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Rapid Communication

A Calcium/Calmodulin-Binding Serine/Threonine Protein Kinase Homologous to the Mammalian Type II Calcium/Calmodulin-Dependent Protein Kinase Is Expressed in Plant Cells

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cDNA fragments corresponding to an apple (*Malus domestica* [L.] Borkh) calmodulin-binding polypeptide have been isolated and characterized. The protein encoded by this messenger contains a serine/threonine protein kinase catalytic domain followed by a calcium/calmodulin-binding regulatory domain, both exhibiting significant sequence similarities to the corresponding regions of the mammalian calcium/calmodulin-dependent protein kinase II subunits. These results confirm a potential regulatory role for calmodulin in phosphorylation-mediated signal transduction events.

The pivotal role played by calcium ions as a second messenger in plant cells is now widely recognized (Trewavas and Gilroy, 1991). In animal cells, free calcium can regulate signal transduction pathways through the activation of calcium- and calmodulin-dependent protein kinases (Colbran et al., 1989). Because various components of the calcium messenger system (like calmodulin and calmodulin-dependent enzymes) have also been found in plants, the identification of calmodulin-dependent protein kinase activities in plant cells has received considerable attention (Allan and Hepler, 1989; Marmé, 1989; Reddy et al., 1991). However, the results of many of these studies have to be reexamined carefully because of the identification of plant CDPKs, which have structural and functional homology to calmodulin and are not calmodulin-dependent (Harper et al., 1991; Roberts and Harmon, 1992). For instance, the similarity between calmodulin and the calcium-binding domain of CDPK may undermine the interpretation of results obtained with calmodulin inhibitors or with antibodies raised against calmodulin. Therefore, the conclusive identification of such a calcium/calmodulin-dependent protein kinase will better define the role of calmodulin.

Molecular cloning and isolation (by heterologous probing) of a plant messenger homologous to the animal calcium/calmodulin-dependent protein kinase would constitute an important step toward this goal. Unfortunately, all the plant protein kinases cloned by the heterologous probing procedure belong to other subfamilies of Ser/Thr protein kinases, such as the cyclic nucleotide- or calcium-phospholipid-dependent subfamilies (Lawton et al., 1989; Biermann et al., 1990; Elliott and Brennan, 1990).

Using radiolabeled bovine calmodulin as a probe to screen an expression cDNA library, an apple (*Malus domestica* [L.] Borkh) cDNA encoding a calmodulin-binding polypeptide has been previously isolated (Watillon et al., 1992). This plant polypeptide appeared to share sequence similarities with the calmodulin-binding region of mammalian CaM kinase II. The RACE protocol (Frohman, 1990) has been used to clone cDNA copies of the 5′ and 3′ regions of the corresponding messenger. The full-length coding sequence of this messenger encodes a polypeptide with a predicted mol wt of 46,500 that exhibits significant sequence homologies with the subunits of the CaM kinase II.

MATERIALS AND METHODS

RNA and DNA Isolation

Poly(A$^+$) mRNA was extracted and purified from micropropagated McIntosh Wijcik apple (*Malus domestica* [L.] Borkh) plantlets as reported earlier (Watillon et al., 1991). Genomic DNA was isolated from apple and *Arabidopsis thaliana* (L.) Heynh. tissue according to the method of Dellaporta et al. (1983) and further purified by cesium chloride gradient ultracentrifugation (Sambrook et al., 1989).

RACE Amplification

Single-stranded cDNA molecules were synthesized from poly(A$^+$) RNA using the Amersham cDNA Synthesis Kit.

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Abbreviations: CaM kinase II, type II calcium/calmodulin-dependent protein kinase; CDPK, calcium-dependent protein kinase; ORF, open reading frame; RACE, rapid identification of cDNA ends.

Fragments of cDNA corresponding to the 5′ and 3′ regions of the CBI messenger were amplified according to the protocol described by Frohman (1990). Amplified fragments were subcloned into the Bluescript plasmid for further characterization.

DNA Sequencing and Sequence Analysis

Double-stranded DNA sequencing by the dideoxy chain-termination method (Sanger et al., 1977) was performed using the T7 DNA polymerase (Pharmacia) according to the manufacturer's instructions. For regions of the messenger amplified by the RACE strategy, sequence information was obtained from three independent clones, using unidirectionally deleted subclones (Henikoff, 1984) as well as specific oligonucleotide primers.

DNA sequences were analyzed using DNAsis software (Pharmacia). Comparisons of the deduced amino acid sequence with the Protein Identification Resource Data Library were performed using PROsis software (Pharmacia).

Genomic DNA Southern Hybridization

Genomic DNA samples (5 μg) from apple and *Arabidopsis* were digested with appropriate restriction endonucleases and electrophoresed through 0.8% agarose gels, followed by transfer onto nylon (Hybond N+, Amersham) filters (Sambrook et al., 1989). High-stringency and low-stringency hybridization procedures were carried out as previously described (Watillon et al., 1992).

RESULTS AND DISCUSSION

Isolation of cDNA Fragments Corresponding to the 5′ and 3′ Regions of the Messenger

The isolation of a 378-bp apple cDNA fragment (CB1) encoding calmodulin-binding polypeptide has been reported elsewhere (Watillon et al., 1992). This cDNA insert (containing an ORF encoding 126 amino acid residues) was flanked by two internal *Eco*RI sites (which did not result from linker addition to the blunt-ended cDNA molecules), and the 5′ and 3′ ends of the coding region were missing. By northern blotting and hybridization with the CB1 probe, the corresponding messenger was estimated to be approximately 2 kb in length.

The RACE protocol, as proposed by Frohman (1990), allows the isolation of cDNA copies of regions of a messenger located upstream or downstream of a region of known sequence. It is especially useful for obtaining full-length sequence information of rare mRNA species. Using this method, cDNA fragments extending toward the 5′ and 3′ ends of the CB1 messenger were successfully isolated and sequenced. The combined nucleotide sequence of these clones, encompassing the sequence of the CB1 378-bp insert, is presented in Figure 1. To avoid sequence errors associated with the use of Taq polymerase in amplification reactions, sequence information was obtained from three independent clones generated by the RACE procedure. At positions 380, 502, 519, and 648 in the sequence, nucleotide substitutions were observed in one of the clones (G, C, T, and G in place of T, T, C, and A, respectively). In each case, only the nucleotide observed in two of three clones was taken into consideration for the sequence presented.

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                                                        GGTCTCT      7
GCTTTGCCTGTTCGATTTCATCAAGCTTTGTTTGTCTTTGTCTTCATCGGAATCGTAACC     67
ATGATACAAGAAACAAGAAGACTTGCAGATGAGTATGAGATATCAGAGATTTTAGGCAGA    127
 M  I  Q  E  T  R  R  L  A  D  E  Y  E  I  S  E  I  L  G  R   (20)

GGAGGGTTCTCTGTGGTCAGAAAAGGCATCAGCAGAAAGTCAAGCAGCAGCAGTGACAAA    187
 G  G  F  S  V  V  R  K  G  I  S  R  K  S  S  S  S  S  D  K   (40)

ACCGATGTGGCAATCAAAACACTCAAAAGGCCGTTTGCACCCTCGAACCCTCCTCCTCTG    247
 T  D  V  A  I  K  T  L  K  R  P  F  A  P  S  N  P  P  P  L   (60)

CCGCCCCACGCCCGGCGGAACGACCAGAATAGCTTTGCTGCTGCCGCTTTCCAAACCCGG    307
 P  P  H  A  R  R  N  D  Q  N  S  F  A  A  A  A  F  Q  T  R   (80)

AAGCAGGTGTCCATATCGAATGTGCTGCTTACAAATGAAATCCTGGTGATGAGGAAGATT    367
 K  Q  V  S  I  S  N  V  L  L  T  N  E  I  L  V  M  R  K  I  (100)

GTTGAGAATGTGTCGCCGCACCCGAATGTGATTGACCTCTATGATGTGTACGAGGATGAG    427
 V  E  N  V  S  P  H  P  N  V  I  D  L  Y  D  V  Y  E  D  E  (120)

AACGGGGTTCACCTTGTGCTGGAGCTATGTTCTGGAGGGGAACTGTTTGATAGGATTGTG    487
 N  G  V  H  L  V  L  E  L  C  S  G  G  E  L  F  D  R  I  V  (140)

AAGCAAGAAAGGTATTCTGAGGTTGGAGCTGCAGCTGTGGTGAGGCAGATTGCCCAAGGC    547
 K  Q  E  R  Y  S  E  V  G  A  A  A  V  V  R  Q  I  A  Q  G  (160)

TTGGCGGCTCTTCACCGCTCGAATATTGTTCACCGGGACTTGAAGCCGGAAAACTGCCTC    607
 L  A  A  L  H  R  S  N  I  V  H  R  D  L  K  P  E  N  C  L  (180)

TTCTTGGACAACACCGTTGATTCTCCGTTGAAGATTATGGATTTTGGGCTGAGTTCCGTG    667
 F  L  D  N  T  V  D  S  P  L  K  I  M  D  F  G  L  S  S  V  (200)

GAGGAGTTCACTGACCCTGTTGTGGGACTGTTTGGTTCCATAGACTATGTGTCACCAGAG    727
 E  E  F  T  D  P  V  V  G  L  F  G  S  I  D  Y  V  S  P  E  (220)

GCTCTTTCTCAGGGGCAAGTAACTTCCAAGAGCGATATGTGGGCTCTTGGTGTAATCTTA    787
 A  L  S  Q  G  Q  V  T  S  K  S  D  M  W  A  L  G  V  I  L  (240)

TATATCCTCCTCTCCGGGTACCCACCTTTTATTGCTCAGTCGAATCGGCAAAAGCAACAG    847
 Y  I  L  L  S  G  Y  P  P  F  I  A  Q  S  N  R  Q  K  Q  Q  (260)

ATGATTATGGCTGGAGAATTCAGTTTCTATGAGAAAACCTGGAAGGGATTTCTTTGTCAG    907
 M  I  M  A  G  E  F  S  F  Y  E  K  T  W  K  G  F  L  C  Q  (280)

CCAAAGCAATTGATTTCGAGCCTCCTCAAAGTCGACCCTGACAAGAGACCTAGTGCTCAA    967
 P  K  Q  L  I  S  S  L  L  K  V  D  P  D  K  R  P  S  A  Q  (300)

GAGCTTCTGGACCATCCATGGGTTGTCGGTCTTTCAGCCAGAGAGGATCAAATGGATGCT   1027
 E  L  L  D  H  P  W  V  V  G  L  S  A  R  E  D  Q  M  D  A  (320)

GAGATTGTATCACGACTGCAGAGTTTTAATGCTCGGCGCAAACTCCGTGCTGCAGCAATA   1087
 E  I  V  S  R  L  Q  S  F  N  A  R  R  K  L  R  A  A  A  I  (340)

GCAAGCGTGTGGACAAGCTCGATTTTCTTAAGGACAAAGAAGCTAAAATCGTTACTAGGA   1147
 A  S  V  W  T  S  S  I  F  L  R  T  K  K  L  K  S  L  L  G  (360)

TCTTATGACCTTAAACCAGACGAAATCAAGAATCTGAGTTCACATTTTAAGAAAATATGT   1207
 S  Y  D  L  K  P  D  E  I  K  N  L  S  S  H  F  K  K  I  C  (380)

GTAAAAGGTGACAATGCTACTCTTTCTGAATTCAGGTTGAGTTGCAAGCGACTGATCCAA   1267
 V  K  G  D  N  A  T  L  S  E  F  R  L  S  C  K  R  L  I  Q  (400)

CTGAGATTGGTCTCGATGTCATTAAGAGCCGAGCCAGCTAGATTATGAATCATAGGCTTA   1327
 L  R  L  V  S  M  S  L  R  A  E  P  A  R  L  *               (415)

CTGGTGGGGAGTTCGTCCCTGCACCGTTTGTTACTTGCGTGATATGTTTATACATTGTGG   1387
AAATGGCGACTTGGCGAAAGATGGAATCAATTTCCAAGTTTTGCCGTTGTTTCTTATTTA   1447
TTTTAAACAAGCGTCTGTGGTTTCTGGTATATCATATGATATGTTTATTTGATAGAACCT   1507
ATCTTTGTTGAGTGGCTGCTTTAGTAGCTGTATGTCAACAAACTAATATGTAGCTGTTTG   1567
TGGGGTTTAGACGGCTCCTTTCCGCCTTCCGAGTGTTGTATATGACCCTTGTCATTTTAC   1627
TTTGGAGCAGTATAAAAGGAGCATGCTTTTATGGCCTTTCCTTGTTGCTA(31)         1707
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Figure 1. Nucleotide and derived amino acid sequence of combined CB1 and 5′ and 3′ RACE-amplified cDNA inserts. Amino acid residues are numbered in parentheses beginning with the presumptive initiation Met codon. Stop codon is indicated by an asterisk.

This sequence contains a 1245-bp ORF starting with the first ATG codon at nucleotide position 68 and ending at nucleotide 1312, before a TGA stop codon. The sequence (AACCATGAT) surrounding this ATG is distantly related to the plant initiation consensus sequence (AACAATGGC) published by Lütcke et al. (1987), nucleotide positions −4, −3, and −2 being conserved. The estimated mol wt of the protein as deduced from the amino acid sequence derived from the nucleotide sequence of the ORF is 46,500.

As previously reported, a computer-assisted homology search had revealed a significant homology score between the 126-amino acid polypeptide encoded by the CB1 insert and the α, β, γ, and δ subunits of CaM kinase II (Bennett and Kennedy, 1987; Lin et al., 1987; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). The homologies between the deduced amino acid sequence of the full-length apple polypeptide and the α and β subunits of rat CaM kinase II are

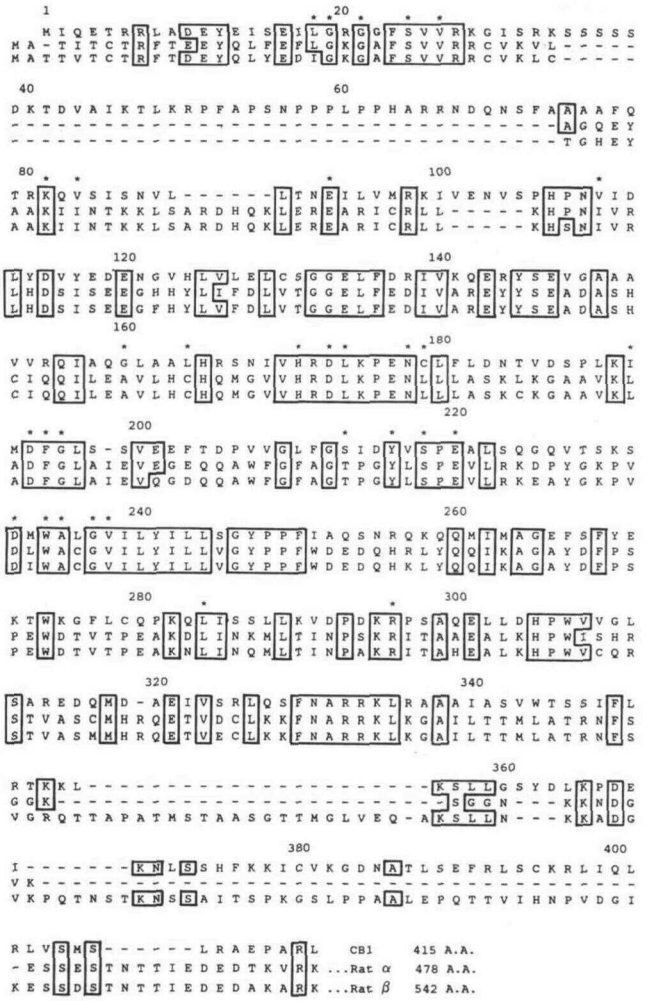

Figure 2. Alignment of the deduced amino acid sequences of the apple polypeptide encoded by the CB1 messenger and the α and β subunits of rat CaM kinase II (Bennett and Kennedy, 1987; Lin et al., 1987). Identities between the apple polypeptide and either of the rat enzyme subunits are boxed. An asterisk is placed above the invariant or nearly invariant residues, characteristic of the I through XI catalytic subdomains of protein kinases (Hanks et al., 1988), which are effectively present in the apple sequence.

presented in Figure 2. Noticeably, 31 of 33 invariant or nearly invariant residues characteristic of the catalytic subdomains I to XI of protein kinases (Hanks et al., 1988) are present at their expected locations in the apple polypeptide. Based on the presence of these diagnostic residues as well as on the calcium-dependent calmodulin-binding activity of the CB1 polypeptide (Watillon et al., 1992), it is concluded that the cDNA inserts we have isolated encode a plant calcium/calmodulin-dependent Ser/Thr protein kinase.

In particular, this plant protein kinase shares significant sequence similarities (not limited to the invariant residues) with the various subunits of mammalian CaM kinase II. For instance, in the region spanning the catalytic subdomains I through XI of protein kinases (Hanks et al., 1988), which corresponds to amino acid positions 1 to 311 in the alignment, the apple polypeptide shares 39% sequence identity with the rat α subunit. If conservative substitutions are permitted, the homology score is then 56%. The region corresponding to amino acid positions 327 through 347 in the alignment has been identified, in the animal CaM kinase II variants, as the calmodulin-binding domain (Colbran et al., 1989). In this region, the apple polypeptide is 38% identical to the rat α subunit, and, if conservative replacements are considered, the extent of sequence homology reaches 76%. However, in this respect, it is worth noticing that the Thr^{286} residue of CaM kinase II α subunit, which is known to play a key role in the autophosphorylation process regulating the activity of the mammalian enzyme (Thiel et al., 1988), is not present at the corresponding location (amino acid position 322, occupied by an Ile residue) in the apple polypeptide. This might reflect a major difference in the regulation of the activity of this plant enzyme versus its animal counterpart.

Southern Detection of Corresponding Genomic Fragments in Apple and *Arabidopsis*

A ^{32}P-labeled 624-bp fragment corresponding to nucleotides 245 to 868 (Fig. 1) was hybridized (under high-stringency conditions) to *Bam*HI, *Eco*RI, and *Hin*dIII-digested McIntosh Wijcik genomic DNA (Fig. 3A). The autoradiogra-

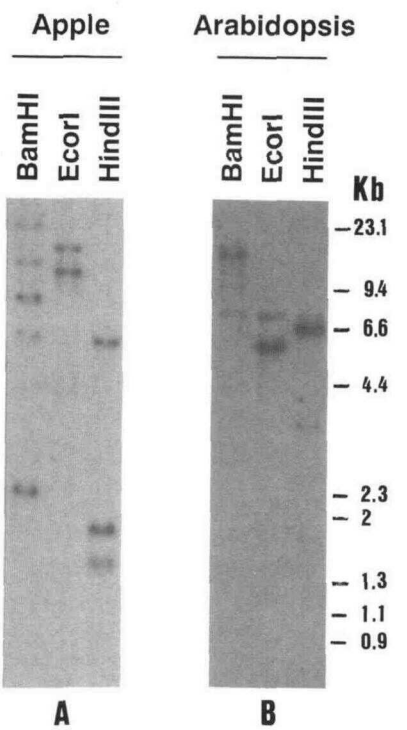

Figure 3. Southern blot detection of CB1-corresponding fragments in apple and *Arabidopsis* genomes. Aliquots (5 μg) of apple (A) and *Arabidopsis* (B) genomic DNA were digested with *Bam*HI, *Eco*RI, and *Hin*dIII, electrophoresed through a 0.8% agarose gel, and transferred onto a nylon membrane. High-stringency and low-stringency hybridizations with a CB1-specific ^{32}P-labeled cDNA probe were carried out as previously described (see "Materials and Methods").

phy of the Southern blot revealed the presence of a small number of major hybridizing bands (only two with *Eco*RI). This observation strongly suggests the presence of a very small number of copies of this gene in the apple genome. When the same probe was hybridized to *Bam*HI, *Eco*RI, and *Hin*dIII-digested DNA from *Arabidopsis* (Fig. 3B) under lower-stringency conditions, only one major hybridizing fragment was detected in each case. This result seems to indicate the existence of only one copy of the corresponding gene in the *Arabidopsis* genome.

Taken together, these results confirm that a messenger encoding a calcium/calmodulin-binding protein kinase homologous to the mammalian CaM kinase II is expressed in plant cells and that this protein could thus be implicated in signal transduction processes. Despite the existence of calcium-dependent but calmodulin-independent protein kinases (CDPKs) in plant tissues, this finding allows us to consider a potential regulatory role for calmodulin in various plant protein phosphorylation events.

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LITERATURE CITED

Allan E, Hepler PK (1989) Calmodulin and calcium-binding proteins. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 15 (Molecular Biology). Academic Press, San Diego, pp 455–484

Bennett MK, Kennedy M (1987) Deduced primary structure of the β subunit of brain type II Ca^{2+}/calmodulin-dependent protein kinase determined by molecular cloning. Proc Natl Acad Sci USA **84:** 1794–1798

Biermann B, Johnson EM, Feldman L (1990) Characterization and distribution of a maize cDNA encoding a peptide similar to the catalytic region of second messenger dependent protein kinases. Plant Physiol **94:** 1609–1615

Colbran RJ, Schworer CM, Hashimoto Y, Fong Y-L, Rich DP, Smith MK, Soderling TR (1989) Calcium/calmodulin-dependent protein kinase II. Biochem J **258:** 313–325

Dellaporta S, Wood J, Hicks J (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Elliott DC, Brennan PA (1990) Using the polymerase chain reaction in studying the structure of plant protein kinase C. *In* DD Randall, DG Blevins, eds, Current Topics in Plant Biochemistry and Physiology, Vol 9. University of Missouri, Columbia, pp 383–397

Frohman MA (1990) RACE: Rapid amplification of cDNA ends. *In* MA Innis, DH Gelfand, JJ Sninsky, TJ White, eds, PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp 28–38

Hanks SK, Quinn AM, Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:** 42–52

Harper JF, Sussman MR, Schaller GE, Putnam-Evans C, Charbonneau H, Harmon AC (1991) A calcium-dependent protein kinase with a regulatory domain similar to calmodulin. Science **252:** 951–954

Henikoff S (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:** 351–359

Lawton MA, Yamamoto RT, Hanks SK, Lamb CJ (1989) Molecular cloning of plant transcripts encoding protein kinase homologs. Proc Natl Acad Sci USA **86:** 3140–3144

Lin CR, Kapiloff MS, Durgerian S, Tatemoto K, Russo AF, Hanson P, Schulman H, Rosenfeld MG (1987) Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase. Proc Natl Acad Sci USA **84:** 5962–5966

Lütcke H, Chow K, Mickel F, Moss K, Kern H, Scheele G (1987) Selection of AUG initiation codons differs in plants and animals. EMBO J **6:** 43–48

Marmé D (1989) The role of calcium and calmodulin in signal transduction. *In* WF Boss, DJ Morré, eds, Second Messengers in Plant Growth and Development. AR Liss, New York, pp 57–80

Reddy ASN, Wang ZQ, Czernik AJ, Poovaiah BW (1991) Multifunctional calcium/calmodulin-dependent protein kinase in plants (abstract No. 434). Plant Physiol **96:** S-67

Roberts DM, Harmon AC (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 375–414

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Thiel G, Czernik AJ, Gorelick F, Narlin AC, Greengard P (1988) Ca^{2+}/calmodulin-dependent protein kinase II: identification of threonine-286 as the autophosphorylation site in the α subunit associated with the generation of Ca^{2+}-independent activity. Proc Natl Acad Sci USA **85:** 6337–6341

Tobimatsu T, Fujisawa H (1989) Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J Biol Chem **264:** 17907–17912

Tobimatsu T, Kameshita I, Fujisawa H (1988) Molecular cloning of the cDNA encoding the third polypeptide (γ) of brain calmodulin-dependent kinase II. J Biol Chem **263:** 16082–16086

Trewavas A, Gilroy S (1991) Signal transduction in plant cells. Trends Genet **7:** 356–361

Watillon B, Kettmann R, Boxus P, Burny A (1991) Characterization of two gene transcripts modulated by cytokinins in micropropagated apple (*Malus domestica* [L.] Borkh) plantlets. Plant Physiol **96:** 479–484

Watillon B, Kettmann R, Boxus P, Burny A (1992) Cloning and characterization of an apple (*Malus domestica* [L.] Borkh) cDNA encoding a calmodulin-binding protein domain similar to the calmodulin-binding region of type II mammalian Ca^{2+}/calmodulin-dependent protein kinase. Plant Sci **81:** 227–235