Mediation of flowering by a calmodulin-dependent protein kinase

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Abstract A calmodulin-dependent protein kinase (MCK1) appeared important in regulating flowering in tobacco. The expression of modified *MCK1* that lacks the C-terminal including calmodulin-binding domain upsets the flowering developmental program, leading to the abortion of flower primordia initiated on the main axis of the plant and, as well, caused the prolongation of the vegetative phase in axillary buds. The abortion process of flowers began first in the developing anthers and subsequently the entire flower senesces. In axillary buds the prolonged vegetative phase was characterized by atypical elongated, narrow, twisted leaves. These results suggested a role for calmodulin-dependent protein kinase homologs in mediating flowering.

Keywords: calmodulin-dependent protein kinase, transgenic tobacco, flower.

In plants, Ca^{2+}-mediated processes are regulated by interactions of Ca^{2+} with targets, such as two well-characterized plant Ca^{2+} target proteins, calcium-dependent protein kinases (CDPKs) and calmodulin (CaM)[1,2]. Calcium may affect kinase activity in a number of ways, including interacting directly with protein kinases (CDPKs), or indirectly via CaM, as with calmodulin-dependent protein kinases (CaMKs). Both types of interactions generally lead to a phosphorylation change of the target substrate proteins. Many CDPKs have now been characterized both biochemically and molecularly and great effort has been expended in the characterization of their physiological and developmental roles in plants[3—6]. Likewise, with regard to the Ca^{2+}-binding regulatory protein CaM, data indicate its involvement in a wide variety of plant developmental and environmental responses through CaM-binding proteins[1,7,8], but in plants there is a little information on specific functions for CaM-binding proteins, including CaMKs[9—14]. In contrast, in yeast and mammals data have shown CaMKs to be key components of CaM-mediated signal transduction. In these organisms, CaMKs have been identified and are reported to mediate many cellular processes including gene transcription, the cell cycle, neuronal memory, carbohydrate metabolism, cytoskeletal function and long-term potentiation[15]. In plants, while several protein kinases have been investigated in relation to a role in plant development[16—18], little information is available on the functions of CaMKs. Indeed, until quite recently, this group of kinases had not been reported in plants[9,12,13]. Previously, it is proposed that some products shift from leaves to the

apex to initiate the flower with its four whorls of organs. The investigations of flowering have resulted in the formulation of the ABC model[19,20] and network model[21] which proposes that the four flower whorls are determined by an interaction between several groups of genes. Interactions of these genes, and their expression as the four whorls, are believed to occur after the apex undergoes "transition" to flowering and sequentially switches on the development of the floral primordia. However, what events mark this transition, and how this transition is regulated, are largely a matter of speculation and need further investigation. In this paper we present evidence supporting a role for CaMK in this transition process.

1 Material and methods

1.1 Constructs and transformation

Three primers Xba5P (GCT CTA GAC GGA GGG CCG GAA CCC AAA CCC TAA C), Sac3F (GGC GAG CTC TTT AGT GTC GTC TTG TAT TTG AAC CCC GTA TCG TG) and Sac3P (GGC GAG CTC TTA AAT TTG CCT TTG TTC ATC TCG TAA CCA AGG GTG AG) were designed. Xba5P/Sac3F and Xba5P/Sac3P were used to synthesize DNA fragments as a full length of *MCK1* (*MCKf*) or a truncated *MCK1* (*MCKt*) respectively by using pMCK1[12] as the template in the polymerase chain reactions. The *Xba*I/*Sac*I digested DNA fragments were then cloned into pPMB7066[22] and confirmed by DNA sequencing. Two constructs, pMCKf and pMCKt were obtained by replacing the *gus* gene of pBI121 with the above inserts. While pMCKf has the sequence encoding the full 625 amino acids of MCK1, the pMCKt sequence encodes only for N-terminal 443 amino acid residues of MCK1. After confirmation by sequencing analysis for the ligation junction, the constructs were introduced into *Nicotina tabacum* W-38 through the mediation of *Agrobacterium tumifaciens* strain LBA4404. Regenerated putative transgenic plants were transferred to soil and grown in greenhouse.

1.2 Nucleic acid analyses

DNA/RNA isolation and Southern/Northern blot were done as previously described[11,12]. The probe was made with maize MCK1 by random primer extension and hybridization was done under high stringency condition.

1.3 Histology

Tissue was fixed at 4℃ in a 0.1 mol • L^{-1} potassium phosphate buffer (pH 6.8) containing 2.5% paraformaldehyde and 0.5% glutaraldehyde, then dehydrated in a graded ethanol series, embedded in paraffin and sectioned at 15 μm. Sections removed paraffin and rehydrated were stained with Sharman's stain.

2 Results

2.1 Termination of flower development on the main axis

To explore the possible role of CaMKs in plant development, a biochemically and molecu-

larly characterized maize CaMK, MCK1, was used[11]. MCK1 has high homology with CaMKs in yeast and animals[12] and shares 68.1% identity and 79.6% similarity at the amino acid level with a recently isolated homolog from tobacco (data not shown). For this work, truncated *MCK1* (*MCKt*) lacking the C-terminal region was placed under control of the CaMV35S promoter. Absence of the C-terminal region, which includes the regulatory domain, allows for the constitutive activation of CaMK, irrespective of the presence of calcium or CaM[23,24].

Transformation of tobacco with MCKt was mediated by *Agrobacterium tumifaciens* and was followed by the regeneration of numerous independent putative transformants. When transplanted to soil, the vegetative development of transformed plants was initially indistinguishable from control plants grown from wild-type control seeds. Among total 40 transformants, their flowers are all initiated at the tip of the main axis, as in control plants. But in most transformants (60%), flowers formed on the main axis failed to develop completely and aborted. Transformants whose flowers all aborted displayed the most severe phenotype, as contrasted with transformants whose most, but not all, flowers abort (less severe phenotype), thus permitting some seed set (fig. 1 (a)).

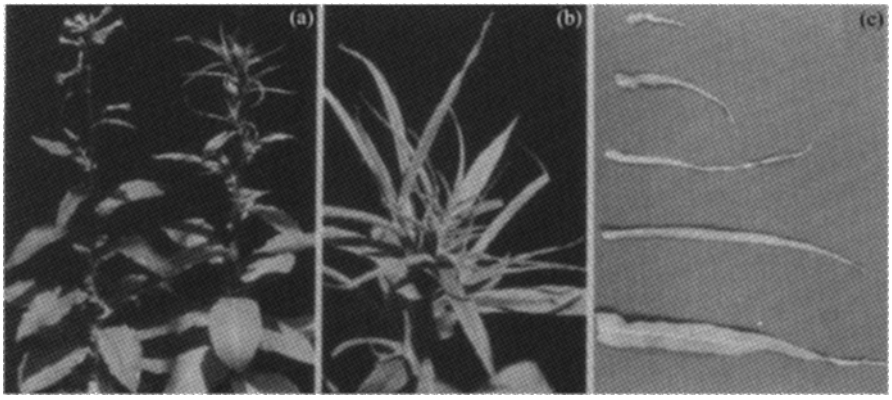

Fig. 1. Wild-type and transformed tobacco plants. (a) Left: indicating typical leaves and flower formation on the main axis of wild-type tobacco; right: showing the most severe phenotype of transformant whose flowers aborted at the terminal portion of the main axis and clusters of morphologically atypical, narrow, elongated leaves appeared at nodes; (b) enlargement of transformant node; (c) atypical transformant leaves.

Using full length *MCK1*, out of 32 transformants, only about 10% plants showed a changed phenotype, and this phenotype was typically less severe than that of plants transformed with *MCKt*. This is a reasonable result since in plants transformed with *MCK1* the expressed protein is still regulated by both calcium and CaM therefore less likely to cause an altered phenotype[10].

The first indication of the termination of flower development was that, in the maturing anthers, the regions destined to produce microspores were senescent (fig. 2 (a)—(c)). Senescence was subsequently observed in the immature carpels, and shortly thereafter the entire flower abscised (fig. 2 (d), (e)). Finally, the growth of main axis ceased.

2.2 Growth of axillary buds of transformants

Axillary buds of wild-type plant typically produce several leaves, the axis elongates and

Fig. 2. Longitudinal histological sections of tobacco flowers. (a) Immature wild-type tobacco flower. Arrow points at the developing anther sac, ×43. (b) An immature flower from a transformant showing the most severe phenotype. Early stages in flower abortion are detected first in the anther (arrow), ×43. (c) The same as in (b), but higher magnification of anther showing senescence in region where pollen will form. ×97. (d, e) Later stages in the abortion of transformant flower showering pronounced senescence of the anthers ("A") and early stages of senescence (narrow) in the ovary ("O"), ×45.

flowers form. With regard to the transformants, the axillary buds were initiated after the terminal flower abscised. However, these buds produced 20—30 very atypical leaves characterized by their elongated, narrow, twisted morphology (fig. 1 (b), (c)), compared with only 5—10 leaves produced by axillary buds of wild type plants. Moreover, such deformed leaves frequently appeared chlorotic. On the other hand, histological sections of apices producing the aberrant leaf type showed them to be indistinguishable from vegetative apices of control plants (fig. 3 (a), (b)). These data showed that CaMK had different effects on terminal and axillary flowers. Although the expression of *MCKt* interrupted the development of terminal flowers on the main axis, in axillary buds it played a different role by prolonging the vegetative phase. In contrast to its effects on axillary buds, the *MCKt* expression does not affect the leaf morphology on the main axis.

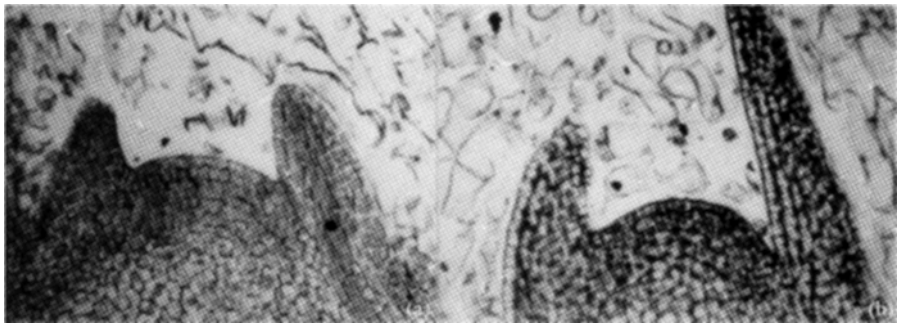

Fig. 3. Longitudinal sections of axillary apices. (a) Wild-type apex producing normal leaves, ×80. (b) Transformant apex producing twisted, narrow, elongated leaves, ×80.

2.3 Southern and Northern analyses of the inserted *MCKt*

To monitor the insertion of *MCKt*, DNA was isolated from four independent transformants and probed by maize *MCK1* (fig. 4 (a)). Different patterns of Southern blot indicated that *MCKt* had inserted at different sites of the transformants, i.e. the phenotype changes of transformants was not due to the insertion activation/inactivation of the genes at the insertion sites. Furthermore, Northern blot analysis demonstrated that the level of *MCKt* expression correlated with the severity

of change in the phenotype: plants showing the greatest change in phenotype (most severe phenotype) also showed the highest level of *MCKt* mRNA (fig. 4 (b), (c)). These data indicated that the changed phenotype of transformants resulted from the expression of inserted *MCKt*.

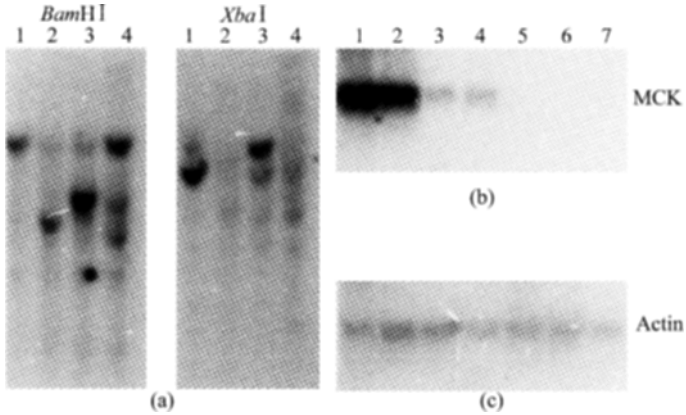

Fig. 4. (a) Southern blot analyses. Genomic DNAs isolated from four independent transgenic plants and digested with *Bam*H I and *Xba* I were blotted and probed with *MCK1* under high stringency condition; (b, c) Northern blot analyses. RNAs isolated from independent transgenic plants were blotted and hybridized with either the *MCK1* probe under moderate stringency conditions (b), or with the maize actin probe under moderate stringency conditions (c). 1, 2, the most severe phenotype; 3, 4, less severe phenotype; 5, 6, transformed plants, but showing only wild-type phenotype; 7, W-38 control plant.

3 Discussion

In this study, we investigated the function of a calmodulin-dependent protein kinase MCK1 in plant development. Our work suggests a role for this kinase in floral development. The expression of *MCKt* did not affect the initiation of terminal flowers on the main axis, but rather upset the terminal flower development program. This was contrasted with the effects of MCKt on axillary buds. In the transformants the vegetative phase of axillary buds was prolonged and characterized by morphologically unusual, atypical leaves (fig. 1 (a)—(c)).

How might MCKt promote or favor the vegetative state? Weigel and Nilsson[25] have hypothesized that floral induction consisted of two parallel pathways. One of which affected the competence of the meristem to respond to the flower meristem identity genes, such as *LEAFY*. Thus, one suggestion as to the regulatory control of MCK1 is that like *LEAFY*, it affects the competence of meristems to respond to the flower stimulus. Also like *LEAFY*, *MCK1* affects differently the lateral and terminal shoot apices. Thus, in tobacco we suggest that inherent developmental differences between the main and the lateral apices may account for phenotypic differences in *MCKt* expression and differences in flower initiation and development on the main and axillary shoots.

It is not clear why *MCKt* expression causes the abortion of already initiated flowers on the main axis. But the unusual leaf type characteristic of axillary shoots is never produced on the main axis, again supporting the view that terminal and lateral apices differ developmentally. But *MCKt* may in some ways affect the main axis, since flower development is terminated in transformants.

An earlier investigation[16] reported the probable involvement of a different family of kinases (serine /threonine and tyrosine) in events of flower development. Liu et al.[26] showed that chimeric CaMK (CCaMK) was expressed in a stage-specific manner during anther development. The appearance of mRNA coincided with meiosis (bud sizes between 0.5 cm and 1.0 cm) and became undetectable at later stages of anther development. Poovaiah et al.[27] analysed the distribution of CCaMK cloned from tobacco (*Nicotina tabacum* L. cv. Xanthi) and lily (*Lilium longiflorum* Thumb. cv. Nellie White). Their results elucidated that CCaMK could first be detected in pollen mother cells and reach a peak around the tetrad stage of meiosis, but was not expressed at later stages of microspore development. Results also showed that CCaMK was detected in the pollen sac and its localization was restricted to the pollen mother cells and tapetal cells. Through immunohistochemical localization of CaMK, our previous work[28] also showed that the distribution of CaMK demonstrated the characteristic of stage- and tissue-specification during flower development. This characteristic suggests that CaMK could play a role in sensing transient changes in free Ca^{2+} concentration in target cells, thereby regulating developmental events in the flower. In our experiments, the activity of *MCKt* is no more regulated by the presence of Ca^{2+}/CaM, so the expression of *MCKt* cannot be controlled normally by Ca^{2+}/CaM. That may cause the transition, needed by flowering, to be disturbed and the normal flowering events to be disrupted. Our results of histological sections were consistent with the location of CaMK described by the above reports, and the abort tissue of flower was exactly the distribution site of CaMK. These results proved from different angles that CaMK might play an important role in flower development.

In summary, MCK1 may be a component of, or affect component(s) of the signal transduction pathway(s) leading to flowering. Our experiments showed that normal flower development needs a precise regulation of this kinase. Through introducing this kind of gene or its products into plant, it is possible to regulate the time of flower development.

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References

1. Roberts, D. M., Harmon, A. C., Calcium-modulated proteins: Targets of the intracellular signals in higher plants, Ann. Rev. Plant Physiol. Plant Mol. Biol., 1992, 43: 375—414.
2. Sun, D. Y., Bian, Y. Q., Zhao, B. H. et al., The effects of extracellular calmodulin on cell wall regeneration of protoplasts and cell division, Plant Cell Physiol., 1995, 36: 133—138.
3. Hrabak, E. M., Dickmann, L. J., Satterlee, J. S. et al., Characterization of eight new members of the calmodulin-like domain protein kinase gene family from *Arabidopsis thaliana*, Plant Mol. Biol., 1996, 31: 405—412.
4. Huang, J. F., Teyton, L., Harper, J. F., Activation of a Ca^{2+}-dependent protein kinase involves intramolecular binding of a calmodulin-like regulatory domain, Biochemistry, 1996, 35: 13222—13234.
5. Yoo, B. C., Harmon, A. C., Intramolecular binding contributes to the activation of CDPK, a protein kinase with a calmodulin-like domain, Biochem., 1996, 35: 12029—12037.
6. Saijo, Y., Hata, S., Sheen, J. et al., cDNA cloning and prokaryotic expression of maize calcium-dependent protein kinases,

Biochem. Biophys. Acta, 1997, 1350: 109—114.
7. Neuhaus, G., Bowler, C., Kern, R. et al., Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways, Cell, 1993, 73: 937—952.
8. Yang, T., Poovaiah, B. W., Molecular and biochemical evidence for the involvement of calcium/ calmodulin in auxin action, J. Biol. Chem., 2000, 275(5): 3137—3143.
9. Watillon, B., Kettmenn, R., Boxus, P. et al., Calcium/calmodulin-binding serine/threonine protein kinase homologous to mammalian type II calcium/calmodulin-dependent protein kinase is expressed in plant cells, Plant Physiol., 1993, 101: 1381—1384.
10. Baum, G., Lev-Yadun, S., Fridmann, Y. et al., Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants, EMBO J, 1996, 15: 2988—2996.
11. Lu, Y. T., Dharmasiri, M. A. N., Harrington, H. M., Characterization of a cDNA encoding a novel heat-shock protein that binds to calmodulin, Plant Physiol., 1995, 108: 1197—1202.
12. Lu, Y. T., Hidaka, Y., Feldman, L. J., Characterization of a calcium /calmodulin-dependent protein kinase homolog from maize roots showing light-regulated gravitropism, Planta, 1996, 199: 18—24.
13. Lu, Y. T., Feldman, L. J., Light-regulated root gravitropism: a role for, and characterization of a calcium/calmodulin-dependent protein kinase homolog, Planta, 1997, 203: S91—S97.
14. Tang, J., Wu, S. P., Bai, J. et al., Extracellular calmodulin~binding proteins in plants: Purification of a ~21KD calmodulin-binding protein, Planta, 1996, 198: 510—516.
15. Braun, A. P., Schulman, H., The multifunctional calcium-calmodulin-dependent protein kinase: from form to function, Physiology, 1995, 57: 417—445.
16. Roe, J. L., Rivin, C. J., Sessions, R. A. et al., The *Tousled* gene in *Arabidopsis thaliana* encodes a protein kinase homolog that is required for leaf and flower development, Cell, 1993, 75: 939—950.
17. Tregear, J., The generation of a plant, in Environmental Plant Biology Series: Embryogenesis (eds. Wang, T. L., Cuming, A.), (Scotland, UK), Oxford: Bio Scientific Publishers Ltd., 1993, 77—88.
18. Roe, J. L., Durfee, T., Zupan. J. R. et al., TOUSLED is a nuclear serine/threonine protein kinase that require a coiled-coil region for oligomerization and catalytic activity, J. Biol. Chem., 1997, 272: 5835—5845.
19. Weigel, D., Meyerowitz, E. M., The ABC's of floral homeotic genes, Cell, 1994, 78: 203—209.
20. Yanofsky, M. F., Floral meristems to floral organs: Genes controlling early events in *Arabidopsis* flower development, Ann. Rev. Plant Phyiol. Plant Mol. Biol., 1995, 46: 67—88.
21. Mendoza, L., Alvarez-Buylla, E. R., Dynamics of the genetic regulation of network for *Arabidopsis thaliana* flower morphogenesis, Journal of Theoretical Biology, 1998 193: 307—319.
22. Mett, V. L., Lochhead, L. P., Reynolds, P. H. S., Copper-controllable gene expression system for whole plants, Proc. Natl. Acad. Sci. USA, 1993, 90: 4567—4571.
23. Kapiloff, M. S., Mathis, J. M., Nelson, C. A. et al., Calcium/calmodulin-dependent protein kinase mediates a pathway for transcriptional regulation, Proc. Natl. Acad. Sci. USA, 1991, 88: 3710—3716.
24. Palnas-Silva, M. D., Means, A. R., Expression of a constitutive form of calcium/calmodulin dependent protein kinase II leads to arrest of the cell cycle in G2, EMBO J, 1992, 10: 501—517.
25. Weigel, D., Nilsson, O. A., Developmental switches sufficient for flower initiation in diverse plants, Nature, 1995, 377: 495—500.
26. Liu, Z., Xia, M., Poovaiah, B. W., Chimeric calcium/calmodulin-dependent protein kinase in tobacco: Differential regulation by calmodulin isoforms, Plant Mol. Biol., 1998, 38: 889—897.
27. Poovaiah, B. W., Xia, M., Liu, Z. et al., Developmental regulation of the gene for chimeric calcium /calmodulin-dependent protein kinase in anthers, Planta, 1999, 209: 161—171.
28. Yang, W., Liang, S., Lu, Y., Immunohistochemical localization of Ca^{2+}/calmodulin-dependent kinase in tobacco, Chinese Science Bulletin, 2001, 46(3): 230—233.