its distinct C-terminal domain (CTD) with the tubulins. CKL6 phosphorylated soluble tubulin as well as microtubule polymers in vitro, and endogenous CKL6 activity was found within the tubulin/MAPs-enriched subcellular fraction. Through mutagenesis analyses, Ser-413 and Ser-420 within the C-terminal end of Arabidopsis tubulin β3 were mapped to be major CKL6 phosphorylation sites in vitro. Organization of the cortical microtubules was found to be altered in the gain-of-function or dominant-negative mutants produced by an ectopic expression of wild-type CKL6 or a kinase-inactive (KI) mutant form of CKL6. Changes in cortical microtubule organization were apparently correlated with abnormal anisotropic cell growth and cell morphogenesis in these plants. We propose that the tubulin phosphorylation by CKL6 may play a role in regulating interphase microtubule dynamics in plant cells.

RESULTS

Association of CKL6 with Cortical Microtubules through the CTD

Insight into the association of CKL6 with the cortical microtubules was gained by examination of transgenic Arabidopsis plants that express CKL6:GFP under the control of the 35S promoter (Fig. 1A). The cytoskeleton, reminiscent of the cortical microtubules, in addition to the punctate structures that were previously reported by transient expression assays (Lee et al., 2005), were labeled by CKL6:GFP in the transgenic plants. Association of CKL6:GFP with the microtubules was also consistent in transgenic tobacco BY-2 calls (Fig. 1A). We hypothesized, based on its unique sequence, that the CTD may contain a specific signal for the targeting of CKL6 to the cytoskeletons. To test this possibility, we produced transgenic Arabidopsis that express the CTD fused alone to GFP (GFP:CTD) under the control of the 35S promoter. Examination by confocal microscopy showed that the GFP:CTD displays a pattern reminiscent of the cortical microtubule cytoskeleton in various cell types, including the epidermal cells of the cotyledon and hypocotyl (Fig. 1B).

To confirm that CTD associates with cortical microtubules, we tested the susceptibility of the cytoskeletal components that are labeled with GFP:CTD to a microtubule inhibitor, amiprophosmethyl (APM). As a positive control for this drug treatment, Arabidopsis transgenic seedlings that express GFP-tagged Arabidopsis tubulin α6 (GFP:Tua) under the control of the 35S promoter (Lu et al., 2005) were employed (Fig. 1C). Cortical microtubules labeled with GFP:Tua in hypocotyl epidermal cells were depolymerized within 30 min after APM treatment, and the fluorescent signals were detected in the cytoplasm. The cytoskeleton components that are labeled with GFP:CTD were also effectively depolymerized upon APM treatment. These results demonstrated that the CTD associates with the cortical microtubules. Consistently, the cytoskeletal pattern labeled with CKL6:GFP was also depolymerized in a similar experiment (data not shown).

Molecular Dissection of the CTD

The CTD did not show any homology to known microtubule-binding domains predicted in the public databases. To determine whether the CTD contains a region that was essential for the microtubule association, we conducted a deletion mutagenesis in which a

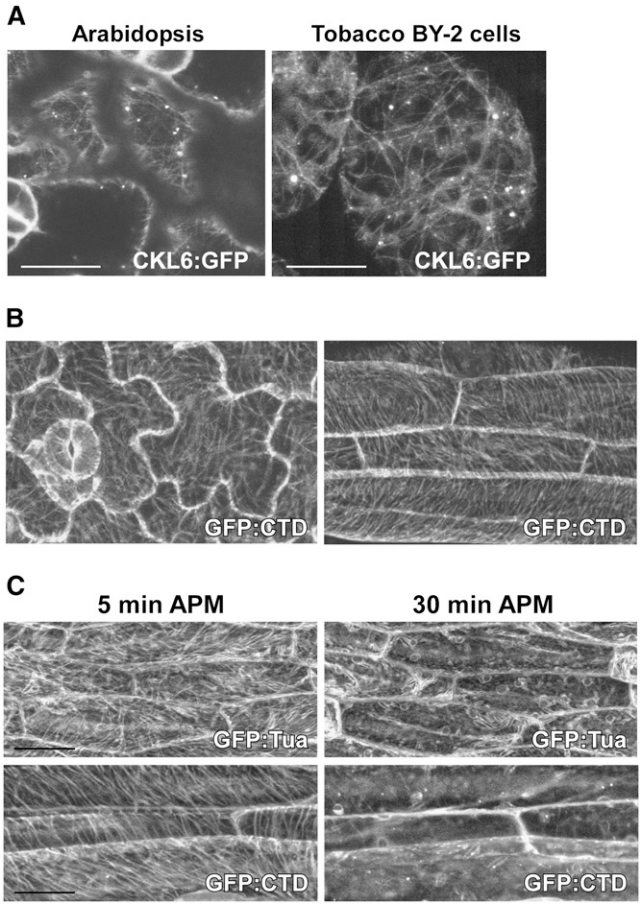

Figure 1. Association with cortical microtubules by the CTD and CKL6 in vivo. A, Association of CKL6:GFP with cortical microtubules in epidermal cotyledon cells of a transgenic Arabidopsis seedling and in transgenic tobacco BY-2 cells. Confocal images were taken from the green channel to detect fluorescent signals produced by GFP-tagged CKL6. Bars = 20 μm. B, Cytoskeletal patterns exhibited by GFP:CTD in the cotyledon (left) or hypocotyl (right) epidermal cells of transgenic Arabidopsis seedlings at 7 d after germination. C, AMP treatment shows the disruption of cortical microtubules that are labeled by GFP:Tua (top) or GFP:CTD (bottom). Images were taken at 5 and 30 min of incubation after application of 100 μM APM. Bars = 10 μm.

series of N-terminal and C-terminal deletion mutants of the CTD (residues 302–479) were generated and tagged with GFP (Fig. 2). Each construct was then qualitatively analyzed for its ability to interact with the microtubules by examining their localization patterns in transient expression assays (Fig. 2A). Deleting up to approximately 50 amino acid residues from either terminus did not affect the ability of the truncated mutants to associate with microtubules ($CTD_{351–479}$; Fig. 2C). Further deletions from either end, however, resulted in a significant ($CTD_{302–396}$; Fig. 2D) or complete ($CTD_{397–479}$; Fig. 2E) loss of microtubule localization. Fluorescent signals produced by these constructs were mostly dispersed into the cytoplasm. These results suggested that an important region required for the interaction with microtubules might reside within the amino acid residues 351 to 434. We then examined whether this minimal region was sufficient for the interaction with microtubules by examining the localization pattern of $CTD_{351–434}$. Expression of GFP-tagged $CTD_{351–434}$ showed that it retained the microtubule-binding property, although to a lower extent than the full-length CTD (Fig. 2B). This region, containing 84 amino acid residues, showed unique sequence features that were highly basic, having a predicted pI of 11.88, and had high Ser (20%), Arg (17%), and Gly (10%) contents (data not shown). It also contained multiple repeats that were similar to the Gly-Ser-Arg repeat domain, which was shown to be important for an interaction of the microtubule actin cross-linking factor with the microtubule (Sun et al., 2001). Collectively, these results demonstrate that CTD contains a region similar to a previously characterized Gly-Ser-Arg repeat domain that is sufficient for the association with the cortical microtubules.

In Vitro Interaction of CTD and CKL6 with Tubulins

Next, to gain insight into the molecular basis for the microtubule association with CTD, we performed yeast two-hybrid screening as described (Kim et al., 1997) using an Arabidopsis cDNA library to isolate potential interaction partners of the CTD (O. Lee and J.-Y. Lee, unpublished data). Among several candidate interaction partners isolated, tubulin β3 (At5g62700) was found to be a specific interaction partner. This result suggested that CTD may interact with the microtubules in vivo through a direct binding to tubulins. To further substantiate their direct interaction, we performed in vitro pull-down assays by employing the CTD fused to the glutathione *S*-transferase (GST; GST:CTD) as bait molecule and purified bovine tubulin dimers as prey (Fig. 3A). Control pull down performed using GST as bait showed the absence of nonspecific binding between this protein and bovine tubulins under our experimental conditions. GST:CTD, however, was shown to bind bovine tubulins, demonstrating their specific interaction.

The yeast two-hybrid screening, which isolated Arabidopsis tubulin β3 as a specific interaction partner for the CTD, predicts a possibility that CTD may preferentially interact with tubulin β. To test this possibility, recombinant Arabidopsis tubulins α6 and β3 were produced in *Escherichia coli* as fusions to the maltose-binding protein (MBP), and the soluble recombinant proteins were affinity purified. Arabidopsis tubulin α6 was chosen simply because this gene was used as a microtubule marker in our study and was thought to be a reasonable choice given the high degree of sequence identity among the six Arabidopsis tubulin α family members (Supplemental Fig. S1). Control pull down employing GST showed the absence of nonspecific binding, as expected (Fig. 3A). In contrast, GST:CTD was able to bind both MBP:Tubα and MBP:Tubβ. Lack of interaction with MBP supported a specific interaction between GST:CTD and both

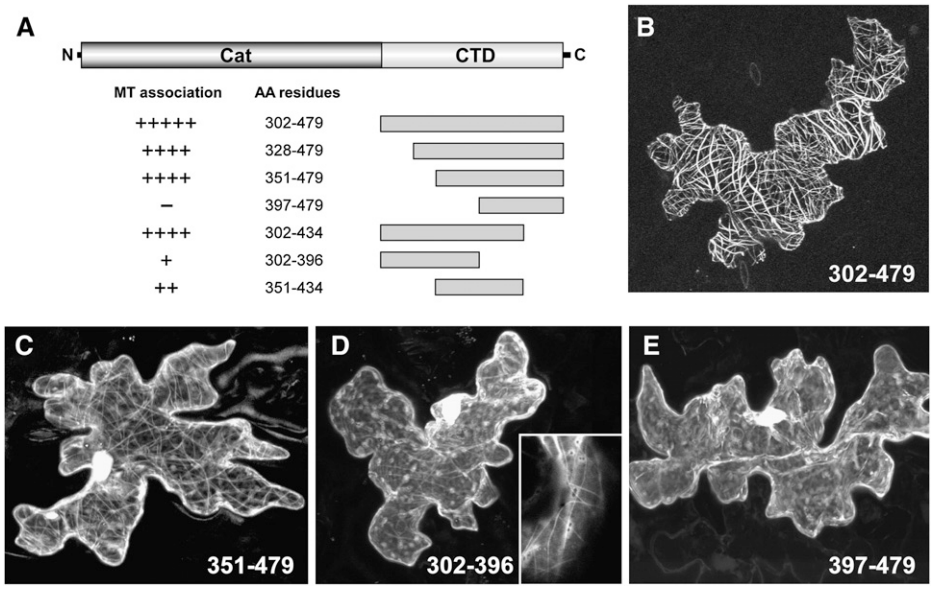

Figure 2. The CTD contains a subdomain important for the association with microtubules (MT). A series of deletion mutants derived from the CTD was produced to map the microtubule-associating subdomain within the CTD. Microtubule association of GFP-tagged CTD mutants was assessed by localization studies in epidermal leaf cells of Arabidopsis. Representative images are shown to support the qualitative analysis summarized in the diagram. All images are three-dimensional images reconstructed from a series of optical Z-sections. A, A diagram illustrating a series of CTD deletion mutants and the extent of their microtubule association, as qualitatively determined in comparison with the full-length CTD. AA, Amino acids. B, GFP:$CTD_{302–479}$ (full length). C, GFP:$CTD_{351–479}$. D, GFP:$CTD_{302–396}$. E, GFP:$CTD_{397–479}$.

tubulin isoforms. Association of the CTD with microtubules in vivo and its interaction with tubulins in vitro suggested that the full-length enzyme would also interact with the tubulins. To test this idea, we performed pull-down assays employing GST:CKL6 as prey and MBP:Tubα or MBP:Tubβ as bait (Fig. 3B). GST:CKL6 specifically interacted with both MBP:Tubα and MBP:Tubβ, whereas GST on its own did not interact with either of them. Consistently, the control pull down employing MBP as bait supported the specificity of the interaction between GST:CKL6 and the recombinant tubulins. Together, these in vitro binding data support the in vivo localization studies. More important, they confirm the direct interaction of CKL6 with tubulins and the role of CTD in this interaction.

CKL6 Phosphorylates Tubulin and Microtubule in Vitro

Based on the above results showing the direct interaction between tubulins and CKL6, it would be conceivable that the tubulins might be substrates of the enzyme as well. To further test whether tubulins are substrates of CKL6, we next performed in vitro phosphorylation assays by employing bovine tubulin dimers and recombinant tubulins as substrates. CKL6 phosphorylated bovine tubulin dimers (Fig. 4A) as well as TMV MP, a known substrate of CKL6 (Lee et al., 2005). Since both tubulin isomers interacted with CKL6 (Fig. 3), we then tested whether both tubulin isomers are good substrates for CKL6. Surprisingly, tubulin β but not tubulin α was strongly phosphorylated by CKL6 (Fig. 4B). This result indicated that the specific phosphorylation by CKL6 is most likely on a region unique to tubulin β. It is conceivable that the phosphorylation of tubulin dimers could affect the microtubule assembly dynamics. Phosphorylation of microtubule polymers by CKL6 could also provide a mechanism for fine-tuning the stability of the microtubule filaments or bundles. As an initial step toward testing this hypothesis, we examined whether CKL6 can phosphorylate tubulin polymers. In this experiment, preformed bovine microtubules and tubulin dimers were allowed to be phosphorylated by CKL6 in kinase assays followed by sedimentation by high-speed centrifugation to separate microtubule polymers from the soluble fraction (Fig. 4C). Bovine serum albumin was included as a negative control for the CKL6 phosphorylation and the sedimentation. This assay showed that CKL6 can also phosphorylate microtubule polymers in addition to soluble tubulins, establishing a framework that the regulation of microtubule dynamics could involve phosphorylation of tubulins by CKL6.

Ser-413 and Ser-420 of Tubulin β Are Putative CKL6 Phosphorylation Sites in Vitro

To determine which amino acid residues are phosphorylated by CKL6 in vitro, we produced a series of

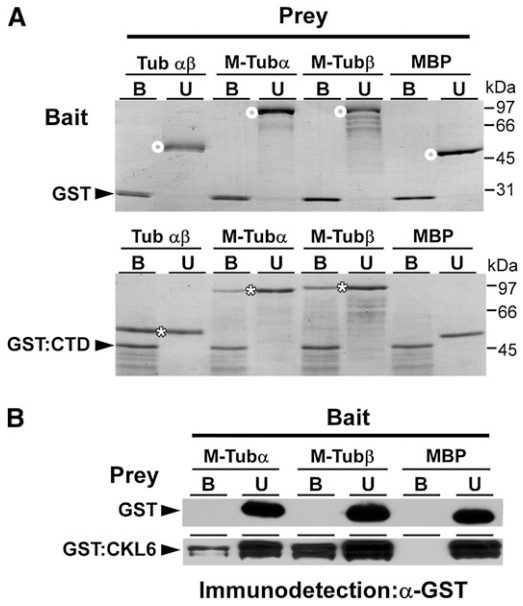

Figure 3. In vitro interaction of CTD and CKL6 with tubulins. A, Control pull-down assays were performed using 1 μg of GST immobilized on glutathione-agarose beads as bait per assay (top). Fractions containing each prey protein bound (B) or unbound (U) to GST were separated on 12% SDS-PAGE gels and stained with Coomassie Brilliant Blue. Prey molecules examined included bovine tubulin dimers (Tub $\alpha\beta$), recombinant Arabidopsis tubulin α6 and β3 fused to MBP (M-Tubα and M-Tubβ, respectively), and MBP. One microgram of prey protein was used in each assay. White circles indicate the size of each prey protein expected to be detected in the bound fraction if pulled down by the bead-bound GST. Pull-down assays employing 1 μg of GST:CTD as bait show specific interaction between GST:CTD and tubulins (bottom). White asterisks indicate the prey molecules that are bound to GST:CTD. B, Pull-down assays performed employing GST or GST:CKL6 as prey for M-Tubα, M-Tubβ, or MBP. The bait proteins (1 μg each) were immobilized on maltose-agarose beads and incubated with 1 μg of prey proteins. Bound and unbound fractions were collected followed by separation on 12% SDS-PAGE gels and electroblotting onto nitrocellulose membranes. GST or GST:CKL6 in each fraction was detected by specific antibodies to GST.

truncation and substitution mutants derived from the Arabidopsis tubulin β3 and investigated their ability to be phosphorylated by CKL6 (Fig. 5). To select potential Ser/Thr substrate sites unique to tubulin β, the sequences of tubulin α and tubulin β genes from Arabidopsis and bovine were compared (Supplemental Fig. S1). Primary structures of tubulin α and tubulin β share a high degree of sequence similarity except for their C termini, which predicts that this domain of tubulin β is a highly probable target region to contain the CKL6 phosphorylation site(s). Consistent with this notion, a deletion of the CTD-spanning amino acid residues 343 to 450 of tubulin β (ΔC343) abolished the phosphorylation by CKL6 (Fig. 5, A and B).

The canonical phosphorylation site for CK1 is defined as a Ser/Thr residue following negatively charged amino acid residues (Gross and Anderson, 1998). A close examination of the sequence comparison to find the consensus Ser/Thr residues meeting this requirement for negative charge within the CTD that are conserved only in tubulin β but not in tubulin α isoforms led to the selection of seven Ser/Thr residues: Thr-366, Ser-382, Thr-386, Thr-399, Ser-413, Ser-420, and Thr-429 (Fig. 5A; Supplemental Fig. S1). All of these except Ser-420 were absolutely conserved among the tubulin β genes. As for Ser-420, three Arabidopsis tubulin β isoforms, tubulin β4, β5, and β9, contained Ala instead of Ser at residue 420 (Fig. 5C). As bovine tubulin β contains Ser at this site as well, we decided to include this site as a target site to examine. To produce nonphosphorylatable mutants, each Ser/Thr residue was altered to Ala by employing point mutagenesis. Phosphorylation assays employing these missense mutants showed that the Ala substitution at Ser-413 or Ser-420 led to a significant reduction in CKL6 phosphorylation by approximately 60% or approximately 40%, respectively, compared with the wild-type tubulin β (Fig. 5B). This mutant phosphorylation screening suggested that those two sites, which both conform to the consensus CK1 phosphorylation sequence, are potential in vitro phosphorylation sites for CKL6. Next, to test whether Ser-413 and Ser-420 are the major sites or whether a third site exists, we produced a double mutant, $S_{413/420} \rightarrow A_{413/420}$, and examined the effect of this mutation on the phosphorylation. This experiment demonstrated that more than 90% of phosphorylation is lost in the double phospho-mutant, suggesting that these two sites are likely two major CKL6 phosphorylation sites in vitro (Fig. 5B).

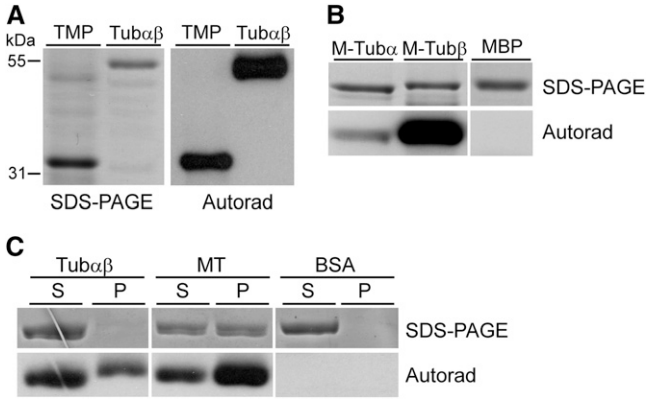

Figure 4. Phosphorylation of tubulins by CKL6. A, Phosphorylation of bovine tubulin dimers (Tub$\alpha\beta$) by CKL6. Phosphorylation of TMV MP (TMP) was included as a positive control. B, Phosphorylation of recombinant tubulin monomers. C, Phosphorylation of microtubule polymers (MT) by CKL6. Phosphorylation reactions were centrifuged at 100,000*g* for 15 min to separate soluble (S) fraction from the pellet (P) fraction. Soluble tubulin dimers and bovine serum albumin (BSA) were included as positive and negative controls for the phosphorylation and sedimentation, respectively.

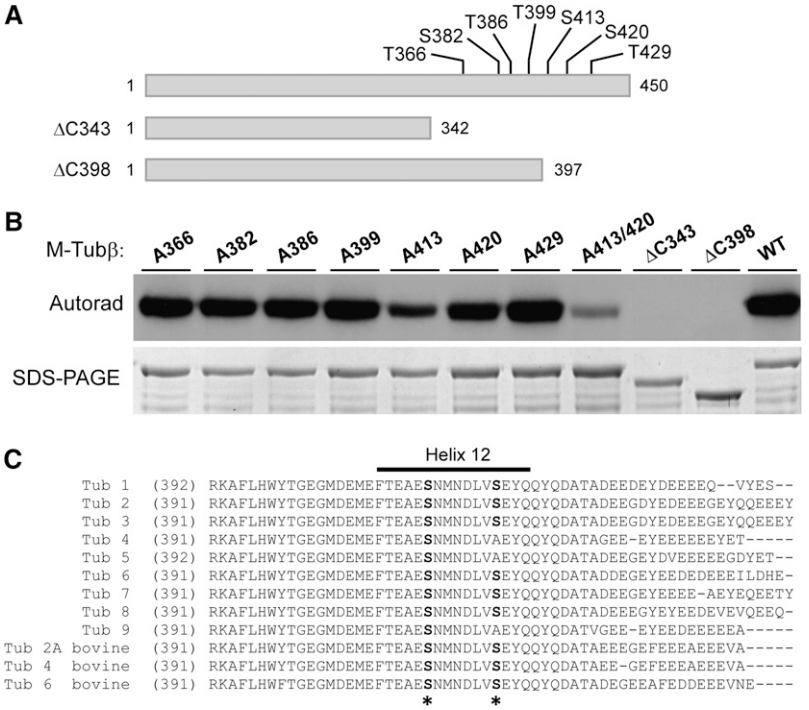

Figure 5. In vitro phosphorylation site mapping for tubulin β3. A, A schematic diagram illustrating the positions of substitution and truncation mutants of Arabidopsis tubulin β3. B, Phosphorylation assays employing the recombinant tubulin β3 mutants fused to MBP as substrates for CKL6. C, Amino acid sequence alignment of tubulin β isoforms from Arabidopsis and bovine at the C-terminal end, showing the conserved phosphorylation sites Ser-413 and Ser-420 (asterisks) within helix 12.

We did not pursue further identifying the residue(s) that is responsible for the residual phosphorylation remaining in $S_{413/420} \rightarrow A_{413/420}$, because the significance of this level of phosphorylation is difficult to assess and is beyond the scope of our study. However, an insight into the potential site(s) was gained by examining another deletion mutant, Tuβ3ΔC_{398}, which lacks amino acid residues 398 to 450 (Fig. 5A). Phosphorylation assays employing this mutant showed that it completely lacks CKL6 phosphorylation sites. Within the deleted region of this truncation mutant, only one Thr residue at position 409 was found to be a probable phosphorylation site that was not included in our mutagenesis. This site was not considered in our analysis because residue 409 is also conserved in tubulin α isoforms as Ser, but its potential as a significant phosphorylation site remains to be elucidated (Supplemental Fig. S1; see "Discussion").

Subcellular Fractions Enriched with Microtubule Polymers/MAPs Contain CKL6-Like Kinase Activity

To biochemically examine the association of endogenous CKL6 with the microtubules, we performed phosphorylation assays using a subcellular fraction enriched with the solubilized microtubules. We reasoned that CKL6 activity would be detected if it cofractionates to some extent with tubulins that are depolymerized from the cytoskeleton pellet. To prepare crude cytoskeleton pellet, Arabidopsis cells were protoplasted followed by conversion of the protoplasts into cytoskeletons, as described elsewhere (Hussey et al., 1987; Chan et al., 1996). Tubulins and MAPs enriched in a crude cytoskeleton were then solubilized by depolymerizing microtubules from the cytoskeletal pellet fraction. Immunoblotting analysis employing anti-tubulin confirmed the enrichment of tubulins in both of these fractions (Fig. 6A; Supplemental Fig. S2). Moreover, an accumulation of CKL6 within the solubilized tubulin/MAPs fraction was verified by an immunoblotting analysis employing CKL6-specific polyclonal antibodies (α-CKL6) that were raised against a CKL6-unique peptide derived from the C-terminal region. α-CKL6 detected both recombinant GST:CKL6 and native CKL6 present within the tubulin/MAPs fraction, as expected (Fig. 6A).

Next, we performed phosphorylation assays employing the solubilized tubulin/MAPs fraction as a source for the native CKL6 and bovine tubulin dimers, MBP-Tubα and MBP-Tubβ, the tubulin β phospho-mutant ($S_{413/420} \rightarrow A_{413/420}$), or TMV MP as substrates (Fig. 6B). Consistent with the phosphorylation data produced by employing recombinant CKL6 (Fig. 5), the tubulin/MAPs fraction was able to specifically phosphorylate bovine tubulins and tubulin β but not tubulin α. Moreover, the kinase within the tubulin/MAPs fraction was not able to phosphorylate the CKL6 phospho-mutant $S_{413/420} \rightarrow A_{413/420}$, although it phosphorylated a known CKL6 substrate, TMV MP. These results establish that the substrate preference of the native CKL6 within the tubulin/MAPs fraction conforms not only to the consensus sites of CK1 but also to the substrate preference of recombinant CKL6. Collectively, these data provide strong evidence that the tubulin/MAPs fraction contains CKL6 and are consistent with the association of CKL6 with the microtubules.

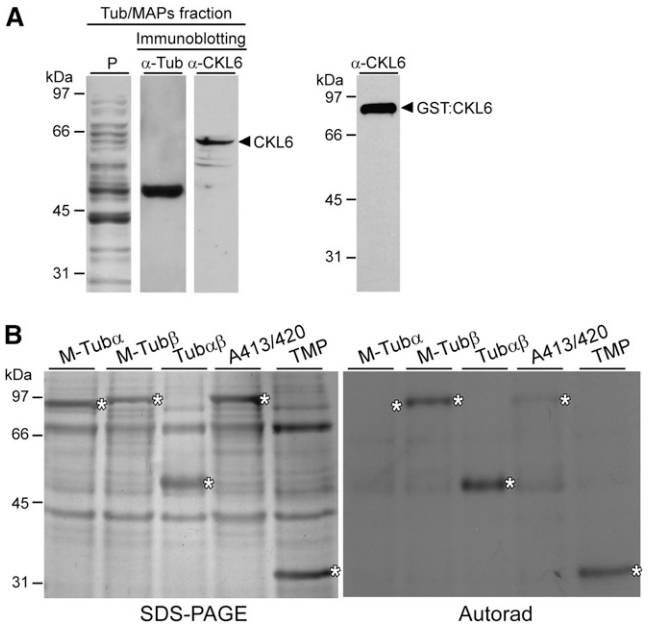

Figure 6. CKL6 activity contained within the tubulin/MAPs-enriched subcellular fraction. A, Western analysis showing an accumulation of CKL6 within the tubulin/MAPs-enriched subcellular fraction. Western blotting was performed employing CKL6-specific antibodies (α-CKL6) or tubulin-specific antibodies (α-Tub) against the solubilized tubulin/MAPs-enriched fraction. Recombinant GST:CKL-6 was included as a positive control for α-CKL6. Lane P, Protein profile of the tubulin/MAPs fraction separated on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue. B, Phosphorylation assays were performed in reactions employing the solubilized tubulin/MAPs fraction as a kinase source and recombinant tubulins α and β (M-Tubα and M-Tubβ, respectively), bovine tubulin dimers (Tub$\alpha\beta$), phospho-mutant $S_{413/420} \rightarrow A_{413/420}$ (A413/420), and TMV MP. White asterisks mark the position of each substrate.

A Potential Role for CKL6 in Cortical Microtubule Organization

The association of CKL6 with cortical microtubules in vivo and tubulin phosphorylation in vitro suggests a potential role for CKL6 in microtubule regulation. To investigate whether an altered expression level of active or inactive CKL6 affects microtubule organization or pattern in Arabidopsis cells, we introduced GFP:Tua by crossing into the transgenic plants that ectopically express wild-type CKL6 or the KI mutant form of CKL6. The CKL6-KI mutant was produced by altering two amino acid residues, Lys-42 and Asp-132, to Arg-42 and Asn-132, respectively. The respective mutation in each residue in animal CK1 members was shown to result in dominant-negative, kinase-inactive mutants (Zhu et al., 1998; Peters et al., 1999). We confirmed by phosphorylation assays using recombinant enzymes that the mutation at Lys-42 and Asp-132 in CKL6 results in an inactive kinase retaining less than approximately 5% catalytic activity compared with that of the wild-type enzyme, as expected (data not shown). This result validates the use of this mutant as a kinase-inactive CKL6 mutant.

Employing the F1 seedlings that resulted from the crosses between GFP:Tua and CKL6 or CKL6-KI lines, microtubule patterns of seedlings labeled with GFP:Tua were examined by confocal microscopy in comparison with those of control seedlings that express GFP:Tua alone (Fig. 7). Epidermal leaf pavement cells of wild-type Arabidopsis showed randomly arrayed cortical microtubules (Fig. 7A). This pattern was found to be altered in the CKL6 and CKL6-KI seedlings. Microtubule filaments labeled with GFP:Tua in CKL6 cells appeared shorter, and their array was more randomized than that of the wild-type cells, forming a web-like network (Fig. 7B). In contrast, the cortical microtubules in CKL6-KI cells were more organized than those in the wild-type cells, forming long parallel microtubule bundles (Fig. 7C). Similarly, opposite alterations of microtubule organization in CKL6 and CKL6-KI cells were also observed in hypocotyl epidermis (Supplemental Fig. S3A).

Epidermal leaf pavement cells of wild-type seedlings form jigsaw puzzle shapes, displaying extended lobe regions and narrow neck regions (Fig. 7D). The shape and expansion of pavement cells in CKL6 seed-

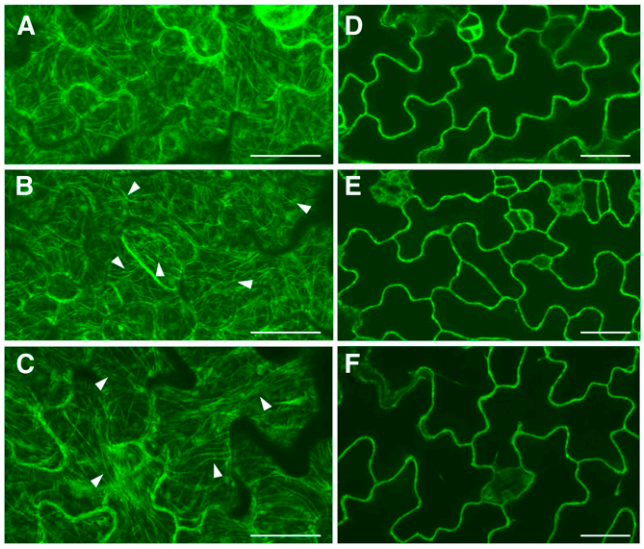

Figure 7. Cellular phenotypes induced by overexpression of CKL6 and CKL6-KI. A to C, Confocal images showing the cortical microtubule patterns labeled with ectopically expressed GFP:Tua in epidermal leaf cells of 6-d-old Arabidopsis seedlings. Z-series of 1-μm-thick optical sections were captured and projected as three-dimensional images. A, Control pavement cells. B, Pavement cells of an F1 seedling expressing CKL6 and GFP:Tua. C, Pavement cells of an F1 seedling expressing CKL6-KI and GFP:Tua. Arrowheads indicate the abnormal array patterns of the microtubules. Bars = 25 μm. D to E, Confocal images of a single optical section showing the shapes of epidermal cells. Expanded areas including the cells shown in A to C are presented. D, Control epidermal cells. E, Epidermal cells of an F1 seedling expressing CKL6 and GFP:Tua. F, Epidermal cells of an F1 seedling expressing CKL6-KI and GFP:Tua. Bars = 25 μm.

lings exhibited lobes that are somewhat short and underdeveloped, creating overall a less intricate interdigitation pattern compared with the wild-type cells (Fig. 7E; Supplemental Fig. S3B). The CKL6 seedlings also exhibited an overall growth inhibition compared with wild-type seedlings (Supplemental Fig. S3C). Pavement cells of CKL6-KI seedlings formed lobes slightly more stretched from the neck regions, causing irregularly pointed and angular lobes that are more deeply interdigitated in some cells compared with the wild-type cells (Fig. 7F). The overall growth of the CKL6-KI seedlings was quite similar to that of wild-type seedlings (Supplemental Fig. S3C). To further support its role in cell morphogenesis and development, we attempted an analysis of CKL6 loss-of-function mutants; however, no putative T-DNA insertion lines available publicly were CKL6 knockout mutants (G. Ben-Nissan and J.-Y. Lee, unpublished data). Down-regulation of *CKL6* mRNA in Arabidopsis by gene silencing did not visually affect cellular or growth phenotypes. We interpreted this result to imply that there may be a functional redundancy between *CKL6* and other *CKL* genes among 14 members in Arabidopsis, or that the level of *CKL6* reduction in the silencing lines was not sufficiently low to induce a strong knockdown phenotype.

Together with the in vitro data, the results above collected by analyzing gain-of-function and dominant-negative mutants suggest a potential role for CKL6 in the regulation of microtubule organization and anisotropic cell expansion and in cell shape formation.

DISCUSSION

In this study, we found that CKL6 plays an important function in regulating the cortical microtubule organization/dynamics and phosphorylates tubulins in vitro. Regulation of mitotic microtubules through the phosphorylation of MAPs had been demonstrated, but little is known about the regulation of interphase microtubules in plants. To our knowledge, neither the direct regulation of microtubules by tubulin phosphorylation nor a protein kinase that associates with the cortical microtubules had been demonstrated in plant systems. Our data provide new molecular and cellular evidence that CKL6 may play such a role by directly modulating microtubule dynamics or organization by phosphorylating the tubulins.

Remodeling of cortical microtubule arrays is thought to involve regulated changes in microtubule assembly and disassembly rates, typically through modulating the activities of MAPs. One of the regulatory mechanisms underlying this process includes reversible phosphorylation of the MAPs. Various MAPs and microtubule effecter molecules, including kinesin motor proteins, small GTPases, microtubule tip-binding proteins, and tubulin-binding proteins, were shown to be modulated by phosphorylation. A highly abundant MAP in neurons, tau, is regulated by phosphorylation (Westermann and Weber, 2003; Fourest-Lieuvin et al., 2006; Verhey and Gaertig, 2007) and promotes microtubule polymer formation and its stability. *Xenopus* XMAP215, a major regulator of microtubule dynamics, and its plant homolog, MOR1, promote microtubule polymerization. The polymerizing activity of XMAP215 can be suppressed upon phosphorylation by the cyclin-dependent kinase Cdk1 in vitro (Vasquez et al., 1999). A temperature-sensitive *mor1* mutation in Arabidopsis MOR1/GEM1 showed abnormally short cortical microtubules at a restrictive temperature, suggesting its involvement in the dynamic instability of microtubules (Hussey and Hawkins, 2001; Whittington et al., 2001). Whether it undergoes phosphorylation by Cdk1 is not yet known. In plants, phosphorylation of NtMAP65-1 by a MAP kinase has been shown to play an important role in regulating the progression of cytokinesis (Sasabe et al., 2006). It is conceivable that phosphorylation of a specific MAP(s), microtubule stabilizer or destabilizer, and/or tubulins, building blocks of microtubules, may facilitate the reorganization of cortical microtubule arrays or control the dynamic properties of microtubule assembly and disassembly. Future investigation will be necessary to address to what extent CKL6 is involved in this process.

Our studies performed employing site-directed mutagenesis coupled with in vitro phosphorylation assays determined that two Ser residues, Ser-413 and Ser-420, of tubulin β are in vitro phosphorylation sites preferred by CKL6. Simultaneous substitutions of these sites led to a great reduction in phosphorylation of the mutant (Fig. 5B). A residual activity in this mutant, which is absent in the ΔC398 mutant, could be explained by the presence of an alternative or a third site. A candidate for such a site among Ser or Thr residues within the region deleted in the ΔC398 mutant is Thr-409. This site is also conserved among tubulin α isoforms as Ser instead of Thr. It is tempting to speculate whether it is this residue that contributed to the basal level of phosphorylation shown in tubulin α. A future investigation will be necessary to explore the possibility that this residue can be a third phosphorylation site and its functional significance.

The phosphorylation sites Ser-413 and Ser-420 are found within the 12th helix at the CTD of tubulin β (Fig. 5C), which together with the immediately following C-terminal tail is exposed to the outer surface of the microtubule (Nogales et al., 1998). Due to the exposure to the outer surface, the CTD is thought to be involved in the binding of MAPs and motor proteins and to be a target for various posttranslational modifications, including polyglutamylation, polyglycylation, detyrosination, acetylation, palmitoylation, and phosphorylation (Westermann and Weber, 2003; Verhey and Gaertig, 2007). Phosphorylation at the C-terminal tail of tubulin β has been reported previously in some organisms, although the responsible kinase has not yet been identified. In terms of the biological function, this phosphorylation was impli-

cated in the microtubule cold adaptation in Antarctic ciliate species (Pucciarelli et al., 1997) and in the differentiation of a mouse neuroblastoma cell line (Gard and Kirschner, 1985). In plant systems, studies on posttranslational modifications of tubulins are largely lacking. However, recent studies highlight the important roles of the tubulin structure in organizing cortical microtubule arrays and in influencing the growth direction of expanding cells. An extensive mutant screening based on the abnormal helical growth phenotype of mutated Arabidopsis plants resulted in a collection of tubulin missense or truncation mutants (Ishida et al., 2007). These mutants showed altered cortical microtubule arrays that correlated with the skewed growth phenotype of the target tissues. This study suggested that the microtubule assembly and dynamics are highly sensitive to changes in the secondary structure of tubulin isomers. It is possible that reversible posttranslational modification of tubulins may bring about a similar effect on the tubulin structure and assembly property.

Molecular dissections performed in our study showed that the unique sequence of the CTD is essential for the association of CKL6 with microtubules. A mammalian CK1 member, CK1δ, can be considered a mammalian homolog of CKL6 in terms of both phylogenetic prediction and the similar subcellular distribution. CK1δ was shown to associate with the interphase microtubules and vesicular structures derived from the Golgi (Behrend et al., 2000). Intriguingly, this dynamic localization of CK1δ is controlled only by the catalytic activity and is independent of its CTD (Milne et al., 2001). As for the CKL6-microtubule interaction, however, the CTD plays an essential role via its novel microtubule-binding domain. It is possible that the mechanism underlying the targeting of CKL6 may have evolved to support a specific biological function of CKL6 in the regulation of interphase microtubules or under a unique cellular environment of the cortical microtubule organization in plant systems.

The formation of jigsaw puzzle cell shapes is thought to be the result of interplay between microtubules and microfilaments (Fu et al., 2005; Smith and Oppenheimer, 2005). Specifically, microtubules are thought to be essential in the lobe formation by restricting cell expansion at the neck region between lobes, while F-actin accumulates at the outgrowing lobe regions. The Rho family of small GTPases and their novel effecter molecules are involved in regulating the lobe formation of epidermal pavement cells by modulating microfilaments and microtubules (Fu et al., 2005). The molecules acting upstream and downstream of these proteins have yet to be identified. Aberrant microtubule organization and cell morphologies induced by CKL6 support the contention that dynamic cortical microtubule organization is a critical factor for determining differential cell growth rates and shape formation. Our observation further indicates that CKL6 activity might play a regulatory role in this process by potentially modulating the microtubule stability or array organization. Demonstration of in vivo phosphorylation of tubulins at the presumed sites identified in this study and potential phosphorylation of MAPs, in addition to tubulins, by CKL6 will be essential future tasks to further elucidate the molecular mechanism underlying the relationship between CKL6 and microtubule regulation.

The evolutionary conservation of CK1 throughout all eukaryotes predicts that it has a fundamental role in plant cells even though the biological role of CK1 is not yet well documented in plants. Our finding that CKL6 is involved in microtubule regulation provides new insight into a basic cellular function of this family of kinase. Based on multifunctional characteristics of CK1 members in general as demonstrated in other organisms, it would be plausible that CKL6 is involved in multiple cellular processes in addition to the microtubule regulation. In this view, full understanding of the cellular function of CKL6 may require a comprehensive study of its potential role in membrane trafficking and the intercellular trafficking of macromolecules in conjunction with the regulation of cortical microtubules.

In summary, our study provides new insight into an important biological role for CKL6 in the regulation of interphase microtubule organization and anisotropic cell growth in plants.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana* ecotype Columbia-1) plants were grown under 16-h/8-h light/dark cycles. *Agrobacterium tumefaciens* cells (C58C1) transformed with binary vector plasmids were used to transform Arabidopsis plants by dipping the inflorescences in a bacterial suspension (Clough and Bent, 1998). Transgenic plants were selected based on resistance to the herbicide BASTA. Stable homozygous transgenic lines with single T-DNA insertions were screened by scoring genetic segregation ratios and used to produce hybrid F1 lines by reciprocal crossing. Arabidopsis seedlings were grown on 1% agar plates containing 0.5× Murashige and Skoog salts (Sigma) for 4 to 13 d. Overexpression lines were produced by transforming Arabidopsis plants or BY-2 tobacco (*Nicotiana tabacum*) cells with 35S::CKL6-GFP, 35S::CKL6, or 35S::CKL6-KI.

Plasmid Construction

The construction of various DNA cloning vectors that express fluorescent fusion proteins under the control of the cauliflower mosaic virus 35S promoter was described by Lee et al. (2005). A series of deletion mutants of CKL6 were cloned by amplifying specific fragments by PCR with a high-fidelity Taq polymerase followed by restriction enzyme digestion and ligation into the desired expression vectors. Each clone was verified by DNA sequencing prior to further experiments. Kinase-inactive CKL6 mutants were produced by PCR-based point mutagenesis. The K42R mutant was constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). For construction of the D132N mutant, a PCR fragment was amplified from pdGN-CKL6 with a forward primer containing the desired nucleotide changes (GAC to AAC), the *Bsm*I restriction enzyme site at the 5′ end, and a reverse primer corresponding to the C-terminal end of CKL6. The PCR fragment was then digested with *Bsm*I and *Eco*RI and replaced the corresponding region in the wild type, full-length CKL6 cDNA clone in pBluescript (Stratagene). The K42R D132N double mutant was produced by the same strategy but by replacing the equivalent region in the K42R mutant cDNA in pBluescript with the *Bsm*I and

*Eco*RI restriction fragments containing the D132N mutation. The CKL6 open reading frame or the resulting mutant cDNA was subcloned into a binary vector pMLBart to produce CKL6 or CKL6-KI transgenic Arabidopsis, respectively.

Confocal Imaging

Confocal images were acquired as described previously (Lee et al., 2005). Briefly, excised leaves were infiltrated with water and placed in a Lab-Tek II chambered coverglass system (no. 1.5 thickness; Nalge/Nunc International). Confocal images were acquired on either an inverted Zeiss LSM 510 NLO or Zeiss LSM5 DUO laser scanning microscope equipped with a META detector for spectral imaging, using the Zeiss 40X C-Apochromat lens (numerical aperture 1.2). Data acquisition was as follows. The 488-nm laser line of a 25-mW argon laser (LASOS) with a 500- to 530-nm band-pass emission filter was used for GFP. Images were captured as single optical sections or as a Z-series of optical sections. For renderings, three-dimensional data sets were displayed as single maximum intensity projections generated using Zeiss LSM software version 3.2 or 4.2.

In Vitro Binding Assays

Full-length open reading frame clones encoding Arabidopsis tubulin α6 and β3 were constructed by reverse transcription-PCR. Recombinant proteins were expressed and affinity purified as described (Lee et al., 2005). Purified bovine tubulins were purchased from Cytoskeleton. GST pull-down assays were performed basically as described (Rexach and Blobel, 1995). Bound and unbound fractions were separated on SDS-PAGE gels followed either by Coomassie Brilliant Blue staining or immunoblotting.

Phosphorylation Assays

Phosphorylation assays were performed as described by Lee et al. (2005). Briefly, recombinant CKL6 fused to GST was expressed in *Escherichia coli*, and affinity-purified proteins were used in 50-μL reaction volumes. The assay mixture contained 0.2 μg of enzyme (or 5 μL of tubulin/MAPs fraction), 1 μg of substrate, 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM EGTA, and 100 μM ATP mixed with 2 μCi of [γ-^{32}P]ATP (3,000 Ci mmol^{-1}). Following 15 min of incubation of the kinase assay mixture at 30°C, 12 μL of 6× SDS gel loading buffer was added to stop the reaction, and 25 to 40 μL of the mixture was separated on 10% SDS-PAGE gels. For the microtubule phosphorylation, 2.5 μg of preformed microtubule polymers (Cytoskeleton), bovine tubulins (Cytoskeleton), or bovine serum albumin was used as substrate. Following the phosphorylation by GST:CKL6, the reactions were centrifuged at 100,000*g* for 15 min to separate soluble and pellet fractions and resolved on SDS-PAGE gels. The gels were then stained with Coomassie Brilliant Blue, dried, and exposed to x-ray film for 2 to 7 h.

Tubulin β3 Mutagenesis

All of the selected sites were mutagenized by changing one nucleotide (A or T→G) in the first position of the target codons or two nucleotides (AG→GC) in the first and second positions for some Ser codons. The mutagenesis was done using the GeneTailor kit (Invitrogen) according to the protocol provided by the supplier. First, overlapping primers, one of which contained the target mutation, were synthesized for all of the selected sites. Second, 1 μg of pMBP:Tuβ3 DNA plasmid was methylated by DNA methylase at 37°C for 1 h. Then, the target mutant DNA was produced in PCR by amplifying the template DNA, using each overlapping primer set and a high-fidelity Taq polymerase. Last, the mutated DNA was transformed into an *E. coli* host (DH5α) followed by plasmid DNA isolation and sequence confirmation. The resulting tubulin β mutants were then expressed in *E. coli* (BL21, DE3/pLys) as MBP fusions, affinity purified using maltose agarose, and used in phosphorylation assays.

Preparation of the Microtubule/MAPs-Enriched Subcellular Fraction

Arabidopsis suspension cultured cells were used to prepare the microtubule/MAPs-enriched subcellular fraction basically as described elsewhere (Hussey et al., 1987; Chan et al., 1996). Briefly, Arabidopsis cells were maintained by subculturing weekly in Murashige and Skoog medium supplemented with hormones. For the preparation of the tubulin/MAPs-enriched subcellular fraction, the cultured cells were harvested at 5 d after transfer and treated with protoplasting buffer containing 20 mM MES, pH 5.7, 1.5% Cellulase R10, 0.4% Macerozyme R10, 0.4 M mannitol, and 20 mM KCl. The cytoskeleton pellet was prepared by extracting the protoplasts with PIPES buffer containing 50 mM PIPES, pH 6.9, 0.4 mannitol, 5 mM EGTA, 5 mM $MgSO_4$, 10% DMSO, 0.05% Nonidet P-40, and a mixture of protease inhibitors followed by washing with 2% Triton extraction buffer. Soluble tubulin/MAPs fraction was prepared by extracting the Triton-washed cytoskeleton pellet at 4°C with a microtubule-depolymerizing buffer containing 5 mM imidazole, pH 7.5, 3 mM $CaCl_2$, 1 mM ATP, 1 mM dithiothreitol, and protease inhibitors followed by a high-speed centrifugation.

Antibodies

CKL-6-specific polyclonal antibodies were raised in rabbits against a CLK-6 peptide, 351-RRNVRGPSPHQNHT-364, derived from the C-terminal region and affinity purified. Monoclonal tubulin antibodies (DM1A) and horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse were used according to the protocols provided by the supplier (Sigma).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY943845 (CKL6) and NP_568960 (TUB3).

Supplemental Data

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

Supplemental Figure S1. Amino acid sequence alignments showing conserved residues among Arabidopsis tubulin α and β isoforms and bovine tubulin β isoforms and Ser/Thr sites selected for Arabidopsis tubulin β3 mutagenesis.

Supplemental Figure S2. Preparation of the tubulin/MAPs-enriched fraction from Arabidopsis suspension cultured cells.

Supplemental Figure S3. Phenotypical analyses showing altered cortical microtubule pattern, cell shape, and seedling growth in transgenic plants that overexpress CKL6 or CKL6-KI.

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