A Plant-Specific Subclass of C-Terminal Kinesins Contains a Conserved A-Type Cyclin-Dependent Kinase Site Implicated in Folding and Dimerization

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Cyclin-dependent kinases (CDKs) control cell cycle progression through timely coordinated phosphorylation events. Two kinesin-like proteins that interact with CDKA;1 were identified and designated KCA1 and KCA2. They are 81% identical and have a similar three-partite domain organization. The N-terminal domain contains an ATP and microtubule-binding site typical for kinesin motors. A green fluorescent protein (GFP) fusion of the N-terminal domain of KCA1 decorated microtubules in Bright Yellow-2 cells, demonstrating microtubule-binding activity. During cytokinesis the full-length GFP-fusion protein accumulated at the midline of young and mature expanding phragmoplasts. Two-hybrid analysis and coimmunoprecipitation experiments showed that coiled-coil structures of the central stalk were responsible for homo- and heterodimerization of KCA1 and KCA2. By western-blot analysis, high molecular mass KCA molecules were detected in extracts from Bright Yellow-2 cells overproducing the full-length GFP fusion. Treatment of these cultures with the phosphatase inhibitor vanadate caused an accumulation of these KCA molecules. In addition to dimerization, interactions within the C-terminally located tail domain were revealed, indicating that the tail could fold onto itself. The tail domains of KCA1 and KCA2 contained two adjacent putative CDKA;1 phosphorylation sites, one of which is conserved in KCA homologs from other plant species. Site-directed mutagenesis of the conserved phosphorylation sites in KCA1 resulted in a reduced binding with CDKA;1 and abolished intramolecular tail interactions. The data show that phosphorylation of the CDKA;1 site provokes a conformational change in the structure of KCA with implications in folding and dimerization.

Although cell division is elementary to growth, the process itself only claims a small part of the complete plant cell cycle period. During that short time, the microtubular cytoskeleton undergoes major transitions and, consecutively, a preprophase band, spindle, and phragmoplast are formed (Vantard et al., 2000; Hasezawa and Kumagai, 2002). These microtubule (MT) arrays are the basis for scaffolds along which chromosomes are aligned and separated into daughter nuclei, and cell wall material is transported to the site where the new cell plate emerges. The exact order of events demands for a perfect orchestration of the action of many proteins. Phosphorylation is an important regulatory mechanism in the control of MT organization during the mitotic processes. Plant A-type cyclin-dependent kinases (CDKs), such as CDKA;1 in Arabidopsis, are the principal regulators of the orderly progression of cell cycle. CDKA;1 is associated with MTs in dividing and interphase cells (Stals et al., 1997; Hemsley et al., 2001; Weingartner et al., 2001) and is involved in the organization of the cytokkeleton during cell division. Short association of the CDKA;1-cyclin B complex to the preprophase band causes disintegration of this structure before nuclear envelope breakdown (Hush et al., 1996). Treatment of metaphase cells with inhibitors of CDKs results in abnormal spindles with chromosomes not aligned at the metaphase plate (Binarová et al., 1998). Therefore, CDK plays a major role in regulation of some of the steps that lead to microtubular rearrangements in dividing cells.

In yeast and animal cells, CDK regulates MT organization and function by controlling the activity or distribution of multiple proteins that are involved in MT arrangement and transport activities. CDK phosphorylates MT-associated proteins, which are important for MT dynamics and stability (Cassimeris, 1999; Andersen, 2000). MT-based motors, such as kinesinlike proteins (further referred to as kinesins), are one of the targets phosphorylated by CDK (Liao et al., 1994; Blangy et al., 1995). Kinesins belong to a large class of...
conserved genes that fall into nine subfamilies. In general, the different classes are involved in separate cellular processes related to cytoskeleton organization and intracellular transport (Moore and Endow, 1996). In dividing cells, they are implicated in the organization and stabilization of the spindle and phragmoplast structures, chromosome movement, and vesicular transport to the site of division (Reddy, 2001). The majority of kinesins consist of a motor domain with a catalytic core that binds MTs and hydrolyzes ATP to generate force and a tail domain that interacts with the cargo (Vale and Fletterick, 1997). The conformational organization and the quaternary structure of kinesins vary, depending on the subfamily, and reflect the wide range of functions with which they are associated and the complex regulatory mechanisms to which they are subjected as well. Although some kinesins operate as monomers, many form homo- or heterodimers, heterotrimer, or bipolar homotetramers (Reilie, et al., 2001). Oligomerization usually takes place by means of the interactions of a series of coiled coils that are located in the stalk domain. In addition to oligomerization, kinesins also have complex folding properties that provide the driving force to bind cargo or to control the activity of the motor domain. Several kinesins carry one or more putative CDK consensus Ser/Thr phosphorylation sites. For example, the bimC kinesin subfamily contains a phosphorylation site around a conserved sequence motif, called the bimC box. Phosphorylation of the embedded Thr in the human bimC kinesin Eg5 is a prerequisite for Eg5 to localize to the mitotic spindle and to ensure the formation of a bipolar organization of the spindle (Blangy et al., 1995; Sawin and Mitchison, 1995). Oligomerization usually takes place by means of the interactions of a series of coiled coils that are located in the stalk domain. In addition to oligomerization, kinesins also have complex folding properties that provide the driving force to bind cargo or to control the activity of the motor domain.

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We report the isolation and characterization of two Arabidopsis kinesin-like proteins that interact with CDKA;1, designated KCA1 and KCA2 (acronym for kinesin CDKA;1 associated). KCA1 and KCA2 are unusual kinesins in that they are unique to plants and possess an N-terminal motor domain that is most similar to that of the C-terminal subfamily of kinesins. We show that KCA1 and KCA2 dimerize through a coiled-coil region in the center and fold intramolecularly through interactions within the tail domain. These conformational properties were regulated by a phosphorylation-dependent control mechanism that involves a putative CDKA consensus phosphorylation site.

RESULTS
Cloning of KCA1 and KCA2

We screened a cDNA λ phage library with a partial kinesin fragment TH65 that had been isolated previously in a two-hybrid screen with CDKA;1 (De Veylder et al., 1997). Partial cDNAs were isolated that were elongated to a putative full-length cDNA of 3,816 bp by RACE-PCR and designated KCA1 (Arabidopsis gene At5g10470). Recently, the same TH65 fragment has been isolated in two other two-hybrid screens with the geminivirus AL1 protein and the Arabidopsis katanin p60 subunit as baits (Kong and Hanley-Bowdoin, 2002; Bouquin et al., 2003). A second, homologous cDNA was isolated from the library, corresponding to a gene designated KCA2, also on chromosome 5 (gene At5g65460). The comparison of the cDNA sequences with genomic DNA revealed a conservation of their gene structures, consisting of 23 exons that encoded putative proteins with 81% identity and 89% similarity. The deduced protein sequences carried an N-terminally located motor domain with the signature sequence SKLSLVDLAGSE and an ATP-binding motif or P-loop, which are the most common features of kinesin motor domains. The motor domain was preceded by a short stretch of approximately 140 amino acids that contained a coiled coil and a neck sequence carrying a conserved GN motif that is confined to minus-end-directed kinesins (Fig. 1B). The KCA proteins consisted of two additional domains, a coiled-coil region at the center, designated stalk domain, and a C-terminal tail domain containing a single coiled coil, a nuclear localization signal (NLS), and two Gly-rich regions of low complexity commonly present in kinesins (Fig. 1, A and B). The structural organization of the KCA kinesins was more closely related to the tripartite structure of plus-end-directed kinesins that have an N-terminally located motor domain. Because of sequence similarity with the group of C-terminal kinesins, KCA were tentatively classified within that group (Kim and Endow, 2000). Database sequence comparison of the tail domain to other kinesins and proteins indicated that although KCA1 and KCA2 were conserved in higher plant species, they did not share significant homology with any other currently described protein domain. A recent phylogenetic analysis of kinesin motor domains separates KCA1 and KCA2 in a distinct subclass with the minus-end kinesin KCBP from...
Arabidopsis as the closest relative (Dagenbach and Endow, 2004). Because KCA1 is potentially phosphorylated by CDKA;1, the presence of consensus CDK phosphorylation sites (S/T-P-X-K/R) was searched for. The tail contained putative A-type CDK phosphorylation sites at positions 698, 849, and 853 in KCA1 and at positions 827 and 831 in KCA2 (Fig. 1B). A single SPGR site in the N-terminal part of the tail domain was fully conserved in KCA-like sequences of other species (Fig. 1C).

Transcriptional Expression Patterns of KCA1 and KCA2

The presence of KCA mRNA transcripts was analyzed in different plant organs and developmental stages by reverse transcription (RT)-PCR with genespecific primers. DNA fragments of the expected size corresponding to KCA1 or KCA2 transcripts were visualized after hybridization (Fig. 2A). KCA1 and KCA2 mRNA were detected in young seedlings and in log-phase cell suspension cultures. KCA1 transcripts were more abundant and required a shorter film exposure to be visualized. Expression of KCA1 and KCA2 transcripts was found in all the organs tested (roots, leaves, stems, and flowers) and was more elevated in roots and flowers. Steady-state expression levels were observed for the ACT2 gene, which served as a loading control.

To determine the transcriptional activity of KCA1 and KCA2 during the course of the cell cycle, an Arabidopsis cell suspension culture was synchronized at G1/S by applying aphidicolin (Menges and Murray, 2002). Samples were prepared from 1-h intervals after

![Figure 1. Secondary structure and domain organization of the Arabidopsis KCAs. A, Coiled-coil domains of KCA1 (dotted line) and KCA2 (dashed line) predicted by the algorithm of Lupas et al. (1991). The abscissa represents the amino acid residue number and the ordinate the probability of coiled-coil formation. B, Composition of KCA1 and KCA2; the N-terminal head contains the motor domain (light gray box), preceded by a neck region (black box) with the residues -GN-. Coiled-coil domains (dark gray boxes) were found in the centrally located stalk domain. Additional coiled-coils were present in the head and tail domain. The C-terminal tail carries a NLS sequence -HKRRK- and two hinge regions (H). KCA1 and KCA2 share two adjacent consensus CDK phosphorylation sites (T/S-P-x-K/R) in the tail domain (black arrowheads). An additional phosphorylation site is present in the tail of KCA1 (gray arrowhead). C, Alignment of the conserved CDK phosphorylation sites -SPGR- (underlined) in KCA-related kinesins in Glycine max (GlymaKCA, accession no. AW200832), Oryza sativa (OrysaKCA, accession no. AQ794870), and Solanum tuberosum (SoltuKCA, accession no. BF053293).

![Figure 2. Expression analysis of KCAs in different organs and in synchronized Arabidopsis cells. A, Semiquantitative RT-PCR analysis of the transcript levels of KCA1 and KCA2 mRNA in 3-week-old Arabidopsis organs, 1-week-old seedlings, and 3-d-old cell suspension. RNA was extracted from roots (R), leaves (L), stems (St), flowers (F), seedlings (S), and cell suspensions (C). The Arabidopsis gene actin 2 (ACT2) was used as loading control. B, KCA mRNA levels during cell cycle. Aphidicolin-treated Arabidopsis MM2d cells were released from the G1/S block and RNA samples analyzed by RT-PCR (top panel) at the indicated succeSSive time points. The metaphase/anaphase index is shown in the bottom panel.](https://www.plantphysiol.org/content/135/4/1419/F1.large.jpg)
drug release and RNA subjected to RT-PCR analysis. The transcriptional levels of KCA1 and KCA2 were constant throughout the cell cycle (Fig. 2B). This is consistent with the ubiquitous expression pattern revealed by immunoblot and immunolocalization by Kong and Hanley-Bowdoin (2002).

**Interaction of KCA with CDKA;1**

The CDKA;1-binding sites of KCA1 and KCA2 were determined by two-hybrid analysis whereby positive interactions were defined by the ability to grow on medium without His. The different KCA1 and KCA2 fragments used for the analysis are presented in Figure 3A. The CDKA;1-binding sites were mapped to the head domain (KCA11497 and KCA2186-307), the centrally located stalk region (KCA1195-926, KCA11660-866, KCA21425-617, and KCA21425-664), and the N-terminal part of the tail domain (KCA11660-862 and KCA21655-864). In addition, the full-length KCA1 protein showed CDKA;1-binding affinity (Fig. 3A). The interaction strength of the individual peptide fragments

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**Figure 3.** Identification of CDKA;1-binding sites in KCA1 and KCA2. A, The various KCA fragments fused with the GAL4 DNA-binding domain (pGBT9) are shown at left. The CDKA;1 gene was cloned in frame with the GAL4 transcription activation domain (pGAD424). Cotransformants (+) were spotted from an equal cell suspension culture on selective medium (−Leu, −Trp, and −His). As a negative control, the pGBT9-KCA fragments were cotransformed with the empty pGAD424 vector (−) and spotted on the same medium. Different concentrations of 3-AT were tested. The associated numbers correspond to the maximum dose of 3-AT (in mM) that allowed growth. B, Coimmunoprecipitation of 35S-Met-labeled HA-tagged CDKA1 with different 35S-Met-labeled c-Myc-tagged versions of KCA1 (KCA11-497, KCA11660-862, and KCA11875-1273; left) and KCA2 (KCA21425-617, KCA21655-864, and KCA21855-1267; right). c-Myc-KCA fragments (circles) were immunoprecipitated with anti-c-Myc antibody (left column of each panel) or with an anti-HA antibody after mixing with the HA-CDKA1 (middle column of each panel). As a positive control, HA-CDKA1 (arrow) was incubated with the anti-HA antibody (right column of left panel). Molecular markers indicate protein size in kD at the left of each panel. C, Western blotting of p10CKS1At affinity purifications of wild-type BY-2 cells (WT) and transgenic lines carrying GFP-KCA11-1273 (tail), GFP-KCA11-1273 (motor), and noninduced (−) and induced GFP-KCA11-1273 (KCA1). Thirty micrograms of protein from crude extract (CE) and p10CKS1At supernatant (SN) was loaded on gel. The p10CKS1At pellet corresponds to purified protein from 300 µg of crude extract. In the lower panel, CDKA is detected after stripping and probing the membrane with an anti-PSTAIRE antibody (1:2,500). The right panel shows a western blot of 30 µg GFP-KCA11-1273 protein extracts from control cells (Co) and cells grown for 24 h in the presence of 10 mM vanadate (Van). Membranes were developed with polyclonal anti-GFP antibody (1:1,000). Arrows indicate the positions of the fusion proteins. Molecular size markers (kD) are shown on the left.
and full length was estimated by including the HIS3 competitive inhibitor 3-aminotriazole (3-AT) in the growth medium. The strongest interactions were observed with KCA fragments that included the conserved CDK phosphorylation site SPGR, with the exception of the complete tail domain (KCA1 660-1,273) that did not interact with CDKA;1. These data indicate that the three separate KCA domains contribute to the interaction with CDKA;1 and that the C-terminal part of the tail has an inhibitory effect on the binding with CDKA;1.

The KCA-CDKA;1 interaction was also analyzed by coimmunoprecipitation experiments using a coupled transcription-translation system in which the KCA1 and KCA2 fragments were tagged with c-Myc and CDKA;1 with hemagglutinin epitope (HA; Fig. 3B). In the control experiment, the c-Myc-tagged peptides precipitated with a c-Myc monoclonal antibody, confirming the correct synthesis of the KCA peptide fragments. Next, the c-Myc- and HA-tagged translation products were mixed and pulled down with monoclonal anti-HA antibodies. The KCA1 peptide fragments containing either the N-terminal tail (KCA1 660-862) or the head (KCA1 1-497) cosedimented with HA-CDKA;1, whereas the fragment containing the C-terminal tail domain (KCA1 677-1,273) did not (Fig. 3B, left panel). Similar results were obtained with peptide fragments of KCA2. The stalk (KCA2 425-615) and the N-terminal part of the tail (KCA2 555-864) were pulled down together with HA-CDKA;1, whereas the C-terminal tail (KCA2 855-1,260) was not (Fig. 3B, right panel). The results confirmed the KCA and CDKA;1 interactions that had been revealed by the two-hybrid analysis. None of the KCA fragments was able to bind with the anti-HA antibody in the absence of HA-CDKA;1 (data not shown).

To investigate the association of KCA1 with CDKA;1 in vivo, CDK-protein complexes were purified from Bright Yellow-2 (BY-2) transgenic cell cultures that produced green fluorescent protein (GFP)-tagged versions of the full-length and fragments containing the motor (KCA1 1-497) or the tail domain (KCA1 660-1,273; Fig. 3C). Extracts of the transgenic cultures and control wild-type BY-2 cells were mixed with p100\(^{KCA1}\)CDKA;1 affinity beads. Crude extract, pellet, and supernatant were analyzed by western blot and developed with polyclonal GFP antibody. As shown in Figure 3C, GFP-fusion products corresponding to the predicted molecular mass were detected in the separated crude BY-2 extracts. Compared to the noninduced protein extract (—), three kinesin-related protein products were present in the preparations from dexamethasone-induced cells producing the full-length kinesin GFP-fusion protein. The 160-kD band corresponded to the intact GFP-KCA1 protein, whereas the smallest 40-kD protein resulted from degradation or prematurely arrested translation. The high molecular mass band at approximately 250 kD may represent a GFP-KCA1 dimer. The presence of kinesin dimers in denaturing polyacrylamide gels has been reported before (Fontijn et al., 2001). Pull-down assays with p100\(^{KCA1}\) beads indicated a strong interaction of CDKA;1 with KCA1, confirming the interaction between the full-length construct and CDKA;1 in two-hybrid experiments (Fig. 3, A and C). The head also interacted with CDKA;1, whereas the tail GFP fusion did not cosediment and remained in the soluble fraction. In light of the two-hybrid interaction data and the coimmunoprecipitation experiments, KCA1 seems to be arranged into multiple folding configurations with differential CDKA;1-binding capacities. Together, three CDKA;1-binding sites were revealed by these experiments, one in the motor domain, one in the stalk, and one in the N-terminal half of the tail.

**Localization of KCA1 in BY-2 Cells**

An important property of kinesin molecules is their ability to attach to MTs either for transport or control of MT organization (Walczak, 2003). To investigate the in vivo protein localization, we fused KCA1 and KCA subdomains to the C-terminal end of GFP and transformed tobacco BY-2 suspension cells. The GFP-KCA1 fusion product (KCA1 1-1,273) resided in the cytoplasmic space excluded from the vacuoles and the nucleus (Fig. 4A). Scanning of the cell periphery and center by confocal optical sectioning did not reveal specific labeling of the cortical or endosomal array, indicating that the full-length GFP-fusion protein did not bind the interphase microtubular structures. By contrast, the N-terminal half of KCA1 containing the motor domain (KCA1 1-497) associated with MTs (Fig. 4B). Cortical and endocytosplasmic MTs were brightly fluorescent in freshly transformed calli. These observations demonstrated that the motor domain as a separate entity exhibited MT-binding activity. Some kinesins, such as the calmodulin-binding kinesin KCBP, carry a nucleotide-independent MT-binding site outside of their motor domain to facilitate MT sliding (Narasimhulu and Reddy, 1998; Kao et al., 2000). Therefore, we analyzed the KCA1 tail domain (KCA1 660-1,273) and expressed it as a GFP-fusion protein in BY-2 cells. Fluorescence was observed in the cytoplasm and in the nucleus without association with microtubular structures (Fig. 4C). Surprisingly, both the motor and the tail GFP-fusion proteins were present in the nucleus, although the motor accumulated at much higher concentrations than the tail (Fig. 4C). Putative nuclear localization signals were identified in the tail domain but not in the motor (Fig. 1). CDKA;1 also concentrates in the nucleus without a classic NLS-targeting signal and associates tightly with interphase chromatin (Weingartner et al., 2001). In addition, it binds MTs similarly to what we observed with the GFP-fused KCA1 motor domain (Geelen and Inzé, 2001). Because CDKA;1 interaction with chromatin is resistant to mild nonionic detergent extraction, we performed a similar extraction procedure on BY-2 cells that produced the GFP-motor or the
GFP-tail domains. Figure 4, D to I, shows that GFP-CDKA;1 and GFP-motor were retained in the nucleus, whereas free GFP (data not shown) or the GFP-tail was readily and completely removed upon the detergent washes. Therefore, the motor protein must be tightly bound to the cellular matrix, presumably as part of a protein complex that possibly also contains the CDKA;1 protein.

The full-length GFP-KCA1 fusion protein was followed during cell division (Fig. 4J). Throughout mitosis, the fusion protein remained in the cortical cytoplasm and the cytoplasmic strands, and it invaded the unrestricted space of the spindle in metaphase and anaphase cells (Fig. 4J). Fluorescence was diffuse and did not reveal fibrous structures, indicating that the fusion protein did not attach to MTs (Fig. 4J). Once the daughter chromosomes were separated, GFP-KCA1 fluorescence accumulated at the midline of the emerging phragmoplast where Golgi-derived vesicles accumulate to form the cell plate (Fig. 4I). In a second stage of cell plate development, concomitant with expansion of the phragmoplast, fluorescence was most bright at the leading edges (Fig. 4J). Reduced fluorescence was observed at the center of the centrifugally expanding phragmoplast, where the cell plate starts to mature and MTs are depolymerized.

In contrast to the findings of Kong and Hanley-Bowdoin, GFP-KCA1 did not concentrate in the nucleus, nor did it associate with condensed chromosomes in metaphase cells. As the N-terminal domain in front of the motor domain may be implicated in nuclear targeting or chromosome binding, we analyzed the subcellular localization of a C-terminal fusion of KCA1 in BY-2 cells. KCA1-GFP was excluded from the nucleus and vacuoles (Fig. 4K). In the cytoplasm, it was associated with a reticulated network resembling the endoplasmic reticulum (ER; Fig. 4K). During division (Fig. 4L), KCA1-GFP was distributed to the polar sides of the spindle and the midline of the phragmoplast reminiscent to the subcellular localization of an ER-targeted marker in BY-2 cells (Saint-Jore et al., 2002).

Figure 4. Intracellular localization of GFP-fused KCA derivatives in BY-2 cells. A, Localization of GFP-KCA11-1,273 (full length) in the cytoplasm and exclusion from the nucleus. B, Labeling of the cytoplasmic and cortical MTs (inset) by the motor domain (GFP-KCA11-497). The motor domain is abundantly present in the nucleus. C, Presence of the tail domain (GFP-KCA660-1,273) throughout nucleus and cytoplasm.

D to I, Detergent extraction of BY-2 cells carrying GFP-CDKA;1 (D, E), GFP-KCA11-497 (F, G), and GFP-KCA1660-1,273 (H, I). Images were taken before (D, F, and H), and after (E, G, and I), extraction with Triton X-100 (0.1%). GFP-CDKA;1 and GFP-KCA11-497 were removed from the cytoplasm but remained attached to the nuclear matrix. By contrast, GFP-KCA1660-1,273 was removed both from the nucleus and the cytoplasm.

J, Time series of N-terminally tagged GFP-KCA1 fluorescence throughout cell division. At metaphase (0′) and anaphase (5′), GFP-KCA1 is present in the cytoplasm of the cell cortex and spindle region. During cytokinesis (16′–41′), GFP-KCA1 labels the midline (arrow) and the forming cell plate (arrowhead). Cell plate labeling decreases when the cell plate has reached the mother cell wall (84′). K, Association of C-terminally tagged KCA1-GFP to a reticulate network at the cell periphery. L, Time series of KCA1-GFP subcellular localization throughout the cell cycle. Interphase (0′), metaphase (21′), early cytokinesis (92′), late cytokinesis (99′), and completed cell plate (114′). Bar = 10 μm.
KCA1 and KCA2 Display Complex Oligomerization

Several observations suggested that the KCA kinesins adopt different folding configurations with distinct properties in terms of interaction with CDKA;1 and in relation to their subcellular localization. Firstly, the N-terminal part of the tail domain could bind CDKA;1 only when the C-terminal part was not included. The inhibitory activity of the C-terminal part of the tail was not evident when the full-length proteins were tested, indicating that these had taken on an alternative configuration immune to control by the tail domain. Secondly, the full-length KCA1 GFP-fusion product was excluded from the nucleus while the head and tail as separate GFP fusion fragments entered the nucleus. Thirdly, MTs were not labeled with GFP fused to full-length proteins but with the GFP-head fusion product. Therefore, we examined the intra- and intermolecular interactions of KCA1 and KCA2 peptides by two-hybrid and immunoprecipitation assays.

Full-length KCA and peptide fragments containing the complete or part of the central coiled-coil region resulted in yeast growth (combinations pGBT-KCA1 473-866 and pGAD-KCA2 425-637, with pGBT-KCA2 425-864), indicating that both KCA1 and KCA2 could form homodimers (Fig. 5A). Evidence for heterodimerization through the central coiled-coil region followed from yeast growth when the stalk domains of both kinesins were tested against each other (combinations of both pGBT-KCA2 425-617 and pGBT-KCA2 425-864 with pGAD-KCA1 473-866).

Two-hybrid interactions were also observed between the N-terminal and the C-terminal halves of the tail of KCA1 (combination pGBT-KCA1 660-862 with pGAD-KCA1 875-1,273) and KCA2 (combination pGBT-KCA2 425-864 with pGAD-KCA2 855-1,267), pointing out that the tail domains had a tendency to fold onto themselves. A similar type of interaction also occurred between the N- and C-terminal tail domains of KCA1 and KCA2 (combinations pGBT-KCA1 660-862 with pGAD-KCA2 855-1,267 and pGBT-KCA1 875-1,273 with pGAD-KCA2 425-864). The folding of the KCA tails probably occurred via bending of two predicted hinge regions that were present in the tail domain (Fig. 1B). The tail fragment upstream of the first hinge region was essential for the interaction (Fig. 5), whereas that downstream of the second hinge did not interact in two-hybrid tests, indicating that the tail fragment between the two hinges was responsible for the interactions observed (combination pGBT-KCA1 1,067-1,273 and pGAD-KCA1 875-1,273, and pGBT-KCA2 1,052-1,267 with pGAD-KCA2 425-864, and the reciprocal combinations). The tail interactions of KCA1 and KCA2 were confirmed by coimmunoprecipitation assays (Fig. 5B). Protein fragments containing the N-terminal part of the tail of KCA1 or KCA2 pulled down a KCA1 fragment containing sequences downstream of the first hinge region (Fig. 5B).

Site-Directed Mutagenesis of the Conserved CDKA;1 Phosphorylation Site Implicated in Kinesin Folding

We showed that KCA1 and KCA2 formed homodimers and that both proteins can occur in a folded conformation. In addition, both proteins bind to CDKA;1 and contain CDKA;1 phosphorylation sites.
in the tail domain. Hence, we investigated whether CDKA;1 phosphorylation was implicated in KCA dimerization and folding.

Western blot of BY-2 cells transformed with GFP fused to the full-length KCA1 protein (Fig. 3C) revealed two high molecular mass bands, one of which probably represents a dimeric form of GFP-KCA1. To test the role of phosphorylation in dimer formation, a phosphatase inhibitor was applied to the cells. BY-2 cells transformed with GFP-KCA1 were treated with vanadate (10 mM; Brown et al., 1999), and equal amounts of proteins of control nontreated and treated cells were loaded on a polyacrylamide gel and blotted (Fig. 3C). The concentration of the 250-kD band increased, at the expense of the 160-kD band (Fig. 3C). These results show that phosphatase inhibition favored the formation of KCA dimers.

To investigate whether CDKA;1 phosphorylation could have implications for CDKA;1 binding and KCA folding, we introduced mutations in the putative phosphorylation sites at positions 698 to 701 (TPNK) and 841 to 848 (SPGR/SPVR) in the pGBT-KCA1660-862 sequence that contains the N-terminal tail of KCA1. Thr (T698) and Ser (S841 and S845) were replaced by either an Ala (A) as a nonphosphorylatable residue or by a Glu (E) that mimics the phosphorylated residue (Table I). We assessed the effects these changes had on the ability to interact with either CDKA;1 or with the KCA tail by means of two-hybrid analysis. Replacement of T698 by either an A or E had no consequences on the interaction with CDKA;1. On the contrary, substitution of the consensus sequences further downstream, S841 or S841/S845 (S841 and S845 double substitution) with E residues, disallowed the yeast strain to grow on selective medium. Replacement of these residues by an A had little or no effect. These results indicated that CDKA;1 binding was sensitive to the phosphorylation status of residues S841 and S845.

The same mutagenized KCA1660-862 fragments were tested against the C-terminal tail fragments KCA1875-1,273 and KCA2855-1,267 in the pGAD vector. Alteration of T698 in either A or E had no effect on the tail interactions. However, substitution of S841 into an E residue strongly reduced the interaction with the C-terminal tail regions of both KCA1 and KCA2, while replacement of both S841 and S845 completely abolished the interaction (Table I). Changing these residues by an A did not alter the growth of yeast. The results suggest that phosphorylation at the consensus sequences 841-844 and 845-848 in KCA1 and 827-830 in KCA2 influences the protein conformation that has important consequences concerning their activity.

**DISCUSSION**

CDKA governs control over the progression of the cell division processes through the interaction with several partners and selective phosphorylation of target proteins. Despite considerable efforts, only few potential targets phosphorylated by CDKA complexes have been identified in plants so far (Reindl et al., 1997; Nakagami et al., 1999; Boniotti and Gutierrez, 2001). The kinesin KCA1 is a novel candidate target that is phosphorylated in a cell cycle kinase-dependent manner in insect cells (Kong and Hanley-Bowdoin, 2002). We report on the interaction of KCA1, and a highly homologous protein KCA2, with CDKA;1 and the role of a conserved CDKA phosphorylation site in dimerization and folding.

KCA1 and KCA2 share high sequence identity and have a conserved structural organization that is reminiscent to classic kinesin molecules. The N-terminal region contains an ATP-loop and an MT-binding site and is most similar to the motor domain of the C-terminal subfamily of kinesins (Kim and Endow, 2000). This class of kinesins typically has a conserved neck sequence that precedes the core motor domain, which is sufficient and necessary to direct these molecules toward the minus-end of MTs (Vale and Hanley-Bowdoin, 2000).

**Table 1. Mutational analysis in the stalk region of the KCA1**

<table>
<thead>
<tr>
<th>Point Mutations</th>
<th>CDKA;1</th>
<th>KCA1875-1,273</th>
<th>KCA2855-1,267</th>
<th>Empty Vector</th>
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<tbody>
<tr>
<td>T698 S841</td>
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</table>

Point mutations were introduced at the CDKA;1 phosphorylation sites 698 to 701 (TPNK), 841 to 844 (SPGR), and 845 to 848 (SPVR) into the KCA1875-1,273 fragment in the pGBT9 backbone. The nucleotide changes resulted in nonphosphorylatable or phosphorylation mimicry of CDKA;1 sites. The resulting amino acid residue substitutions in the pGBT9-KCA1875-1,273 fragment were shown on the left. The mutated pGBT9-KCA1875-1,273 vectors were tested in two-hybrid assays against the CDKA;1, KCA1875-1,273, and KCA2855-1,267 in the pGAD vector and against the empty pGAD424 vector to check for self-activation (last column). As control, the nonmutated pGBT9-KCA1875-1,273 vectors were used (first row), + and −, Positive and negative interactions, respectively. The increments in + correspond to the different 3-AT concentrations (0, 5, 10, and 15 mM) that allowed growth on the selective medium.
Fletterick, 1997). The same motif has been found in that of KCA1 and KCA2, suggesting that these kinesins confer minus-end-directed motility.

The calmodulin-binding protein KCBP is a minus-end kinesin that belongs to the C-terminal clad, which is nearest to that containing KCA1 and KCA2 (Dagenbach and Endow, 2004). The KCA kinesins are peculiar because they consist of N-terminal motor proteins that are all of plant origin. We searched the public available databases for KCA-like sequences and found representatives from plant species only. Therefore, the KCA kinesins seem to have evolved to a separate class of kinesins unique to plants. To prove that KCA kinesins are able to bind MTs, we have demonstrated that the GFP-tagged KCA1 head was associated with cortical and endocytic MTs in BY-2 cells. The association with MTs occurs when the motor domain is fused to GFP independently from the tail domain and is not observed with a fusion containing the full-length protein. Thus, MT-binding activity is strictly regulated through an activity residing in the C-terminally located tail domain.

Binding of MT with KCBP is controlled by calcium through the interaction with calcium calmodulin (Narasimhulu et al., 1997), which binds next to the motor domain near the C-terminal end, thereby preventing the MT binding of KCBP and disallowing stimulation of motor ATPase activity (Deavours et al., 1988). The KCA1 and KCA2 sequences do not contain a predicted calmodulin-binding site, suggesting that MT binding is controlled by another molecule. Alternatively, MT binding and motor activity could also be controlled directly by an interaction between the tail and motor domains, a phenomenon referred to as tail inhibition (Coy et al., 1999). However, two-hybrid experiments with different peptide fragments did not uncover the existence of such a type of interaction in KCA. At this point, we do not know what controls the MT-binding activity. It has been well established that phosphorylation regulates motor activity in a number of cases (Reilein et al., 2001). The stimulation of the human kinesin Eg5, for example, is accomplished by phosphorylation of a Ser in the motor domain. Upon phosphorylation of this particular residue by the cell cycle kinase p34<sub>cdc2</sub>, association with the spindle MTs is stimulated and the bipolar organization of the spindle ascertained (Blangy et al., 1995). In the case of other motor proteins, such as dynein and kinesin II, phosphorylation events control MT-binding and motor activity indirectly by modulating the interacting proteins dynein and kinesin light chain, respectively (Lindesmith et al., 1997; Reese and Haimo, 2000). The Arabidopsis KCBP interacts with a protein kinase KIPK, suggesting that in addition to control by calcium, KCBP targeting is regulated by KIPK-mediated phosphorylation (Day et al., 2000). To test whether phosphorylation is important for the targeting of KCA, we applied drugs that inhibit phosphorylation by BY-2-producing GFP-tagged KCA full-length protein or fragments. No effect on the localization was observed suggesting that modulation of the phosphorylation status of KCA was not crucial for the interaction with MTs (data not shown). Because the MT-binding property of the GFP-KCA head was lost upon continuous propagation of the transgenic BY-2 cell lines, we decided to analyze the properties of the tail domain in more detail.

KCBP carries an MT-binding site in the N-terminally located tail domain that is independent from calmodulin (Narasimhulu and Reddy, 1998). A computer-assisted analysis of the KCA1 and KCA2 tails did not reveal a known MT-binding signature. Instead, we found a NLS motif and hinge regions that are important for the flexibility and, hence, the folding properties of the tail (Kirchner et al., 1999). The GFP-tagged KCA1 tail leads to an accumulation of fluorescence in the nucleus in agreement with the presence of an NLS. However, GFP-tagged full-length KCA1 was totally excluded from the nucleus. Neither the N- nor C-terminally tagged KCA1 entered the nucleus, indicating that the NLS was not activated in these protein fusions. Kong and Hanley-Bowdoin (2002) demonstrated by means of immunolocalization experiments, in which an antiserum was used that did not discriminate KCA1 and KCA2, that abundant KCA epitopes are concentrated in the nuclei of Nicotiana benthamiana leaves and root cells. In addition, they showed accumulation of label at the chromosomes in chemically fixed mitotic cells. We interpret the absence of the full-length GFP-KCA1 protein in BY-2 nuclei and from condensed chromosomes as an inability of these fusion molecules to undergo the necessary refolding and/or interactions that are needed for nuclear import and chromosome binding.

As certain folding configurations may no longer have been possible because of the presence of the GFP moiety, a different subcellular localization of KCA was revealed. C-terminally tagged KCA1-GFP appeared to associate with the ER. It concentrated at the polar sides of the metaphase spindle where ER and Golgi derived organelles are known to congregate (Nebenfuhr et al., 2000; Saint-Jore et al., 2002). KCA antisera also decorated the spindle poles in N. benthamiana immuno-localizations reported by Kong and Hanley-Bowdoin (2002). During cytokinesis, N-terminally tagged GFP-KCA1 labeled the phragmoplast midline, and fluorescence intensity was strongest at the edges of the expanding phragmoplast. This localization pattern is reminiscent to that of Golgi-derived vesicles accumulating at the phragmoplast midline through an MT-dependent transport mechanism, involving the action of one or more kinesins (Smith, 2002). At this point, however, it is uncertain whether KCA1 contributes to the transport of Golgi vesicles. Targeting of vesicular compartments to the midline would require a plus-end-directed kinesin because of the antiparallel organization of the phragmoplast MTs. The AtPAKRP2 kinesin is in that respect a more likely candidate for Golgi-vesicle transport to the growing cell plate (Lee et al., 2001). The ER, to which the KCA1-GFP was
targeted, also accumulates at the phragmoplast midline and may have functions that are unrelated to deposition of cell plate forming vesicles (Nebenfuhr et al., 2000; Saint-Jore et al., 2002). To resolve a possible function in cytokinesis, it will be necessary to investigate cell lines or plants in which KCA is inactivated.

The KCA proteins have been shown to interact with CDKA;1 in two-hybrid assays (De Veylder et al., 1997; Kong and Hanley-Bowdoin, 2002; our results). Because kinesin motor activity is often tightly coupled to regulatory phosphorylation and because the tail domains of KCA1 and KCA2 carry putative CDKA;1 phosphorylation sites, we analyzed the KCA domains responsible for CDKA;1 binding. Two-hybrid experiments and immunoprecipitation assays indicated that the motor domain, the central region, and the tail domain interacted independently with CKDA;1. For two proteins to interact, they have to colocalize at a given point in time. Several studies have shown that CDKA;1 is predominantly nuclear (Stals et al., 1997; Weingartner et al., 2001). We found that the N-terminal motor (head) and the tail fragments were both targeted to the nucleus. GFP-CDKA;1 and the GFP-tagged KCA1 head fragment were attached to the nuclear content in a Triton X-100-resistant manner. This observation points toward a possibility that both proteins are in a complex in interphase nuclei. Further support came from pull-down experiments that show a strong interaction between the KCA1 head and CDKA;1. CDKA;1 has also been shown to interact with interphase and mitotic MTs, presumably because of dynamic association with cellular targets attached to the MT cytoskeleton (Stals et al., 1997; Hemsley et al., 2001; Weingartner et al., 2001; Joubès et al., 2003). Throughout mitosis, GFP-tagged KCA1 remained in the cytoplasm and did not appear to associate with preprophase band, spindle, or phragmoplast MTs. Because immunolocalization data and the GFP-fusion analysis did not indicate a prevailing association of KCA with MTs, other proteins than KCA must be responsible for targeting CDKA;1 to the MTs (Kong and Hanley-Bowdoin, 2002).

The coiled coils in the stalk region have been implicated in kinesin oligomerization that is necessary for proper control of motility and cargo binding (Vale and Fletterick, 1997). The conventional kinesin I from neurons is a typical example that forms homodimers for progressive movement along the MT with at least one motor domain in contact with the MT at all times (Bloom et al., 1988). KCA1 and KCA2 carry three coiled coils in the stalk domain. Two-hybrid and immunoprecipitation experiments suggested that the stalk contributes to homo- and/or heterodimerization of KCA1 and KCA2. Indeed, a doublet protein band with molecular masses corresponding to theoretically predicted weights of homomeric and dimeric fusion protein appeared in western blots from BY-2 extracts that produced GFP-KCA1. In the presence of the broad phosphatase inhibitor vanadate, cell extracts contained more dimers, indicating that phosphorylation events play a role in the control over the ratio of dimers and homomers. The fluorescence pattern was unaltered in the vanadate-treated cells, indicating that dimerization was not sufficient to target KCA to MTs. Because CDKA;1 may phosphorylate the KCA tail to trigger a conformational shift, which, in turn, drives the dimerization of KCA1, it is possible that in the presence of the phosphatase inhibitor, less dimer is cycled back to the monomeric form. Alternatively, CDKA;1 may bind to the stalk domain and compete with stalk-stalk interactions under conditions that are affected by vanadate.

The KCA tail domain carries a CDKA;1 phosphorylation site that is conserved in all KCA-like kinesins found in the publicly available databases. Therefore, this site is the best candidate for a general role in the functioning of the KCA kinesins. Two-hybrid and immunoprecipitation experiments revealed that the N-terminal part of the tail domain interacted with CDKA;1 as well as intramolecularly between the N-terminal part of the tail and a downstream region flanked by the two hinges. These interactions are probably mutually exclusive because the tail, when tested in its entirety, did not bind CDKA;1. The folding of the tail fragment would have prevented an interaction with CDKA;1.

How could CDKA;1 affect the conformational changes and functioning of KCA? The phosphorylation, dimerization, and the internal tail interactions are probably interdependent and may be implicated in phosphorylation-controlled activation and/or binding of cargo. Point mutations in the putative CDKA phosphorylation sites of the KCA1 tail abolished the intramolecular tail interaction. Thus, KCA molecules not phosphorylated at the Ser residues in the tail would have a compact folding conformation. This conformational stage might keep the KCA inactive until modulated by the cell cycle-controlled CDKA;1 kinase. The opening up of the tail would prepare the single KCA molecules to bind the cargo they need to transport. Alternatively to the stimulation of cargo binding upon phosphorylation, it is also possible that the opened-up kinesin tail no longer prevents the homo- or heterodimerization that is driven by the stalk domain.

MATERIALS AND METHODS
Isolation and cDNA Characterization of KCA1 and KCA2
As a DNA phage library of Arabidopsis (L.) Heynh, ecotype Columbia 0 was used to screen for the full-length clone matching fragment TH65 that had been isolated previously in a two-hybrid experiment with CDKA;1 as bait (De Veylder et al., 1997). Two new cDNA fragments were isolated and sequenced that corresponded to the C-terminal region of the KCA1 gene, including a poly(A) tail region of 226 bp. The KCA2 gene was found by sequence homology with KCA1. The complete 5′ terminus of KCA1 and the full length of KCA2 were isolated by RACE-PCR according to the manufacturer’s instructions (CLONTECH, Palo Alto, CA) with the primers 5′-ATGCCGCTAGCTAGAAGTTAACAC-3′ and 5′-GCCCAAAACTTTGTTCCAGATCTCG-3′ for KCA1 and 5′-ATGCGGAGCAGAAAGAGGATCTA-3′ and 5′-GAGATTATCTTCGCTTGTCAG-3′ for KCA2. The reported nucleotide sequences
Sequence Analysis

The Arabidopsis KCA proteins and KCA homologs were aligned by the Clustal method (PileUp) from the GCG Wisconsin package version 10.1 program (Accelrys, San Diego) without penalizing gaps. A set of analysis tools was applied for a compressive sequence interpretation. As BLAST programs, the programs AtBlast (http://www.arabidopsis.org/blast/) (Huala et al., 2001) and WU-BLAST2 (http://dove.embl-heidelberg.de/Blas2/; Altschul et al., 1990) were used to search databases for homologous sequences in Arabidopsis and other organisms. The Arabidopsis sequence map and gene redundancy were studied with different tools from the Munich Institute for Protein Sequences (Martinsried, Germany; http://mips.gsf.de/proj/tml/db/index.html). Protein domains were analyzed with SMART (http://smart.embl-heidelberg.de/; Schultz et al., 2000), general motifs were predicted and estimated with the algorithm of Lupas et al. (1991) by using the program COILS (http://www.ch.embnet.org/software/COILS_form.html).

CDKA;1 Phosphorylation Site Implicated in Kinesin Folding

The CDKA;1 interaction site was mapped by constructing deletion fragments generated by PCR for the two-hybrid assays were used to generate the pBSK-c-Myc and pBSK-HA vectors (Stratagene). The Arabidopsis CDKA;1 was recloned from the pGADCDK/A1 vector in the pBSK-HA vector with EcoRI and BamHI restriction sites. Plasmids were sequenced to verify in-frame cloning with the c-Myc tag and HA tag.

In Vitro Transcription-Translation and Immunoprecipitation

The same KCA deletion fragments generated by PCR for the two-hybrid experiments were used to generate the pBSK-c-Myc and pBSK-HA vectors (Stratagene). The Arabidopsis CDKA;1 was recloned from the pGADCDK/A1 vector in the pBSK-HA vector with EcoRI and BamHI restriction sites. Plasmids were sequenced to verify in-frame cloning with the c-Myc tag and HA tag.

Growth, Transformation, and Fluorescence Microscopy

The KCA1 full-length open reading frame and fragments were cloned behind the open reading frame of enhanced GFP by using the GATEWAY system (Invitrogen). GATEWAY-compatible vectors were designed by inserting the EGFP-coding region and the GATEWAY rfA cassette into the pBin19 backbone. The expression of the fusion was under the control of the cauliflower mosaic virus 35S promoter and 35S polyadenylation signal. For the construction of the inducible vector, a similar strategy was followed. The backbone. The expression of the fusion was under the control of the cauliflower mosaic virus 35S promoter and 35S polyadenylation signal. For the construction of the inducible vector, a similar strategy was followed. The backbone. The expression of the fusion was under the control of the cauliflower mosaic virus 35S promoter and 35S polyadenylation signal.

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inducible expression vector were induced overnight on BY-2 agar containing 10 μM dexamethasone. Callus material was transferred on a slide with a coverslip, and observed with an epifluorescence microscope (Axioplan 2; Zeiss, Jena, Germany) equipped with a fluorescein isothiocyanate filter set. GFP-positive calli were transferred to liquid BY-2 medium with selection and grown as cell suspensions.

Confocal images were taken with a scanning confocal microscope (LSM 510; Zeiss) with argon laser illumination at 488 nm and a fluorescein isothiocyanate filter set. For transmission light images, differential interference contrast optics was used. Images were taken with 25% laser power to reduce photobleaching.

**Detergent Extraction of Cells**

Cells were incubated in liquid BY-2 medium containing 0.1% Triton X-100 (Sigma-Aldrich) for 15 min with gentle agitation. Cells were washed twice in BY-2 medium, transferred to a slide, and covered with a coverslip. The detergent-extracted cells were observed directly with the confocal microscope.

**Protein Gel-Blot Analysis**

Three-day-old liquid cultures of BY-2 transgenic lines, also used for GFP-localization experiments, and wild-type BY-2 cells were ground in liquid nitrogen with a mortar and pestle and homogenized in ice-cold P10 buffer (25 mM Tris-HCl, pH 7.6, 15 mM ethylene glycol-bis(β-aminoethyl) ether-acetate, 1 mM dithiothreitol, 15 mM MgCl2, 85 mM NaCl, 15 mM pN6P, 60 mM glycerol phosphate, 0.1% Nonidet P-40, 1 mM NaF, 0.1 mM Na3VO4, and 100 μL protease inhibitor cocktail; Sigma-Aldrich). The homogenate was centrifuged at 10,000g for 10 min in an Eppendorf centrifuge 5417 at 4°C to remove cell debris. The supernatant was then centrifuged at 14,000g for 10 min. A sample was taken and kept on ice as crude extract. Then, 50 μL of 50% (v/v) p10CKS1At Sepharose beads was added to 300 μg of proteins and incubated at 4°C for 1 h on a rotating wheel. The beads were collected by centrifugation; the supernatant was removed and kept on ice. Beads were washed three times with bead buffer (50 mM Tris-HCl, pH 7.5, 5 mM NaF, 25 mM NaCl, 5 mM EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 0.1 μM benzamidine, and 0.1 mM Na3VO4). Loading buffer (Laemmli, 1970) was added and the samples were heated for 10 min at 95°C. After centrifugation at 14,000g for 4 min, 30 μg crude extract protein, 30 μg p10CKS1At superantigen, and p10CKS1At pellet purified from 300 μg initial crude extract was separated on a 12% gel. The amount protein loaded was verified in a separate gel by Coomassie blue staining. Gels were blotted onto nitrocellulose membranes (Hybond-C super; Amersham Biosciences) in 190 mM Gly and 25 mM Tris with a liquid miniblotting system (Bio-Rad, Hercules, CA) for 1 h. The protein on the blot was detected using a 1:2,500 dilution of cdc2 PSTAIRE antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody at a dilution of 1:10,000. Membranes were stripped at 60°C for 30 min and 5% skim milk (BD Difco; Becton-Dickinson, Franklin Lakes, NJ). For blocking, the membranes were incubated in a blocking buffer containing 3% skim milk for 2 h at room temperature. CDKA protein was detected using a 1:2,500 dilution of antibodies to the bovine brain CDKA/cyclin D complex. J Biol Chem 275: 3409–3416

**Site-Directed Mutagenesis**

Point mutations were introduced by PCR site-directed mutagenesis in the pGTF-KCA1 plasmid with the Advantage polymerase mix (CLON-TECH). The linear PCR product was circularized by ligation. The nucleotide changes were verified by sequencing the KCA1 inserts in two directions.

**Distribution of Materials**

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AX499336 and AX449307.

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**LITERATURE CITED**


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