A Tobacco Calcium/Calmodulin-binding Protein Kinase Functions as a Negative Regulator of Flowering*

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A tobacco calcium/calmodulin-binding protein kinase (NtCBK1) was isolated and identified. The predicted NtCBK1 protein has 599 amino acids, an N-terminal kinase domain, and shares high homology with other calmodulin (CaM)-related kinases. Whereas NtCBK1 phosphorylates itself and substrates such as histone IIIS and syntide-2 in the absence of CaM, its kinase activity can be stimulated by tobacco CaMs. However, unlike another tobacco protein kinase designated NtCBK2, NtCBK1 was not differentially regulated by the different CaM isoforms tested. The CaM-binding domain of NtCBK1 was located between amino acids 436 and 455, and this domain was shown to be necessary for CaM modulation of kinase activity. RNA in situ hybridization showed that NtCBK1 was highly regulated in the transition to flowering. Whereas NtCBK1 mRNA was accumulated in the shoot apical meristem during vegetative growth, its expression was dramatically decreased in the shoot apical meristem after floral determination, and in young flower primordia. The expression of NtCBK1 was up-regulated to high levels in floral organ primordia. Fluctuations in NtCBK1 expression were verified by analysis of tobacco plants expressing green fluorescent protein under the control of the NtCBK1 promoter, suggesting a role of NtCBK1 in the transition to flowering. This conclusion was confirmed by overexpressing NtCBK1 in transgenic tobacco plants, where maintenance of high levels of NtCBK1 in the shoot apical meristem delayed the switch to flowering and extended the vegetative phase of growth. Further work indicated that overexpression of NtCBK1 in transgenic tobacco did not affect the expression of NFL, a tobacco homologue of the LFY gene that controls meristem initiation and floral structure in tobacco. In addition, the promotion of tobacco flowering time by DNA demethylation cannot be blocked by the overexpression of NtCBK1.

Calcium (Ca$^{2+}$) plays important roles as a second messenger in plant developmental and physiological processes via a group of Ca$^{2+}$ target proteins, including CaM and Ca$^{2+}$-dependent protein kinases (CDPKs), two well known Ca$^{2+}$-binding proteins (1, 2). CaM is ubiquitously expressed and has no enzymatic activity of its own. The activities and expression patterns of a variety of CaM-binding proteins including calcium/CaM-dependent protein kinases (CaMks) are key players in Ca$^{2+}$/CaM-mediated signaling pathways in plants (3–5).

In contrast to animal systems, there are only a few reports on CaM-binding protein kinases (CBKs) in plants. To date, CBKs have been isolated from apple (CB1, a homolog of mammalian CaMK II, 6), maize (MCKs, 7, 8), rice (OsCBK, 9), and chimeric CaM-dependent protein kinases (CaMks) from lily, tobacco, and maize (10–12). Whereas all of these CBKs have been shown to have CaM binding activity, only tobacco NtCBK2, rice OsCBK2, and CCaMKs have been shown experimentally to possess kinase activity. CaMks have been shown to have a visinin-like domain and three EF-hand motifs that are necessary for Ca$^{2+}$-dependent autophosphorylation and for maximal activation (10–12). The enzymatic activity of OsCBK, on the other hand, was found to be independent of either calcium or CaM (9).

Considerable attention has recently been paid to the analyses of the expression profiles of plant CBKs (5, 13). These analyses have indicated that CCaMks, MCKs, and OsCBK are regulated temporally and spatially during various stages of plant development (5, 8, 9, 12, 14), as well as by environmental triggers such as light. For example, red light down-regulates the expression of maize ZmCCaMK (15). It has also been reported that overexpression of maize MCK1 in tobacco leads to the abortion of flower primordia on the main shoot axis, implying a role for MCK1 in flower development (16). Because little information is available about the roles of CBKs, their biological functions remain to be elucidated.

The transition from vegetative to reproductive growth is a key event in the life cycle of plants. The transition has been intensively studied (17), and the regulatory networks controlling the process are perhaps best understood in Arabidopsis (18, 19). Environmental factors, including photoperiod (day length), light quality (spectral composition), light quantity (photon flux density), cold temperature (vernalization), and nutrient and water availability play important roles in flowering time control in Arabidopsis (20). Not all plants use the same environmental triggers to bring about a change from vegetative to reproductive growth. For example, tobacco shows important differences in its flowering response when compared with Arabidopsis. Tobacco flowers in response to internal cues, such as plant size or number of vegetative nodes (18), and the

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‡ The abbreviations used are: CDPK, Ca$^{2+}$-dependent protein kinase; CaMK, calcium/CaM-dependent protein kinase; CBK, CaM-binding protein kinase; CCaMK, chimeric CaM-dependent protein kinase; GFP, green fluorescent protein; Ni-NTA, nickel-nitrilotriacetic acid; FITC, fluorescein isothiocyanate; SAM, shoot apical meristem.
duration of the vegetative phase is independent of day length, and regulated by as yet unidentified developmental signals (21, 22). An example of the type of developmental signal that can regulate flowering time comes from overexpression of the Antirrhinum gene CENTRORADIALIS in tobacco, which brings about an extension of the vegetative growth phase and delays the switch to flowering (23). Further investigations on exactly which events mark the transition to flowering and their regulation is needed (24).

Protein kinases have been shown to be involved in many cellular processes, including regulation of metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, differentiation, and responses to a variety of stimuli (25, 26). Several protein kinases, such as protein kinase CK2 and never in mitosis A (NIMA)-like kinase, have been reported to be involved in the regulation of flowering plants. Protein kinase CK2, interacting with circadian clock-associated 1, has been shown to play an important role in the regulation of the circadian clock in Arabidopsis (19). Plants overexpressing CKB3, a regulatory subunit of CK2, increases CK2 activity, resulting in reduced flowering time (27, 28). NIMA-like kinase, on the other hand, interacts with 14-3-3 proteins and SELF-PRUNING, a member of a family of modulator proteins, to regulate tomato shoot architecture and flowering (29).

Here we present evidence for a role of the tobacco Ca2+/H1001/CaM-binding protein kinase NtCBK1 in flowering. NtCBK1 is identified as a CBK based on molecular and biochemical evidence. NtCBK1 binds CaM in a Ca2+/H1001-dependent manner and phosphorylates both itself and substrates. Whereas kinase activity assays indicate that this kinase has enzymatic activity in the absence of CaM, this activity can be stimulated up to 5-fold by CaM. The expression of NtCBK1 in the shoot apical meristem is highly regulated during the transition to flowering as shown by both RNA in situ hybridization and transgenic analyses with GFP driven by the NtCBK1 promoter. Transgenic tobacco plants overexpressing NtCBK1 display a late-flowering phenotype, suggesting that NtCBK1 could function as a negative regulator of flowering. Further work indicated that overexpression of NtCBK1 did not affect flowering by influencing either the expression of NFL, a tobacco homologue of LFY that controls meristem initiation and floral structure, or by blocking demethylation, a treatment that promotes flowering in tobacco.

EXPERIMENTAL PROCEDURES

Tobacco CDNA Library Screening—mRNAs were isolated from tobacco (Nicotiana tabaccum cv. W38) and used for cDNA library construction with a ZAP-cDNA synthesis kit following the manufacturer’s instructions (Stratagene). The cDNA library was screened with plasmid p550 containing a maize CDNA encoding MCK1 (7). Positive recombinant plasmids were sequenced.
Construction of NtCBK1 and Truncated NtCBK1—To define the CaM-binding domain, several truncated constructs were made with the pFastHTb expression vector (Fig. 2A). The cDNAs for the full open reading frame (NtCBK1) and three truncated forms (T1–455, T1–416, and T436–599) of NtCBK1 were amplified with 4 pairs of primers as follows: 5' primer CCGGATCCATGGGGCACTGCTGCAGTAAGG and 3' primer CCGGATCCCTTATCGATGATGTCTTGTGCTTGAACC for NtCBK1; 5' primer CCGGATCCATGGGGCACTGCTGCAGTAAGG and 3' primer CCGGATCCCTTAGAGGTAGATTAACTCTTC for T1–455; 5' primer CCGGATCCATGGGGCACTGCTGCAGTAAGG and 3' primer CCGGATCCCTTATCGATGATGTCTTGTGCTTGAACC for T1–416; 5' primer CCGGATCCAAACGTGCAGCATTGAAGGCTC and 3' primer CCGGATCCTTATAAAACAGGATTTTCCGTCC for T1–455, but lacks the tentative CaM-binding domain. The third truncated form, T436–455, contains the C terminus of NtCBK1 and the tentative CaM-binding domain. The fourth truncated form, T436–599, has the same N-terminal amino acid sequence as T1–455, but lacks the kinase catalytic domain, several truncated constructs were made with the pFastHTb expression vector (Fig. 2A). The cDNAs for the full open reading frame (NtCBK1) and three truncated forms (T1–455, T1–416, and T436–599) of NtCBK1 were amplified with 4 pairs of primers as follows: 5' primer CCGGATCCATGGGGCACTGCTGCAGTAAGG and 3' primer CCGGATCCCTTATCGATGATGTCTTGTGCTTGAACC for NtCBK1; 5' primer CCGGATCCATGGGGCACTGCTGCAGTAAGG and 3' primer CCGGATCCCTTAGAGGTAGATTAACTCTTC for T1–455; 5' primer CCGGATCCATGGGGCACTGCTGCAGTAAGG and 3' primer CCGGATCCCTTATCGATGATGTCTTGTGCTTGAACC for T1–416; 5' primer CCGGATCCAAACGTGCAGCATTGAAGGCTC and 3' primer CCGGATCCTTATAAAACAGGATTTTCCGTCC for T1–455, but lacks the tentative CaM-binding domain. The third truncated form, T436–599, contains the C terminus of NtCBK1 and the tentative CaM-binding domain. PCR products were digested with BamHI and subsequently cloned into the BamHI site of plasmid pFastHTb. After sequencing confirmation, recombinant plasmids were transformed into DH10Bac competent cells containing the bacmid with a mini-att Tn7 target site and helper plasmid. The mini-Tn7 element on the pFastHTb donor plasmid can transpose to the mini-att Tn7 element on the bacmid in the presence of transposition proteins provided by the helper plasmid. Clones containing recombinant bacmid were identified based on the disruption of the lacZ gene. The Sf9 cells were maintained as monolayer cultures in Grace's medium containing 10% fetal bovine serum and transfected by the recombinant bacmid with Cell-line 293 cells. Recombinant virus was harvested after 72 h and identified by X-gal staining. The supernatant was harvested after 72 h and identified by bioluminescence. The recombinant virus for 72 h and harvested at room temperature. Cells were washed once with Grace's medium, resuspended in 5 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride), sonicated for 30 s, centrifuged at 12,000 × g for 10 min. The supernatant was applied to a Ni-NTA resin column pre-equilibrated with buffer A (50 mM potassium phosphate, pH 6.0, 300 mM KCl, 1% glycerol). After extensive washing with buffer B followed by buffer A containing 50 mM imidazole, NtCBK1 was eluted with buffer A containing 200 mM imidazole. The eluted NtCBK1 was dialyzed in 25 mM Tris-HCl, pH 7.5, supplemented with 0.5 mM CaCl₂ for about 6 h, and loaded on a Ma-Sepharose 4B (Amersham Biosciences) column pre-equilibrated with buffer B (25 mM Tris-HCl, pH 7.5, 2 mM CaCl₂). After washing with buffer B plus 200 mM NaCl, elution of NtCBK1 was with buffer C (25 mM Tris-HCl, pH 7.5, 2 mM EGTA). After dialysis in 25 mM Tris-HCl, pH 7.5, for 6 h, purified NtCBK1 was either used immediately or stored at −80 °C prior to SDS-PAGE or enzymatic analyses. Protein concentration was determined by quantification of tryptophan in NtCBK1 (30). All procedures were performed at 4 °C unless stated otherwise.

Preparation of [35S]Labeled and Biotinylated CaM—Tobacco calmodulin (NtCaM1/3/13) cDNAs cloned into the pET15 expression vectors (a gift from Dr. Y. Ohashi, National Institute of Agrobiological Sciences) were used to prepare [35S]-labeled CaM as described previously (32, 33). Bovine CaM was biotinylated as described by Billingsley et al. (34). Briefly, bovine CaM was dialyzed overnight at 4 °C against 0.1 M phosphate buffer, pH 7.4. The bovine CaM was supplemented with 1 mM CaCl₂ and incubated with N-biotinoyl-N-amidocaproic acid-N-hydroxysuccinimide ester (Sigma) dissolved in N,N-dimethylformamide at a final concentration of 1 mM. The incubation was performed for 2 h at 4 °C with constant stirring. The biotinylated CaM was further dialyzed in 0.1 mM phosphate buffer, pH 7.4, for 48 h at 4 °C.
bovine serum albumin as standard (35) and stored in 20% glycerol at 
−20 °C.

**CaM Binding Assay**—NtCBK1 and its truncated forms were sepa-
rated by SDS-PAGE and blotted onto a polyvinylidene difluoride mem-
brane. For biotinylated CaM binding assays, the membrane was 
blocked in 2% bovine serum albumin/Tris-buffered saline (50 mM Tris-
HCl, pH 7.5, 200 mM NaCl, 50 mM MgCl₂, plus 0.5 mM CaCl₂ or 2 mM 
EGTA) and washed three times with Tris-buffered saline for 15 min 
each. After incubation in Tris-buffered saline containing biotinylated 
CaM for 3 h at room temperature and then washing with Tris-buffered 
saline, the membrane was treated with avidin-horseradish peroxidase 
conjugate (Bio-Rad) dissolved in Tris-buffered saline for 1 h. Protein 
bound to biotinylated CaM was visualized by color development with 
4-chloro-1-naphthol and H₂O₂.

**Assay of NtCBK1 CaM Binding Affinity**—CaM binding affinity was 
assayed as described (9, 10). Briefly, purified NtCBK1 protein (4 pmol) 
separated by SDS-PAGE was electrophoretically transferred onto nit-
trocellulose filters and incubated with different concentrations of ³²P-
labeled CaM (0.5 × 10⁶ cpm/µg) in 1 mM CaCl₂ overnight. After washing 
with 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM CaCl₂, the 
radioactivities of the bound CaM on each filter, and free CaM in the 
incubation buffer collected before washing, were measured by liquid 
scintillation counting (Beckman LS 6500). Average background counts 
were subtracted from the counts in NtCBK1 protein samples when 
calculating the specific binding. The dissociation constant Kᵣ was cal-
culated by using SPSS EnzymeKinetics software (SPSS Science, Inc.). 
The data points are expressed as means of the results from three 
independent assays in duplicate.

**Autophosphorylation of NtCBK1**—Autophosphorylation was carried 
out in a 100-µl reaction mixture containing 25 mM Tris-HCl, pH 7.5, 0.5 
mM dithiothreitol, 10 mM magnesium acetate, 100 μM ATP, 10 μCi of 
³²P-ATP (5000 Ci/mmol) plus 0.5 mM CaCl₂ or 0.5 mM CaCl₂, 1 μM 
CaM at 30 °C. For time course assays, 1 µg of NtCBK1 was used in a 
100-µl reaction mixture. Aliquots for the zero time point were taken 
immediately after the addition of NtCBK1 and the reactions were 
terminated by adding 1/5 volume of 5% SDS-PAGE sample buffer. All 
 aliquots were separated by SDS-PAGE with a 10% separating gel. After 
staining with 0.1% Coomassie Brilliant Blue, gels were vacuum-dried 
and exposed to x-ray film at 80 °C. The amount of phosphate trans-
ferred to the enzyme was determined by counting the radioactivities of 
the excised NtCBK1 bands in a liquid scintillation counter. Experi-
ments were repeated three times in duplicate.

**Substrate Phosphorylation Assay**—Substrate phosphorylation as-
says were performed in a 100-µl reaction mixture containing 25 mM
Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 10 mM magnesium acetate, 100 μM ATP, 10 μCi of [γ-32P]ATP (5000 Ci/mmol), 100 μM histone IIIS, or 100 μM syntide-2 plus 0.5 mM CaCl₂ or 0.5 mM CaCl₂, 1 μM CaM with 1 μg of NtCBK1 at 30 °C.

For time course assays with histone IIIS as substrate, aliquots for the zero time point were taken immediately after the addition of NtCBK1 and the reaction was terminated by adding 1/5 volume of 5× SDS-PAGE sample buffer. Aliquots were separated and counted as described above. The experiments were repeated three times in duplicate. For time course assays with syntide-2 as substrate, the experiments with two parallel reactions for different treatments were repeated three times. Aliquots for the zero time point were taken immediately after the addition of NtCBK1 to initiate the reaction and the reaction was terminated by adding 1/5 volume of 5× SDS-PAGE sample buffer. Aliquots from one reaction were separated by SDS-PAGE with a 10% separating gel. The amount of phosphates transferred to the kinase was determined by counting the radioactivities of the excised NtCBK1 bands in a liquid scintillation counter. Aliquots from another reaction were applied to P81 phosphocellulose filters (2 × 2 cm squares, Whatman) for total 32P incorporation of the kinase and syntide-2. Filters were washed four times for 10 min each in 75 mM phosphoric acid, and rinsed in 100% ethanol and then air-dried. 32P incorporation was determined by liquid scintillation counting. The amount of phosphates transferred to substrate syntide-2 was determined by subtracting 32P incorporation of NtCBK1 from total 32P incorporation of NtCBK1 plus syntide-2.

Activations Assay of NtCBK1 by NtCaM Isoforms—The reaction for NtCBK1 substrate phosphorylation was performed as described above. Aliquots (10 μl) were removed and applied to P81 phosphocellulose filters (2 × 2 cm squares, Whatman). Filters were washed four times for 10 min each in 75 mM phosphoric acid and rinsed in 100% ethanol and
RNA was prepared by using TRIzol (Invitrogen). For each sample, 20 μl of DNA was digested with EcoRV and separated by agarose gel electrophoresis.

The predicted size of the cDNA. The deduced amino acid sequence of NtCBK1 consists of 599 residues with a calculated molecular mass of 66.0 kDa.

Cloning of NtCBK1 Promoter—The NtCBK1 promoter region was isolated with an in vitro PCR cloning kit (TaKaRa Biotechnology, Shiga, Japan). Ten micrograms of tobacco genomic DNA was digested with the HindIII restriction enzyme, ligated with the HindIII cassette, and amplified by PCR according to the manufacturer’s instructions. PCR products were cloned into the pH101-GFP vector, and the resulting plasmid was named pNtCBK1-GFP.

Genetic Transformation and Flowering Time Examination—The NtCBK1 cDNA was obtained by PCR with primers: 5′-primer CGGATCATGAGGTTGAGCTGAGGTCGC, and 3′-primer GAGATTTGCTTTGGTGCTTGAAC, using pNtCBK1 as template and cloned into the SK-1. The BamHI-digested DNA fragment was then cloned into the BamHI site of pBlm that was made by adding three restriction digestion sites and deleting the GUS sequence of pBl121 and confirmed by DNA sequencing. Then, both the construct and pH1121 were transferred into Agrobacterium tumefaciens strain LBA4404 and introduced into tobacco. For NtCBK1 RNAi constructs, the coding sequence of either NtCBK1 or NtCBK2 were amplified by PCR and double inserted into BamHI or XhoI/SacI sites with different orientations of pBl1 that was made by placing the fourth intron of Arabidopsis thaliana into the BamHI/XhoI site of pBlm. Regenerated kanamycin-resistant transgenic plants were transferred to soil and grown in the greenhouse. When the terminal flower was formed, all of the transgenic plants that never flowered, the number of nodes per shoot was recorded by counting the number of nodes on the primary shoot from the last leaf (1 cm long) produced to the most basal leaf.

To search for a putative function of NtCBK1, a data base comparison was conducted to identify homology between NtCBK1 and other genes with known functions. NtCBK1 contains all 11 subdomains characteristic of the protein kinase catalytic domain (38), and shares high sequence identity with CBKs and CDPK-related protein kinases, including maize MCK1 (GenBankTM accession number 1839597; 69%) (7), OsCBK1 (GenBankTM accession number AF435452; 59%) (39), and NtCBK2 (GenBankTM accession number AF388282; 56%) (9), carrot CDPK-related protein kinases (GenBankTM accession number 1103386; 59%) (39), and NtCBK2 (GenBankTM accession number AF435452; 59%) (40), suggesting that NtCBK1 could be a Ca2+-dependent protein kinase. NtCBK1 was analyzed with Anthewin software indicated that the amino acid sequence at positions 436–455 of NtCBK1 could form a CaM-binding α-helix domain (33, 42). These structural features strongly suggest that NtCBK1 is a CaM-binding protein kinase.

Characterization of the CaM-binding Domain of NtCBK1—To identify the putative CaM-binding domain of NtCBK1, four expression constructs (NtCBK1, T1–416, T1–455, and T436–599) were made to express full and truncated forms of NtCBK1 (Fig. 2A). Whereas T1–455 contained the N-terminal catalytic domain, and a putative CaM-binding domain (at positions 436–455) at its C terminus, T1–416 had the same amino acid sequences of T1–455, but lacked the C-terminal CaM-binding amino acid residues 417–455. Construct T436–599 encoded the C terminus of NtCBK1 lacking the kinase catalytic domain, but having the N-terminal 20-amino acid residues at positions 436–455 for the putative CaM-binding domain. These constructs were used to infect Sf9 cells, and the expressed proteins purified by Ni-NTA affinity chromatography were used for Western blot analyses with biotinylated CaM. Proteins expressed by NtCBK1, T1–455, and T436–599 bound CaM in a Ca2+-dependent manner, whereas proteins expressed from T1–416 did not bind CaM (Fig. 2B and C), demonstrating a CaM-binding domain within the region from residues 417 to 599.

It has been reported that diverse CaMBPs have different binding affinities with different CaM isoforms, resulting in subtle regulation by different concentrations of either CaM or CaMBPs (43, 44). In tobacco, 13 NtCaMs have been identified.
and placed into three groups based on sequence homology (31).
NtCaM1/2 have high similarity to potato PCaM1 and are placed in group I, NtCaM3–12 are similar to most plant CaMs such as soybean SCaM and are placed in group II, and NtCaM13 is a homologue of soybean SCaM4 and is a member of group III. To explore whether NtCBK1 binds various CaM isoforms with different affinities, NtCaM1, -3, and -13 were selected as representatives of members of the three groups of tobacco CaM isoforms. Four pmol of NtCBK1 was immobilized on membranes and incubated with increasing concentrations of 35S-labeled NtCaM isoforms until binding was saturated. The binding of 35S-labeled NtCaM to NtCBK1 was measured directly by measuring radioactivity in the CaM-NtCBK1 complex. The $K_d$ of NtCaMs for NtCBK1 were estimated to be 26.0 ± 1.6 nM for NtCaM1, 26.3 ± 1.4 nM for NtCaM3, and 23.6 ± 0.8 nM for NtCaM13. The binding of all CaMs to NtCBK1 was completely blocked in the presence of EGTA.

**Enzymatic Properties of NtCBK1**—To establish that NtCBK1 is a protein kinase and to analyze its enzymatic properties, autophosphorylation and substrate phosphorylation by NtCBK1 were analyzed. For these experiments, recombinant NtCBK1 was purified by Ni-NTA and CaM-affinity chromatography and shown to be a single 68,000-dalton polypeptide by SDS-PAGE, consistent with the predicted molecular mass of the fusion protein (Fig. 3A).

Because of the high similarity between NtCBK1 and CDPK/CBK, Ca$^{2+}$ and EGTA were used in the kinase assays. As shown in Fig. 3, NtCBK1 phosphorylated itself, and substrates such as histone IIIS and syntide-2, in the presence of both Ca$^{2+}$ and EGTA. Maximal autophosphorylation activity was achieved in the presence of Ca$^{2+}$/CaM, but autophosphorylation could also be stimulated ~4-fold by Ca$^{2+}$ alone (Fig. 3C). Phosphorylation of histone IIIS and syntide-2 by NtCBK1 was stimulated 3- and 5-fold, respectively, by Ca$^{2+}$ (Fig. 3, D and E). Kinetic parameters of NtCBK1 kinase activity were determined from double-reciprocal analyses of data after phosphorylation of various substrate concentrations in the presence of 100 μM ATP. The $K_m$ for histone IIIS was 16.2 ± 0.7 μM, and for...
syntide-2 it was 9.5 ± 0.4 μM. With histone IIIS as substrate, the $V_{\text{max}}$ was 32.4 ± 1.3 nmol/min/mg in the presence of Ca$^{2+}$, and increased to 87.6 ± 3.4 nmol/min/mg in the presence of CaM. With syntide-2 as substrate, $V_{\text{max}}$ with CaM was 649.5 ± 21.8 nmol/min/mg, and with Ca$^{2+}$ alone it was 115.7 ± 7.2 nmol/min/mg. Capillary electrophoresis analysis, which has been reported to be a successful tool to identify amino acid residues (36), demonstrated that serine residues of NtCBK1 were autophosphorylated, indicating that NtCBK1 was a serine protein kinase (Fig. 4).

Previous work has demonstrated that different CaM isoforms may regulate CaM-binding proteins differentially because of the presence of either 1-5-10 or 1-8-14 CaM-binding motifs. It has also been reported that different CaM isoforms differential modulate the activity of plant CaM-binding protein kinases such as CCaMK and NtCBK2 (12, 40) when assayed with NtCaM isoforms 1, 3, and 13. Unlike CCaMK, the CaM-binding domain of NtCBK1 shares both 1-8-14 (KRAALK-SKALTEEELILY) and 1-5-10 (LSKALTEEEL) motifs, making it likely that the CaM modulated activity of NtCBK1 is different from that of CCaMK. We used the tobacco CaM isoforms NtCaM1, NtCaM3, and NtCaM13 to test this hypothesis. Our results show that whereas the kinase activity of NtCBK1 was stimulated by the tobacco CaMs, activity amplification was the same for all three CaM isoforms, suggesting that regulation of NtCBK1 by CaM is different from that of CCaMK (Fig. 3). This conclusion was supported by the observation that similar $K_a$ values for NtCBK1 activity were obtained when its activity was assayed with the addition of increasing amounts of the three tobacco CaMs in the presence of 0.5 mM Ca$^{2+}$ (Fig. 5). When histone IIIS was employed as a substrate in the presence of NtCaM1, NtCaM3, and NtCaM13, half-maximal $K_a$ of NtCBK1 was 12.6 ± 1.1, 14.1 ± 1.4, and 11.8 ± 0.5 nM, respectively (Fig. 5A). With syntide-2 as substrate, NtCaM1, NtCaM3, and NtCaM13 gave half-maximum $K_a$ values with NtCBK1 of 10.2 ± 0.8, 12.3 ± 1.0, and 12.9 ± 0.3 nM, respectively (Fig. 5B).

To explore a possible role of the CaM-binding domain in the regulation of NtCBK1 activity, protein products from constructs T1–455 and T1–455 were used for kinase assays. Both T1–416 and T1–455 were shown to autophosphorylate and phosphorylate substrates such as histone IIIS either in the absence or presence of Ca$^{2+}$/CaM as effectively as full-length NtCBK1 (Fig. 6), suggesting that the C terminus of NtCBK1 was not absolutely needed for the basal activity of the kinase. However, whereas the kinase activity of T1–455 could be stimulated by CaM, the activity of T1–416 was not, indicating that the CaM-binding domain was necessary for CaM regulation of the kinase activity.

**Expression of NtCBK1 in Tobacco**—To understand the possible roles of NtCBK1, its expression in various tissues of tobacco plants was examined to provide clues to its physiological functions during development. Gene-specific probes from the 3′

![Fig. 6. Phosphorylation assays of truncated forms of NtCBK1. Autophosphorylation and substrate phosphorylation using histone IIIS as substrate of the truncated forms of NtCBK1, T1–455, and T1–416 were performed in the presence of Ca$^{2+}$ or Ca$^{2+}$/NtCaM3 and resolved by SDS-PAGE.](image-url)
The terminal flower on the main shoot axis is the first flower formed, and axillary bud meristems generated at nodes below the main shoot apex subsequently develop into lateral flowers and the same old control plants showing the phase of senescence are photographed.

Furthermore, the expression of NtCBK1 was up-regulated to high levels in floral organ primordia (Fig. 7B, d). These results indicated that while NtCBK1 mRNA accumulated in meristems in the vegetative growth phase and in axillary buds before floral determination, its expression was down-regulated to very low levels in meristems during the floral determination phase. However, NtCBK1 expression subsequently increased to a high level in floral organ primordia as flowers developed. No positive reaction was detected on the sections hybridized with the sense RNA (Fig. 7B, b).

To further confirm this pattern of NtCBK1 expression in the transition to reproductive development in tobacco, 1.2 kb of the 5′-upstream region of NtCBK1, with the predicted TATA box and a putative transcription start site (as shown by a data base search of plant cis-acting regulatory DNA elements, Ref. 46), was isolated from tobacco genomic DNA and fused with the GFP reporter gene as construct pNtCBK1:GFP (Fig. 8A). pNtCBK1:GFP and a construct in which GFP was fused with the 35S promoter were introduced into tobacco by A. tumifaciens-mediated transformation, and GFP expression in the transgenic plants was examined. Fluctuations in GFP fluorescence were not observed in plants in which GFP expression was driven by the 35S promoter (Fig. 8D), however, there was strong fluorescence in axillary bud meristems (Fig. 8B) and very little in young floral primordia (Fig. 8, B and C) in plants in which GFP was under the control of NtCBK1 promoter. When flowers were formed, GFP was strongly expressed in floral organ primordia (Fig. 8C). These results confirmed the changes in NtCBK1 expression in the transition to flowering formation in tobacco, consistent with the pattern found by our in situ hybridization experiments (Fig. 7). These fluctuating changes of NtCBK1 expression in the transition to flowering imply a role of NtCBK1 in the transition to flowering.

A Role of NtCBK1 in the Transition from Vegetative to Reproductive Phase in Tobacco—To further explore the possible role of NtCBK1 in the transition to flowering, transgenic analyses were performed to change the pattern of NtCBK1 expression. Tobacco plants were transformed with full-length NtCBK1 cDNA under the control of the 35S promoter, and the control construct pBI121. When tobacco was transformed with

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**Fig. 8. GFP expression under the control of the NtCBK1 promoter during the transition to flowering.** A, the predicted TATA box and the transcription start site are indicated by the box. B, young floral primordia and axillary bud meristems of transgenic plants expressing the NtCBK1 Promoter-GFP construct. C, the floral primodium and floral organ primodia of NtCBK1 transgenic plants. L, leaf; M, meristem; AM, auxiliary bud meristem; P, floral primodium; FO, floral organ primordia.

untranslated region of NtCBK1 were used for in situ hybridization. The tobacco plant has determinate inflorescences (flowers). The terminal flower on the main shoot axis is the first flower formed, and axillary bud meristems generated at nodes below the main shoot apex subsequently develop into lateral flowers and these are also determinate. This pattern of determination is described as a cymose pattern. During the transition to flowering in tobacco, the development of shoot apical meristem (SAM) can be divided into three phases as shown in Fig. 7A: the vegetative growth phase (Fig. 7A, a), the floral determination phase in which the SAM develops the capacity to form floral shoots (Fig. 7A, b), and the inflorescence expanding phase (Fig. 7A, c) (45). Our results showed that transgenic tobacco plants overexpressing NtCBK1 had high amounts of NtCBK1 during all phases of apical development. The expression of NtCBK1 was strongly concentrated in young leaf primordia and in the SAM in the vegetative growth phase (Fig. 7B, a).

Expression of NtCBK1 in control plants was greatly decreased in the SAM during the floral determination phase (Fig. 7B, b), and in young flower primordia (Fig. 7B, c and d). NtCBK1 mRNA was also abundant in axillary bud meristems that would later differentiate into floral primordia (Fig. 7B, c).
Flowering Time of Transgenic Tobacco Not Expressing NicBK1—RNAi is a powerful tool to inactivate the expression of specific genes. We used this approach to block NicBK1 expression in tobacco and measured time to flowering. The results indicated that time to flowering in transgenic plants was not altered relative to controls (Table II), and NicBK1 expression was below the level of detection by Northern blot analysis. The tobacco kinase NicBK2 that shares 59% identity to NicBK1 was isolated in our laboratory (40) and the transgenic plants overexpressing NicBK2 also displayed a delayed flowering phenotype (data not shown). When NicBK2 expression was blocked by RNAi, the flowering time was not promoted. Then we obtained the plants by crossing transformants in which either NicBK1 or NicBK2 was blocked, respectively, and examined the flowering time of these plants in which the expression of both NicBK1 and NicBK2 was undetected. Our results indicated that the flowering time of these plants was not also changed when compared with control plants (data not shown).

Effect of DNA Demethylation on Flowering Time of the Transgenic Plants—DNA methylation has been shown to play an important role in the repression of the floral transition, and reduced DNA methylation may result in early flowering (47–49). Seeds of NicBK1 transgenic plants, T2–12, T2–57, and T2–61 were treated with different concentrations of 5-azaC that has been widely used for DNA demethylation in plants (47, 48), and flowering time was examined. Whereas 100 μM 5-azaC treatments did not change flowering time of either control and transgenic plants, 200 μM 5-azaC treatments promoted flowering time of both control and NicBK1 transgenic plants (Table III), suggesting that the promotion of tobacco flowering time by demethylation cannot be blocked by the overexpression of NicBK1.

NFL Expression in NicBK1 Transgenic Tobacco—The transition from vegetative to reproductive growth is one of the most important steps in plant development. DNA methylation has been shown to play an important role in the repression of the floral transition, and reduced DNA methylation may result in early flowering (47–49). Seeds of NicBK1 transgenic plants, T2–12, T2–57, and T2–61 were treated with different concentrations of 5-azaC that has been widely used for DNA demethylation in plants (47, 48), and flowering time was examined. Whereas 100 μM 5-azaC treatments did not change flowering time of either control and transgenic plants, 200 μM 5-azaC treatments promoted flowering time of both control and NicBK1 transgenic plants (Table III), suggesting that the promotion of tobacco flowering time by demethylation cannot be blocked by the overexpression of NicBK1.
dramatic developmental changes in plant development. In *Arabidopsis*, many of the genes that control time to flowering are floral meristem identity genes, or genes that interact with floral meristem identity genes (50, 51). LFY, a principal flower meristem identity gene in *Arabidopsis*, is expressed in very young flower primordia, and is involved in regulation of time to flowering (50, 52, 53). Its tobacco homologue, NFL, has been shown to control meristem initiation and floral structure (23, 54, 55). To see if there is a connection between *NtCBK1* and NFL in the regulation of time to flowering in tobacco, the expression of NFL in floral organs of *NtCBK1* transgenic plants was investigated. NFL expression was observed in all floral organs tested (Fig. 12A), suggesting that *NtCBK1* was not regulating time to flowering in transgenic tobacco via a change in the expression of NFL (Fig. 12B).

**DISCUSSION**

In this study, we have characterized *NtCBK1*, a CaM-binding protein kinase from tobacco, having a CaM-binding domain, but lacking a Ca<sup>2+</sup>-binding motif. *NtCBK1* bound CaM in the presence of calcium and lost its binding ability in the absence of calcium. Whereas purified *NtCBK1* showed basal kinase activity in the absence of added Ca<sup>2+</sup> or CaM, it could be fully activated in the presence of tobacco CaMs.

*NtCBK1* differs from the other CBKs previously characterized in plants. Neither CCaMK nor OsCBK require CaM for activation. CCaMK has a calcium-binding, visinin-like domain with three EF-hand motifs and binds CaM, whereas rice OsCBK shows high CaM binding affinity, but CaM is not required for full activation of this kinase (9). By contrast, the kinase activity of *NtCBK1* can be stimulated up to 5-fold by CaM. Furthermore, all three classes of tobacco CaM isoforms are equally effective in stimulating *NtCBK1* activity. This is in sharp contrast to the activation of tobacco kinase *NtCBK2*, whose activity is differentially regulated by the three classes of tobacco CaM isoforms (40). It is likely that the ability of *NtCBK1* to be activated equally by all three tobacco CaMs is related to the fact that the CaM-binding domain of *NtCBK1* possesses both 1-5-10 and 1-8-14 CaM binding motifs. It has been reported that different CaMs regulate their targets based on the types of the motifs present in their CaM-binding domains (56). We speculate that because *NtCBK1* has both 1-5-10 and 1-8-14 CaM-binding domains, it can be activated by several different CaM isoforms.

To investigate the role of *NtCBK1* in plant development, we explored the expression of *NtCBK1* by both Northern blot and *in situ* hybridization, and found that *NtCBK1* was expressed in most plant organs including roots, stems, leaves, and flowers (5). An interesting finding in the present studies was that when shoot apical meristems gave rise to floral primordia, the expression of *NtCBK1* was decreased in the floral primordia, implying a role of *NtCBK1* in flowering. This speculation was supported by experiments with transgenic tobacco plants over-expressing *NtCBK1*. When *NtCBK1* was constitutively expressed in tobacco under the control of the cauliflower mosaic virus 35S promoter, time to flowering was delayed, and *NtCBK1* expression in the shoot apical meristem remained high and did not fluctuate. Our results suggest that reduced expression of *NtCBK1* in the shoot apical meristem leads to the formation of flower primordial. Previous work from our laboratory with MCK1, a putative CaM-binding protein kinase from maize, also supports a role of protein kinases in flower development in tobacco. When MCK1 was overexpressed in tobacco under the control of the 35S promoter, time to flowering was not changed, but the flowers formed on the main axis failed to develop completely and were finally aborted (16).

We also inactivated *NtCBK1* in transgenic plants using RNAi to establish whether this kinase affects time to flowering in tobacco. Transformants in which *NtCBK1* expression is blocked do not display an altered time to flowering as suggested by the overexpression of *NtCBK1* in our experiments. This could be explained by functional gene redundancy. Whereas *NtCBK1* expression is inactivated, its role in the transition to flower can be functionally replaced by other gene(s). In addition to *NtCBK2*, it is possible that there are several other homologues of *NtCBK1* in tobacco and that these share overlapping roles. In fact, there have been many recent reports indicating that genetic loss-of-function analyses could not confirm data obtained by the overexpression of genes. For example, overexpressing either microRNA 172a-1 or microRNA 172a-2 in *Arabidopsis* results in late flowering. One would expect that loss of function alleles of these microRNAs would have the opposite phenotype including early flowering. However, the insertional mutations with either the microRNA 172a-1 or microRNA 172a-2 precursor genes do not change flowering time, suggesting that there is genetic redundancy within the miR172 gene family (57). An alternative explanation for the lack of effect of *NtCBK1* down-regulation on flowering is that *NtCBK1* could play a role in preventing early flowering only, but not promoting early flowering of tobacco plants.

In summary, *NtCBK1*, a CaM-binding protein kinase isolated from tobacco is structurally different from both CCaMK and CDPK, and has different enzymatic properties from both OsCBK and NtCBK2. *NtCBK1* binds to CaM in a Ca<sup>2+</sup>-dependent manner and phosphorylates itself at serine residues. Kinase activity of *NtCBK1* is stimulated with similar efficiencies.
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by all isoforms of tobacco CaMs tested, and its expression is highly regulated in shoot apical meristem during the transition to flowering. Transgenic tobacco plants overexpressing NtCBKI resulted in late flowering or no flowering, suggesting a role of NtCBKI in flowering transition.

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A Tobacco Calcium/Calmodulin-binding Protein Kinase Functions as a Negative Regulator of Flowering

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