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PURIFICATION AND CHARACTERIZATION OF GLUTAMATE DECARBOXYLASE FROM COWPEA

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Key Word Index—*Vigna unguiculata*; Leguminosae; cowpea; enzymology; glutamate decarboxylase; GABA; purification.

Abstract—Glutamate decarboxylase (GAD) was purified 300-fold from green cowpea (*Vigna unguiculata* L.) pods using a combination of PEG precipitation, DEAE cellulose chromatography, hydroxylapatite chromatography, and Q-resin chromatography. The partially purified preparation demonstrated 2 primary bands in SDS-polyacrylamide gel electrophoresis with up to 3 additional minor bands. Cowpea GAD has a pH optimum at between pH 5.5–6.0, and an apparent K_M for glutamate of 3.2 mM at pH 5.8. Both crude GAD preparations and preparations partially purified through the hydroxylapatite step can be stimulated by Ca^{2+} and calmodulin when assayed at pH 5.8. However, the purified enzyme does not show activation by Ca^{2+} and/or calmodulin at pH 5.8 or at pH 7.0. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

γ-Aminobutyric acid (GABA) is a nonprotein amino acid that serves as a major inhibitory neurotransmitter in mammalian central nervous systems [1] and is found in virtually all plant tissues [2]. GABA accumulation in plants has been shown to dramatically increase under a variety of stress-related conditions, including anaerobiosis [3], cytosolic pH reduction [2], cold-stress [4], and heat-stress [5]. The exact function of this metabolite and its role in stress metabolism remain the subject of speculation [6].

Heat stressed or shocked cowpea (*Vigna unguiculata*) cell cultures exhibit increased levels of GABA (> 10-fold) resulting from a 64-fold increase in the rate of GABA synthesis from glutamate [5]. Glutamate decarboxylase (EC 4.1.1.15) is believed to be the enzyme responsible for GABA formation, with a concurrent release of CO_2. However, no significant change in the levels of extractable glutamate decarboxylase (GAD) can be shown in heat stressed cowpea cells compared to unstressed cells [7, Johnson and Locy, unpublished]. This implicates some type of post-translational control of GAD activity in the regulation of GABA levels during heat-stress in cowpea cells.

Recently, glutamate decarboxylase (GAD) from petunia (*Petunia hybrida* L.) petals was shown to bind to calmodulin (CaM) *in vitro* in a calcium-dependent manner [8], and Ling *et al.* [9] reported that fava bean (*Vicia faba* L.) GAD activity was stimulated by Ca^{2+}/CaM, giving evidence that GAD may participate in a Ca^{2+}-mediated signal transduction pathway. More recently, Arazi *et al.* [10] have demonstrated that petunia petal GAD produced as a recombinant protein in *Escherichia coli* has a nearly absolute requirement of Ca^{2+}, CaM for activity at pH 7.0 but not at pH 5.8, and Snedden *et al.* [11] have shown that soybean GAD is stimulated by Ca^{2+}/CaM.

These studies along with the fact that heat shock is known to result in cytoplasmic calcium increases [12, 13], and that in plants CaM-related gene expression is modulated during heat stress [14, 15] have prompted us to undertake the purification and partial characterization of GAD from cowpea (*Vigna unguiculata* L.). A preliminary report of these findings has been previously reported [16].

RESULTS AND DISCUSSION

Purification of GAD from cowpea pods

Cowpea GAD was purified from mature, green pods as outlined below in the Experimental Section. The elution of GAD activity from DEAE cellulose, hydroxylapatite, and the Q column is shown in Figs. 1, 2, and 3, respectively. These purification steps resulted in 300-fold purification of GAD from Cowpea pods (Table 1). SDS-PAGE shows that the preparation contains two substantial bands at 54 and 51 kD (see Fig. 4).

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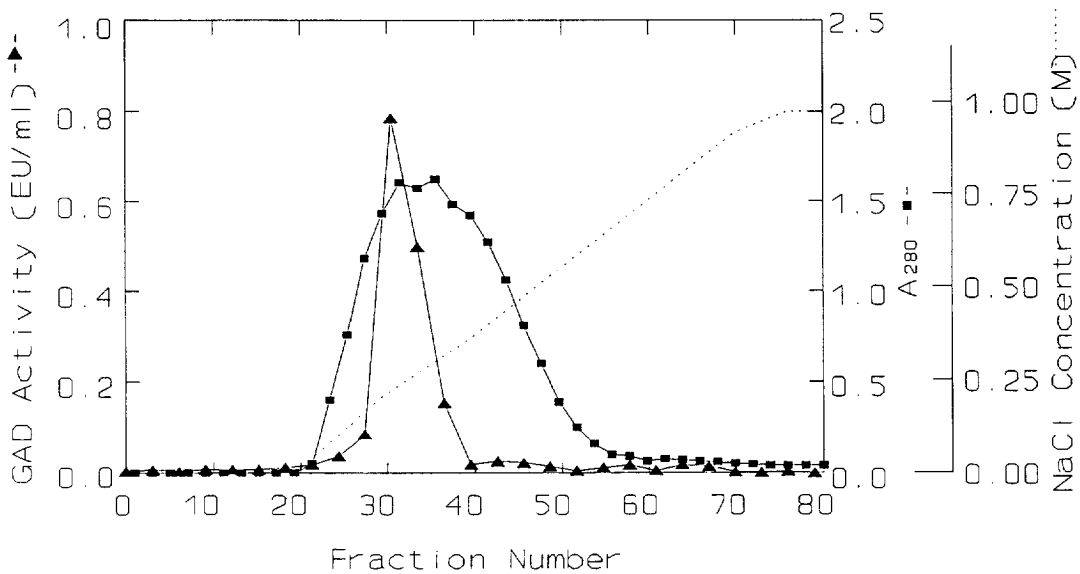

Fig. 1. The elution of GAD activity from DEAE Cellulose. GAD activity is indicated by the closed triangles, absorbance at 280 nm by the closed squares, and the NaCl gradient is shown by the dotted line.

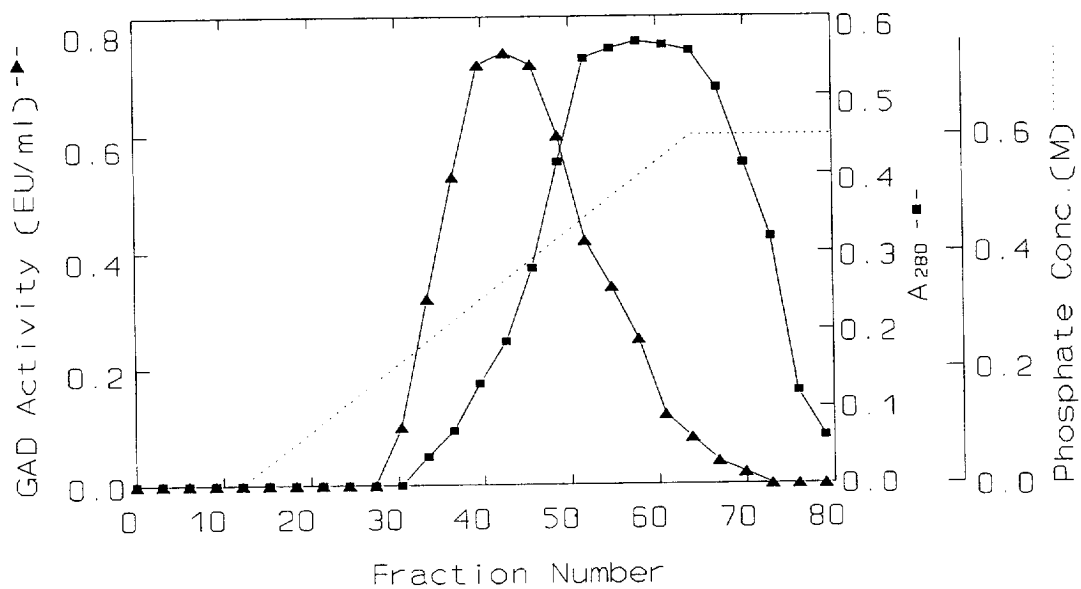

Fig. 2. The elution of GAD activity from hydroxylapatite. The pooled DEAE Cellulose fractions were applied to hydroxylapatite as described in the text. GAD activity is indicated by the closed triangles, absorbance at 280 nm by the closed squares, and the sodium phosphate gradient is shown by the dotted line.

Additional minor bands at 73, 32, and 21 kD are inconsistently observable in most preparations (data not shown). It is possible that these bands are all parts of a GAD complex and that the protein is pure. However, this is unlikely considering that others have reported that GAD consists of multiple identical subunits of 58 kD (squash) or 45.5 kD (potato) [17, 18]. The two major bands at 54 and 51 kD are of similar size to previously determined plant GAD masses. Snedden *et al.* [11] have reported that GAD is sensitive to partial proteolysis during purification. even though purification occurs in the presence of phenylmethylsulfonylfluoride (PMSF), it is possible that the 51 kD band is a proteolytic product of the 54 kD band. This possibility is further evidenced by a lack of activation by Ca^{2+}/CaM in the final purification product. Snedden *et al.* [11] found that GAD that was not protected from proteolysis during purification was insensitive to activation by Ca^{2+}/CaM. It has previously been determined that CaM binding occurs in a 21 amino acid segment at the carboxyl end of petunia GAD [8]. This sequence is not present in *E. coli* GAD, which is not activatable by Ca^{2+}/CaM. The molecular mass of the 21 amino acid sequence is 2.9 kD. Assuming that the 54 kD band on SDS-PAGE represents cowpea GAD, the 51 kD band could represent

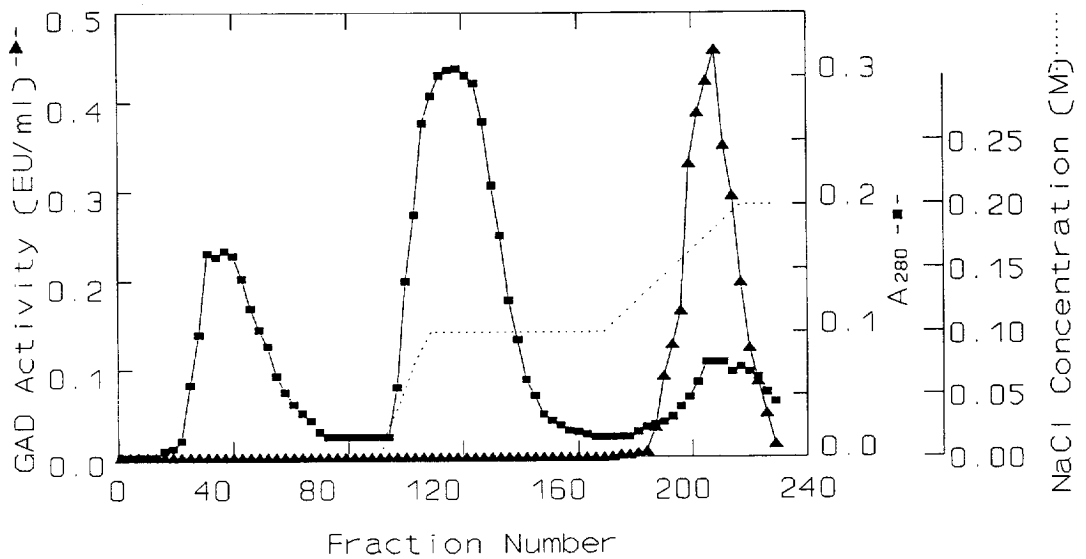

Fig. 3. The elution of GAD activity from Q-resin. The pooled hydroxylapatite fractions were applied to Q-resin column as described in the text. GAD activity is indicated by the closed triangles, absorbance at 280 nm by the closed squares, and the NaCl gradient is shown by the dotted line.

Table 1. Purification table for cowpea pod GAD

Step	Fold purificaiton	Enzyme activity (EU)	Total protein (mg)	Specific activity (EU mg^{-1})	Percent recovery
Crude	1.00	461.1	53940	0.0085	100.0%
12.5% PEG	1.82	124.3	7968	0.0156	26.9%
DEAE cellulose	9.44	126.8	1570	0.0808	27.4%
Hydroxylapatite	110	104.1	110	0.9450	22.5%
Q-resin	301	26.9	10.4	2.57	5.8%

cowpea GAD that is missing a small portion of the protein's carboxyl end and is therefore not activatable by Ca^{2+}/CaM.

Other plant GADs have been purified and studied. Inatomi and Slaughter [19] showed that barley embryos have two forms of GAD, a 256 kD form and a 120 kD form. The 120 kD form was relatively inactive and appeared to spontaneously associate to the 256 kD form after storage at 0°C. Barley root GAD was a single species with a M_w of approximately 310 k [20]. Narayan and Nair [17] purified GAD from potato tubers. This enzyme displayed a M_w of 91 k on native PAGE with a M_w of 43 k on SDS-PAGE. Thus, it appears that potato GAD is a homodimer. GAD from squash was purified by Matsumoto *et al.* [18]. This enzyme was found by gel filtration to have a M_w of 340 k when eluted at pH 5.8. However, when eluted at pH 7.2, GAD eluted as a protein with a M_w of 120 k. Furthermore, SDS-PAGE showed a single GAD band with M_w of 58 k [18]. The 120 kD form demonstrated some enzyme activity but considerably less than the 340 kD form. The authors were unable to detect PLP in the 120 kD form [18]. Squash GAD appears to form a stable, active, multi-subunit complex (340 kD form) at pH 5.8 that dissociates (120 kD form) and inactivates when the pH is raised. It does not appear that native cowpea GAD as prepared here demonstrates multiple interconvertible forms as has been shown in the above cases; rather native cowpea GAD from pods appears to exist as a high M_w complex based on native acrylamide gel electrophoresis and chromatography as Sephacryl S-300 (data not shown). Recently, Baum *et al.* [20] have shown that CaM is a component of a large multisubunit active GAD complex. These authors propose that the presence of CaM in the complex is responsible for the rapid activation response (within 30 sec) of GAD observed under certain stress conditions known to increase cytoplasmic Ca^{2+} [21, 22].

pH Dependence and apparent K_m *for glutamate of purified cowpea GAD*

A pH-dependent activity profile of the enzyme preparation was determined using a two buffer system (see Fig. 5). The pH optimum of cowpea GAD is between 5.5 and 6.0. When the pH-dependent activity profile of the enzyme preparation was determined with the

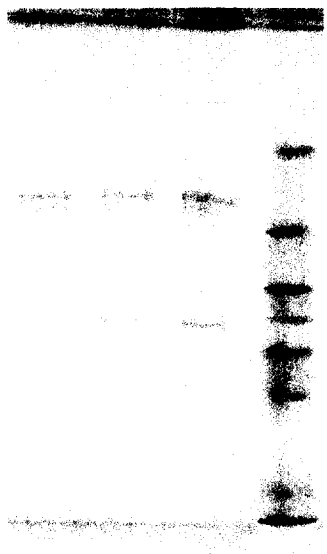

Fig. 4. Silver stained SDS-Polyacrylamide gel of Q-resin purified cowpea GAD. The left three lanes contained 1, 2, and 4 μg of protein (left to right, respectively). The right lane is molecular weight standards (top to bottom: bovine serum albumin, 66,000; ovalbumin, 45,000; glyceraldehyde 3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; chymotrypsinogen, 24,000; trypsin inhibitor, 20,100, and α-lactalbumin, 14,200).

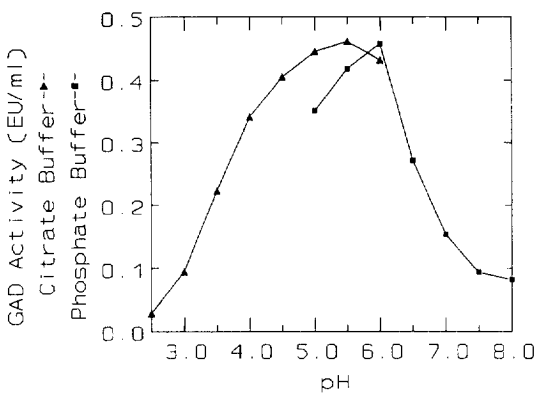

Fig. 5. The pH dependence of GAD activity from Q-resin. The specific activity of GAD was measured at varying pHs using either phosphate buffer (squares) or citrate buffer (triangles).

addition of Ca^{2+}/CaM in the assay mixture, and essentially identical profile was obtained (data not shown).

Using a double reciprocal plot (see Fig. 6), the apparent K_m of cowpea GAD for glutamate was determined to be 3.2 mM. It should also be noted that the enzyme showed significant substrate inhibition at higher glutamate levels. However, these glutamate levels are too high to be physiologically meaningful. Thus, it is not anticipated that cowpea GAD is substrate-inhibited *in vivo*.

Stimulation of cowpea GAD by Ca^{2+}/CaM

The partially purified GAD prepared as described was not Ca^{2+}/CaM stimulated under a variety of conditions (Table 2), including those in which the soybean GAD was stimulated [11]. However, the crude pod extract and samples from purification steps through the hydroxylapatite chromatography did exhibit activation by Ca^{2+}/CaM by as much as 160% at pH 5.8 (see Table 2). Under identical assay conditions, the more purified enzyme was not activated by Ca^{2+} alone (data not shown), by CaM alone (data not shown) or by Ca^{2+}/CaM.

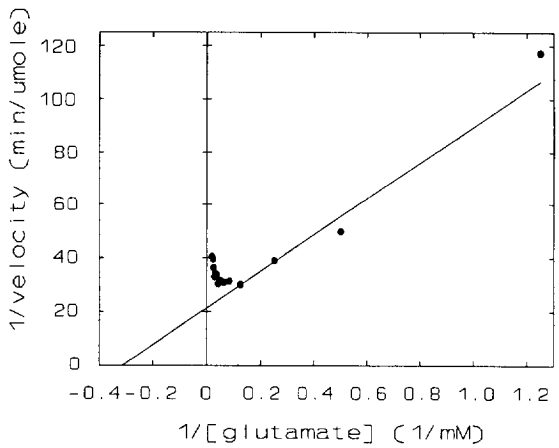

Fig. 6. Lineweaver-Burke plot showing the reciprocal of the initial velocity as a function of the reciprocal of the glutamate concentration.

At pH 5.8, the maximum activation by Ca^{2+}/CaM that could be demonstrated was 164%. This was shown in the hydroxylapatite eluent. Previous reports of Ca^{2+}/CaM activation of GAD were obtained using an assay system at pH 7.0 [9, 11]. Snedden *et al.* [11] found that GAD from soybean had a pH optimum of 5.8 with only 12% of maximum activity at pH 7.0. However, the enzyme at pH 5.8 was not activatable by Ca^{2+}/CaM. Using identical assay conditions, we were unable to show activation of purified cowpea GAD at pH 7.0 (data not shown). However, crude and partially purified cowpea GAD preparations do demonstrate Ca^{2+}/CaM activation at pH 5.8. It is unclear what factors effect this loss of activation during the purification process, but clearly the cowpea enzyme responds to pH differently than does the petunia enzyme [11].

In summary, we have shown that crude and partially purified preparations of cowpea GAD are activatable by Ca^{2+}/CaM at pH 5.8. However, we were unable to demonstrate such stimulation in the more purified preparation of GAD.

EXPERIMENTAL

All chemical reagents were purchased from Sigma Chemical Co. unless otherwise stated, and were reagent grade or better.

Determination of GAD activity. GAD activity *in vitro* was determined by the method of Kitaoka and Nakano [23] with some modifications. The standard

Table 2. Assay of cowpea GAD at different stages of purification in the absence and presence of Ca^{2+}/CaM

Step	No addition (μmol min^{-1} ml^{-1})	Ca^{2+} and CaM (μmol min^{-1} ml^{-1})	Ca^{2+} and CaM/no addition
Crude	0.091	0.186	2.04
Hydroxylapatite	0.116	0.306	2.64
Q-resin	0.678	0.498	0.73

Note: Assays of GAD activity were conducted using the standard assay described in the Materials and Methods or in the same assay containing 500 μM $CaCl_2$ and 200 nM bovine brain CaM.

assay reaction volume consisted of 200 μl containing 50 mM sodium phosphate, pH 5.8, 30 mM L-glutamate, 20 μM pyridoxal-5-phosphate (PLP), and enough protein to produce a reaction rate such that velocity was linear and proportional to the amount of protein added. When assays were done with Ca^{2+} and/or CaM, the assay mixt. also contained 500 μM $CaCl_2$ and/or 200 nM bovine brain CaM. Reactions were incubated at 25° for 30 min. The reaction was terminated by the addition of 200 μl of 200 mM sodium borate, pH 9.0, and 1 ml of 6% phenol soln, followed by immersion in an ice–H_2O bath. After cooling for 2–5 min, 400 μl of 5.25% sodium hypochlorite (Chlorox Laundry Bleach) was added followed by vigorous agitation. The mixt. was placed in a boiling H_2O bath for 10 min., then immediately replaced into the ice–H_2O bath for 20 min. Occasional agitation was necessary to promote colour development during the final cooling. Activity was measured by determining the *A* at 630 nm and comparing to a standard curve.

Purification of GAD from cowpea. All procedures were carried out at 4° unless otherwise noted. Cowpea pods were routinely used, as they contain large amounts of extractable GAD and can be stored at −80° for at least one year without loss in enzyme activity. 500 g of pod tissue were frozen in liquid N_2, placed in a 3.8 l capacity Waring Blender cooled with liquid N_2, and ground to a fine powder. 50 g of insoluble polyvinylpyrrolidone was added, along with an equivalent tissue weight of extraction buffer consisting of 50 mM sodium phosphate, pH 5.8, 200 μM DTT, 200 μM PLP, and 1 mM PMSF. After homogenization, the protein extract was filtered through 4 layers of cheesecloth and centrifuged at 10 000 *g* for 15 min. This crude extract was made 12.5% polyethylene glycol (PEG) by the addition of 50% PEG-8000, incubated with gentle stirring for 30 min, and centrifuged at 12 000 *g* for 15 min. The pellet containing GAD activity was resuspended in a minimal amount of buffer containing 20 mM sodium phosphate, pH 5.8, 200 μM DTT, and 200 μM PLP (standard buffer). The resuspension was centrifuged at 5000 *g* for 10 min. The GAD-containing supernatant was loaded onto a DEAE-cellulose column pre-equilibrated with standard buffer plus 1 mM PMSF. The column was washed with 3 column vols of standard buffer, and bound proteins were eluted with a linear gradient of 0–0.5 M NaCl in standard buffer (see Fig. 1). Those frs containing GAD activity were pooled and diluted once with a soln containing 200 μM DTT and 200 μM PLP. This pool was loaded onto a hydroxylapatite column [24], pre-equilibrated with 10 mM sodium phosphate, pH 5.8, 200 μM DTT, and 200 μM PLP. The column was washed with 3 vols of the same buffer, and bound proteins were eluted with a linear gradient of 10–600 mM sodium phosphate, pH 5.8 (see Fig. 2). Frs containing activity were pooled and desalted into standard buffer using a G-50 Sephadex column. The desalted G-50 eluate was then loaded onto a High-Q Econo-Pac column cartridge (Biorad, Inc). The column was washed with 25 ml of standard buffer followed by 25 ml of standard buffer plus 100 mM NaCl. GAD was eluted from the column by a linear gradient of 100–200 mM NaCl (see Fig. 3). Frs containing GAD activity were pooled and concd using Amicon C-10 microconcentrators.

SDS-PAGE analysis. SDS-PAGE was accomplished according to Laemmli [25]. Protein was visualized using the silver-staining technique [26] or staining with Coomassie Blue.

REFERENCES

1. Nathan, B., Bao, J., Hsu, C.-C., Aguilar, P., Wu, R., Yarom, M., Kuo, C.-Y. and Wu, J.-Y., *Proceedings of the National Academy of Science of the U.S.A.*, 1994, **91**, 242.
2. Crawford, L. A., Bown, A. W., Breitkreuz, K. E. and Guinel, F. C., *Plant Physiology*, 1994, **104**, 865.
3. Tsushida, T. and Murai, T., *Agriculture and Biological Chemistry*, 1987, **51**, 2865.
4. Naidu, B. P., Paleg, L. G., Aspinall, D., Jennings, A. C. and Jones, G. P., *Phytochemistry*, 1991, **30**, 407.
5. Mayer, R., Cherry, J. and Rhodes, D., *Plant Physiology*, 1990, **94**, 796.
6. Narayan, V. S. and Nair, P. M., *Phytochemistry*, 1990, **29**, 367.
7. Heuss-LaRosa, K. L., Ph.D. thesis, Purdue University, 1993.
8. Baum, G., Chen, Y., Arazi, T., Takatsuji, H. and Fromm, H., *Journal of Biological Chemistry*, 1993, **268**, 19610.

9 Ling, V., Snedden, W. A., Shelp, B. J. and Assmann, S. M., *Plant Cell*, 1994, **6**, 1135.
10. Arazi, T., Baum, G., Snedden, W., Shelp, B. and Fromm, H., *Plant Physiology*, 1995, **108**, 551.
11. Snedden, W., Arazi, T., Fromm, H. and Shelp, B., *Plant Physiology*, 1995, **108**, 543.
12. Drummond, I. A. S., McClure, S. A., Poenie, M., Tsien, R. Y. and Steinhardt, R. A., *Molecular Cell Biology*, 1986, **6**, 1767.
13. Calderwood, S. K., Stevenson, M. A. and Hahn, G. M., *Radiation Research* 1988, **113**, 414.
14. Braam, J., *Proceedings of the National Academy of Science of the U.S.A.*, 1992, **89**, 3213.
15. Braam, J. and Davis, R. W., *Cell*, 1990, **60**, 357.
16. Johnson, B. S., Cherry, J. H., Locy, R. D., *Plant Physiology*, 1995, **108**, 295s.
17. Narayan, V. S. and Nair, P. M., *European Journal of Biochemistry*, 1985, **150**, 53.
18. Matsumoto, T., Yamamura, I. and Funatsu, M., *Agriculture and Biological Chemistry*, 1990, **54**, 3001.
19. Inatomi, K. and Slaughter, J. C., *Biochemical Journal*, 1975, **147**, 479.
20. Baum, G., Lev-Yadunm, S., Fridmann, Y., Arazi, T., Katsnelson, H., Zik, M. and Fromm, H., *EMBO Journal*, 1996, **15**, 2988.
21. Ramputh, A.-I. and Bown, A. W., *Plant Physiology*, 1996, **111**, 1349.
22. Roberts, D. M. and Harmon, A. C., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1992, **43**, 375.
23. Kitaoka, S. and Nakano, Y. *Journal of Biochemistry*, 1959, **66**, 87.
24. Muench, K. H., *Proceedings of Nucleic Acid Research*, 1971, **2**, 515.
25. Laemmli, U. K., *Nature*, 1970, **277**, 680.
26. Blum, H., Beier, H. and Gross, H., *Electrophoresis*, 1987, **8**, 93.