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**Calcium- and Calmodulin-Mediated Regulation of
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ABSTRACT

We have taken a molecular approach to clone cellular targets of calcium/calmodulin ($\text{Ca}^{2+}/\text{CaM}$). A ^{35}S -labeled recombinant CaM was used as a probe to screen various cDNA expression libraries. One of the isolated clones from petunia codes for the enzyme glutamate decarboxylase (GAD) which catalyzes the conversion of glutamate to γ -aminobutyric acid (GABA). The activity of plant GAD has been shown to be dramatically enhanced in response to cold and heat shock, anoxia, drought, mechanical manipulations and by exogenous application of the stress phytohormone ABA in wheat roots. We have purified the recombinant GAD by CaM-affinity chromatography and studied its regulation by $\text{Ca}^{2+}/\text{CaM}$. At a physiological pH range (7.0-7.5), the purified enzyme was inactive in the absence of Ca^{2+} and CaM but could be stimulated to high levels of activity by the addition of exogenous CaM ($K_{0.5} = 15 \text{ nM}$) in the presence of Ca^{2+} ($K_{0.5} = 0.8 \text{ }\mu\text{M}$). Neither Ca^{2+} nor CaM alone had any effect on GAD activity. Transgenic tobacco plants expressing a mutant petunia GAD lacking the CaM-binding domain, or transgenic plants expressing the intact GAD were prepared and studied in detail. We have shown that the CaM-binding domain is necessary for the regulation of glutamate and GABA metabolism and for normal plant development. Moreover, we found that CaM is tightly associated with a 500 kDa GAD complex. The tight association of CaM with its target may be important for the rapid modulation of GAD activity by Ca^{2+} signaling in response to stresses.

OBJECTIVES OF THE ORIGINAL PROPOSAL

The long term goal of the proposed research was to unravel Ca^{2+} -modulated signalling networks involved in plant responses to environmental stresses. We proposed to identify, isolate and characterize Ca^{2+} /CaM-regulated proteins that are likely to function under stress conditions, that are regulated by stress-induced Ca^{2+} signals and that are involved in developmental processes crucial to crop production.

RELEVANT DATA, METHODOLOGY, RESULTS AND DISCUSSION

Ca^{2+} as a second messenger in plant cells: calmodulin target proteins

In recent years it has become apparent that Ca^{2+} homeostasis in plant cells is controlled in a manner similar to that in animal cells (reviewed by Bush, 1995). Resting levels of Ca^{2+} in the cytosol are maintained at about 100 nM, whereas the concentrations of Ca^{2+} in the apoplast or in intracellular organelles (e.g. vacuoles, and ER) is in the mM range. New technologies for measuring cytosolic Ca^{2+} have provided important information on the kinetics of the changes in cytosolic Ca^{2+} , its subcellular localization and absolute concentrations (Knight et al., 1991, 1992; Read et al., 1993; Bush, 1995). These studies revealed elevations of cytosolic Ca^{2+} concentrations in response to phytohormones (McAinsh et al., 1990; Gilroy and Jones, 1992; Bush, 1995) as well as in response to abiotic stresses such as cold (Knight et al., 1991), mechanical manipulations (Knight et al., 1991; 1992) and hypoxia (Subbaiah et al., 1994).

How transient changes in cytosolic Ca^{2+} are translated into biochemical processes that switch the plant from one physiological state to another, thus preparing it to cope with a stress situation? Ca^{2+} signals are transduced via Ca^{2+} modulated proteins including metabolic enzymes, structural proteins, or regulatory proteins such as phosphatases and kinases. Alternatively, Ca^{2+} may exert its effects by interacting with Ca^{2+} -transducing proteins, which have no enzymatic activity on their own, such as CaM, a highly conserved protein among all eukaryotes (Vogel, 1994). CaM represents one of the most thoroughly studied Ca^{2+} binding proteins in eukaryotes (reviewed in Weinstein and Mehler, 1994; Crivici and Ikura, 1995; James et al, 1995). Although CaM is a highly conserved molecule, it modulates the activity of a wide diversity of proteins. More than 25 proteins in animals have been shown to be regulated by CaM in a Ca^{2+} -dependent manner including kinases, a phosphatase, nitric-oxide synthase, cyclic-nucleotide phosphodiesterase, Ca^{2+} -pumps, cyclic nucleotide gated ion channels, proteins involved in motility and cytoskeletal function, and a variety of others (Crivici and Ikura, 1995; James et al., 1995). In contrast, until very recently, only a few CaM-binding proteins have been described in plants. Because CaM is a key mediator of Ca^{2+} signals in eukaryotes, identifying its

targets in plants is of primary importance. It is also of interest to consider the evidence that plants possess several CaM isoforms encoded by distinct genes (reviewed by Roberts and Harmon, 1992). For example, in hexaploid bread wheat (*Triticum aestivum*) we have characterized cDNAs corresponding to ten distinct genes encoding three CaM isoforms (Yang et al., 1996).

CaM-binding domains exhibit predicted similarities in secondary structure, but their amino acid sequences display considerable variability. The diversity of CaM targets is largely attributable to the structure of CaM itself. The crystal structure of CaM gives the appearance of a dumbbell-shaped molecule comprised of two globular domains, each possessing two Ca^{2+} -binding sites, which are separated by a long central helix (reviewed in Weinstein and Mehler, 1994 and Vogel, 1994). This helix is interrupted midway by a short, seemingly unstructured, region. It is believed that this region provides the central helix with considerable flexibility in solution and contributes to the ability of CaM to recognize and bind a diverse array of targets which share a few common structural features. These are mostly composed of a colinear stretch of 12-30 amino acids with a basic-amphiphilic α -helix structure, the presence of two residues with large hydrophobic side chains separated by 8-12 amino acid residues, and a net positive charge. The positively charged groups of CaM binding domains are involved in electrostatic interactions with acidic residues in CaM. The binding of Ca^{2+} by CaM induces a conformational change which exposes hydrophobic clefts and thereby facilitates interaction with the hydrophobic residues within its targets amphiphilic helix.

An approach we have taken a few years ago to identify plant calmodulin-binding proteins is based on screening of cDNA expression libraries with ^{35}S -radiolabeled recombinant CaM (Fromm and Chua, 1992; Baum et al., 1993). Using this approach we have isolated several clones encoding CaM-binding proteins from different cDNA libraries prepared from a variety of plant organs, or from plants exposed to different abiotic stresses. Among the analyzed cDNA clones, we identified one coding for the enzyme glutamate decarboxylase (GAD) (Baum et al., 1993) which has become the focus of the BARD project.

Activation of GAD in response to abiotic stresses

GAD catalyzes the α -decarboxylation of L-glutamate to γ -aminobutyrate (GABA) while consuming a proton and releasing a molecule of CO_2 . Most of the interest in GABA metabolism in plants has arisen due to observations that a diverse array of environmental stresses or experimental treatments induce the production of GABA in plant tissues, cell cultures, or isolated cells. Examples of the variety of environmental stimuli that induce GABA production in plants is summarized in Table 1.

Table 1. Examples of abiotic-stress induced changes in GABA production

Stimuli or treatment	Cell or tissue type	Kinetics of GABA production*	Reference
Cold shock	•soybean plants or detached leaves	rapid, within 5 min	Wallace et al., 1984
	• <i>Spirodella</i> fronds	rapid, within 1 min	Baum and Fromm, unpublished
	•asparagus mesophyll cells	rapid, within 15 min	Cholewa et al., 1996
Mechanical manipulation	•soybean leaves	rapid, within 5 min	Wallace et al., 1984
	•asparagus mesophyll cells	rapid, within 1 min	Ramputh and Bown 1996
Hypoxia/Anoxia	•rice seedlings	slow, within 24 hr	Aurisano et al., 1995a
	•rice roots	slow, within 3 hr	Aurisano et al., 1995b
	•radish leaves	slow, within 0.5-2 hr	Streeter and Thompson, 1972
	•tea leaves	slow, within 3 hr	Tsushida and Murai, 1987
	•cotton roots	slow, 24 hr	Guinn and Brinkerhoff, 1970
	•maize root tips	slow, 40 min - 10 hr	Roberts et al., 1992
Water stress	•suspension cultures of tomato cells	slow, 30 min-2 days	Rhodes et al, 1986
	•cotton leaves	slow, 24 hr	Hanower and Brzozowska, 1975
	•germinating mustard seeds	slow, 24 hr	Vandewalle and Olsson, 1983
Heat Shock	•suspension cultures of cowpea cells	slow, 15 min-2 hr	Mayer et al., 1990

Although the function of elevated GABA synthesis during stress remains unclear, the magnitude and rapidity of GABA production in response to external stimuli is often quite dramatic. Certain stimuli appear to elicit a very rapid (within seconds to minutes) induction of GABA synthesis whereas others result in GABA synthesis only after prolonged treatment (hours to days). Soybean leaves which are briefly stimulated by touch, rolling, or crushing show increased GABA levels within 30s of 9-, 11-, and 18-fold, respectively (Ramputh and Bown, 1996). Similarly, detached soybean leaves exhibit an increase in GABA levels of 15-fold within 1 min and reach a saturable level of about 30-fold within 5 min (Wallace et al., 1984). In addition, when intact soybean plants are subjected to an abrupt temperature change from 33° to 6° C, GABA levels in leaf tissue increase within 5 min by about 44-fold (Wallace et al., 1984). In contrast, in response to stresses such as hypoxia and heat shock, stimulation of GABA synthesis is typically observed only after 30 min to several hours (Table 1).

Thus, it is plausible that different modes of regulation of GABA synthesis (i.e. short-term vs. long-term) are evoked by different stimuli.

Given the rapid enhancement of GABA synthesis under conditions such as cold shock or mechanical manipulation, it seems unlikely that this phenomenon is a consequence of changes in transcriptional or translational regulation. Rather, it is more reasonable to suggest that the underlying mechanism in these cases involves an immediate increase in the catalytic activity of GAD. Based upon several lines of evidence, it is likely that transient changes in cytosolic Ca^{2+} are involved in regulating rapid GABA synthesis in response to various stress stimuli.

Regulation of a purified recombinant GAD by Ca^{2+} /CaM

The use of a homogeneously pure 58-kDa recombinant petunia GAD (Snedden et al., 1996) has permitted an examination of GAD's response to Ca^{2+} -CaM *in vitro*. *E. coli* expressing the full-length recombinant petunia GAD were used and GAD purification was performed by a single-step procedure of CaM-affinity chromatography (Snedden et al., 1996). Using purified recombinant GAD we demonstrated that: (i) only the 58-kDa subunit is required for catalytic activity and CaM-responsiveness, (ii) Ca^{2+} exerts influence upon GAD activity only via CaM, (iii) GAD is virtually inactive in the absence of Ca^{2+} and CaM at physiological pH (7.0-7.5). The levels of Ca^{2+} ($K_{0.5} \sim 0.8 \mu\text{M}$) and CaM ($K_{0.5} \sim 15 \text{ nM}$) required for half-maximal activation of recombinant GAD at pH 7.3 (deemed physiological) are in accord with physiological estimations of these signal molecules (Bush, 1995; Roberts and Harmon, 1992) and are similar to values reported for other purified CaM-binding proteins in plants (Roberts and Harmon, 1992) and animals (Crivici and Ikura, 1995). What is perhaps most interesting about the recombinant GAD is that in the absence of Ca^{2+} -CaM, at physiological pH values (7.0-7.5), it displays no activity (Snedden et al., 1996).

Transgenic plants as a tool to study GAD regulation

One *in vivo* method that has helped shed some light on GAD regulation is the use of transgenic plants. Tobacco plants which express the 58-kDa petunia GAD (*GAD* plants) show moderately elevated GABA levels and reduced glutamate levels in leaves and stems and display a normal phenotype (Baum et al., 1996). In contrast, transgenic tobacco plants expressing petunia GAD lacking 27 amino acids from the carboxy-terminus, including the CaM-binding domain, (*GADAC* plants) exhibit a number of severe morphological disorders. *GADAC* plants possess short stems, in which cortex parenchyma cells fail to elongate, associated with very high levels of GABA and low levels of glutamate when compared to wild-type or *GAD* plants (Baum et al., 1996). It is noteworthy that the data are consistent with the hypothesis that the CaM binding domain of GAD is autoinhibitory (Snedden et al., 1996): the loss of the CaM-binding domain in *GADAC* plants, and hence

autoinhibition, results in high levels of GABA and low levels of glutamate. The unusual stem phenotype of *GADΔC* plants is also correlated with the disappearance of high-molecular weight GAD/CaM complexes which are detected in both wild-type and *GAD* plant extracts by using monoclonal antibodies against CaM or GAD. Collectively, the data suggest that the CaM-binding domain of GAD is necessary for the regulation of glutamate and GABA metabolism and that regulation of GAD activity is essential for normal plant development.

Rapid stimulation of GABA synthesis by Ca^{2+} /CaM *in vivo*

If CaM remains associated with GAD *in vivo* (Baum et al., 1996), this provides for a rapid and sensitive Ca^{2+} -sensing and GABA synthesis mechanism. Klee (1986) suggested that association of CaM with its target may be a prerequisite for rapid target activation, because otherwise activation will rely on macromolecular diffusion which is relatively slow. The findings of Arazi et al (1995) and Baum et al. (1996) noted above are certainly consistent with this theoretical consideration. Namely, GAD/CaM complexes may occur in plant cells and are thus able to respond rapidly to external stimuli via Ca^{2+} signaling.

Recently, Aurisano et al. (1995b), reported that rice roots subjected to anoxia, a treatment known to cause both cytosolic acidification (Perata and Alpi, 1993) and an increase in cytosolic Ca^{2+} levels (Bush, 1995, 1996), in the presence of CaM antagonists or Ca^{2+} -channel blockers, did not accumulate GABA to the levels observed in anoxia-treated controls. Similarly, isolated asparagus mesophyll cells incubated with either the Ca^{2+} -channel blocker lanthanum, or the CaM antagonist W7, accumulated less GABA than control cells in response to a cold-shock treatment (Cholewa et al., 1996). In addition, the Ca^{2+} ionophore calcimycin (A23187) was able to induce GABA synthesis in the absence of an external stimulation or any decline in cytosolic pH (Cholewa et al., 1996). Collectively, these findings suggest that Ca^{2+} signals stimulate GABA synthesis *in vivo* by modulating GAD's catalytic activity via CaM.

DESCRIPTION OF THE COOPERATION

The cooperation in the framework of this BARD project was most effective during the early stages of the work, at which time, H. Fromm has initiated his studies of Ca^{2+} /CaM binding proteins. H. Fromm has developed a molecular screening procedure for calmodulin-binding proteins (Fromm and Chua, 1992; Baum et al., 1993) and assisted the co-PI in screening cDNA expression libraries for isolating CaM-binding proteins by providing ^{35}S -labeled CaM. Subsequently, each PI continued studying a different set of CaM-binding proteins. The traveling funds allowed H. Fromm to meet with the co-PI

in the US at the end of the second year of the project. This meeting was important for establishing future research directions.

EVALUATION OF THE RESEARCH ACHIEVEMENTS

The three years of the BARD project mostly focused on the characterization of petunia glutamate decarboxylase as Ca^{2+} /CaM-regulated enzyme. We were the only group to purify a recombinant GAD. In a recent publication (Snedden *et al.*, J. Biol. Chem. 271, 4148-4153, 1996) we demonstrated that the activity of purified GAD is essentially dependent on Ca^{2+} and CaM at physiological pH range. We then provided evidence that CaM is associated with a multisubunit GAD complex in plants, and that disrupting this association results in developmental abnormalities associated with high GABA and low Glu levels (Baum *et al.*, EMBO J 15, 2988-2996). To our knowledge this work provides the only *in vivo* demonstration of a function for CaM binding to a target protein in plants. Moreover, Our studies suggest that GAD is a major CaM-binding protein in plants, playing a crucial role in normal plant development (Baum *et al.*, 1996). The data regarding the role of Ca^{2+} /CaM in GAD regulation, and the apparent tight association of CaM with GAD, provides a working model to explain the extremely rapid activation of GAD in response to certain stresses (within seconds to minutes). Taken together, since we initiated the molecular studies of GAD and its interactions with CaM (Baum *et al.*, 1993), this enzyme has become the most thoroughly studied Ca^{2+} /CaM-regulated enzymes in plants.

CONCLUDING REMARKS

The evidence from *in vitro* and *in vivo* studies indicating that plant GAD is a Ca^{2+} /CaM-regulated enzyme and that CaM is a component of a multisubunit GAD complex are in accord with the observations of rapid activation of GAD in responses to certain stresses. However, plants must be able to cope with various duration of stresses: It is unlikely that a single mechanism controls a metabolic pathway in response to short and prolonged stresses. Indeed, recent studies in our laboratory suggest that under prolonged stress situations GAD gene expression may be involved in modulating the rate of GABA synthesis (Zik *et al.*, 1996). The occurrence of mechanisms for rapid and slow modulation of metabolic pathways in response to stresses is likely a general phenomenon not confined to GAD regulation. Thus, the mechanisms controlling GAD activity may be studied as a paradigm for elucidating short-term and long-term mechanisms of metabolic regulation in plants.

LIST OF PUBLICATIONS

- Snedden AW, Koutsia N, Baum G and Fromm H (1996) Activation of a recombinant petunia glutamate decarboxylase by calcium/calmodulin or by a monoclonal antibody which recognizes the calmodulin-binding domain. *J. Biol. Chem.* 271, 4148-4153.
- Baum G, Lev-Yadun S, Fridmann Y, Arazi T, Katsnelson H, Zik M and Fromm H (1996). Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. *EMBO J.* 15, 2988-2996.
- Snedden WA and Fromm H (*in preparation*) Regulation of the γ -Aminobutyrate-Synthesizing Enzyme, Glutamate Decarboxylase, by Calcium/Calmodulin: A Mechanism for Rapid Activation in Response to Stress (a review).

Remark: No patents resulted from this research

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REFERENCES

- Arazi, T., G. Baum, W.A. Snedden, B.J. Shelp & H. Fromm, 1995. Molecular and biochemical analysis of calmodulin interactions with the calmodulin-binding domain of plant glutamate decarboxylase. *Plant Physiol.* 108: 551-561.
- Aurisano, N., A. Bertani & R. Reggiani, 1995a. Anaerobic accumulation of 4-aminobutyrate in rice seedlings; causes and significance. *Phytochemistry* 38: 1147-1150.
- Aurisano, N., A. Bertani & R. Reggiani, 1995b. Involvement of calcium and calmodulin in protein and amino-acid-metabolism in rice roots under anoxia. *Plant Cell Physiol.* 36: 1525-1529.
- Baum, G., Y. Chen, T. Arazi, H. Takatsuji & H. Fromm, 1993. A plant glutamate decarboxylase containing a calmodulin binding domain. *J. Biol. Chem.* 268: 19610-19617.
- Baum, G., S. Lev-Yadun, Y. Fridmann, T. Arazi, H. Katsnelson, M. Zik & H. Fromm, 1996. Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. *EMBO J.* 15: 2988-2996.
- Bush, D.S., 1995. Calcium regulation in plant cells and its role in signaling. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 46: 95-122.
- Bush, D.S., 1996. Effects of gibberellic acid and environmental factors on cytosolic calcium in wheat aleurone cells. *Planta* 199: 89-99.
- Cholewa, E., A.J. Cholewinski, B.J. Shelp, W.A. Snedden & A.W. Bown, 1997. Cold shock-stimulated γ -aminobutyrate synthesis is mediated by an increase in cytosolic Ca^{2+} not by an increase in cytosolic H^{+} . *Can. J. Bot.* (in press)
- Crivici, A. & M. Ikura, 1995. Molecular and structural basis of target recognition by calmodulin. *Ann. Rev. Biophys. Biomol. Struct.* 24: 85-116.
- Fromm, H. & N.-H. Chua, 1992. Cloning of plant cDNAs encoding calmodulin-binding proteins using ^{35}S -labeled recombinant calmodulin as a probe. *Plant Mol. Biol. Rep.* 10: 199-206.
- Gilroy, S. & R.L. Jones, 1992. Gibberellic-acid and abscisic-acid coordinately regulate cytoplasmic calcium and secretory activity in barley aleurone protoplasts. *Proc. Natl. Acad. Sci. U.S.A.* 89: 3591-3595.
- Guinn, G. & L.A. Brinkerhoff, 1970. Effect of root aeration on amino acid levels in cotton plants. *Crop Sci.* 10: 175-178.
- Hanower, P. & J. Brozowska, 1975. Influence d'un choc osmotique sur la composition des feuilles de cotonnier en acides amines libres. *Phytochemistry* 14: 1691-1694.

- James, P., T. Vorherr & E. Carafoli, 1995. Calmodulin-binding domains: just two faced or multi-faceted? *T.I.B.S.* 20: 38-42.
- Klee, C.B., D.L. Newton, W.-C. Ni & J. Haiech, 1986. Regulation of the calcium signal by calmodulin. *In Calcium and the Cell*, Ciba Foundation Symposium. John Wiley & Sons, Chichester, UK, pp 162-182.
- Knight, M.R., A.K. Campbell, S.M. Smith & A.J. Trewavas, 1991. Transgenic plant aequorin reports the effect of touch and cold shock and elicitors on cytoplasmic calcium. *Nature* 352: 524-526.
- Knight, M.R., S.M. Smith & A.J. Trewavas, 1992. Wind-induced plant motion immediately increases cytosolic calcium. *Proc. Natl. Acad. Sci. U.S.A.* 89: 4967-4971
- Mayer, R.R., J.H. Cherry & D. Rhodes, 1990. Effects of heat shock on amino acid metabolism of cowpea cells. *Plant Physiol.* 94: 796-810.
- McAinsh, M.R., C. Brownlee & A.M. Hetherington, 1990. Absciscic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. *Nature* 343: 186-188.
- Perata, P. & A. Alpi, 1993. Plant responses to anaerobiosis. *Plant Sci.* 93: 1-17.
- Ramputh, A.-I. & A.W. Bown, 1996. Rapid γ -aminobutyric acid synthesis and the inhibition of the growth and development of oblique banded leaf-roller larvae. *Plant Physiol.* 111, 1349-1352.
- Read, N.D., P.S. Shacklock, M.R. Knight & A.J. Trewavas, 1993. Imaging calcium dynamics in living plant cells and tissues. *Cell Biol. Int.* 17: 111-125.
- Rhodes, D., S. Handa & R.A. Bressen, 1986. Metabolic changes associated with adaptation of plant cells to water stress. *Plant Physiol.* 82: 890-903.
- Roberts, D.M. & A.C. Harmon, 1992. Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* 43:375-414.
- Roberts, J.K.M., M.A. Hooks, A.P. Miaullis, S. Edwards & C. Webster, 1992. Contribution of malate and amino acid metabolism to cytoplasmic pH regulation in hypoxic maize root tips studied using nuclear magnetic resonance spectroscopy. *Plant Physiol* 98: 480-487.
- Snedden, W.A., N. Koutsia, G. Baum & H. Fromm, 1996. Activation of a recombinant petunia glutamate decarboxylase calcium/calmodulin or by a monoclonal antibody which recognizes the calmodulin binding domain. *J. Biol. Chem.* 271: 4148-4153.
- Streeter, J.G. & J.F. Thompson, 1972. Anaerobic accumulation of γ -aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). *Plant Physiol.* 49: 572-578.
- Subbaiah, C.C., D.S. Bush & M. Sachs, 1994. Elevation of cytosolic calcium precedes anoxic gene expression in maize suspension-cultured cells. *Plant Cell* 6: 1747-1762.

- Tsushida, T. & T. Murai, 1987. Conversion of glutamic acid to γ -aminobutyric acid in tea leaves under anearobic conditions. *Agric. Biol. Chem.* 51: 2865-2871.
- Tuin, L.G. & B.J. Shelp, 1996. *In situ* [^{14}C]glutamate metabolism by developing soybean cotyledons II. The importance of glutamate decarboxylation. *J. Plant Physiol.* 147: 714-720.
- Vandewalle, I. & R. Olsson, 1983. The γ -aminobutyric acid shunt in germinating *Sinapsis alba* seeds. *Plant Sci. Letters.* 31: 269-273.
- Vogel, H.J., 1994. Calmodulin: a versitle calcium mediator protein. *Biochem. Cell. Biol.* 72: 357-376.
- Wallace, W., J. Secor & L. Schrader, 1984. Rapid accumulation of gamma-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. *Plant Physiol.* 75: 170-175.
- Weinstein, H. & E.L. Mehler, 1994. Ca^{2+} -binding and structural dynamics in the functions of calmodulin. *Ann. Rev. Physiol.* 56: 213-236.
- Yang, T., G. Segal, S. Abbo, M. Feldman & H. Fromm, 1996. Characterization of the calmodulin gene family in wheat: structure, chromosomal location, and evolutionary aspects. *Mol. Gen. Genet.* in press.
- Zik, M., T. Arazi & H. Fromm, 1996. Two isoforms of calmodulin-binding glutamate decarboxylase in *Arabidopsis* differ in organ-specific expression and in response to stress signals. *Plant Physiol. Suppl.* 111: abstract 716.