Identification of a Novel Divergent Calmodulin Isoform from Soybean Which Has Differential Ability to Activate Calmodulin-dependent Enzymes*

(Received for publication, March 13, 1995, and in revised form, June 26, 1995)

Sang Hyyoung Lee†, J ong Cheol Kim†, Mal Soon Lee†, Won Do Heo§, Hae Young Seo¶, Hae Won Yoont, J ohng Chan Hong¶, Sang Yeol Lee¶, J ong Dong Bakh¶, Inhwan Hwang¶, and Moo J e Cho§§

From the †Plant Molecular Biology and Biotechnology Research Center, §Department of Biochemistry, Gyeongsang National University, Chinju 660-701, Korea

Calmodulin plays pivotal roles in the transduction of various Ca\textsuperscript{2+}-mediated signals and is one of the most highly conserved proteins in eukaryotic cells. In plants, multiple calmodulin isoforms with minor amino acid sequence differences were identified but their functional significances are unknown. To investigate the biological function of calmodulins in the regulation of calmodulin-dependent enzymes, we cloned cDNAs encoding calmodulins in soybean. Among the five cDNAs isolated from soybean, designated as SCaM-1 to -5, SCaM-4 and -5 encoded very divergent calmodulin isoforms which have 32 amino acid substitutions from the highly conserved calmodulin, SCaM-1 encoded by SCaM-1 and SCaM-3. SCaM-4 protein produced in Escherichia coli showed typical characteristics of calmodulin such as Ca\textsuperscript{2+}-dependent electrophoretic mobility shift and the ability to activate phosphodiesterase. However, the extent of mobility shift and antigenicity of SCaM-4 were different from those of SCaM-1. Moreover, SCaM-4 did not activate NAD kinase at all in contrast to SCaM-1. Also there were differences in the expression pattern of SCaM-1 and SCaM-4. Expression levels of SCaM-4 were approximately 5-fold lower than those of SCaM-1 in apical and elongating regions of hypocotyls. In addition, SCaM-4 transcripts were barely detectable in root whereas SCaM-1 transcripts were as abundant as in apical and elongating regions of hypocotyls. In conclusion, the different biochemical properties together with differential expression of SCaM-4 suggest that this novel calmodulin may have different functions in plant cells.

Calmodulin, a highly conserved and ubiquitous protein in eukaryotes, mediates Ca\textsuperscript{2+} signals to various target proteins (1). A variety of regulatory enzymes and proteins such as protein kinases, ion channels, Ca\textsuperscript{2+} pumps, nitric oxide synthetase, inositol trisphosphate kinase, cyclic nucleotide phospho-

diesterase, and NAD kinase are known to be regulated by Ca\textsuperscript{2+} and calmodulin (2-4). While a great deal of information has been known for biological roles of calmodulin in animal cells, very little is known about the roles of calmodulin in plant cells. This is mainly due to the absence of purified calmodulin-dependent enzymes and/or their genes in plants. As an effort to investigate the biological role(s) of calmodulin in plants, calmodulin genes in various plant species have been cloned and characterized recently (4). Interestingly, in Arabidopsis, cDNAs encoding multiple calmodulin isoforms have been isolated although the degree of sequence divergence is minor, only 6 amino acid differences between the two most divergent isoforms among Arabidopsis calmodulins (5-7). This is very notable because, in animal cells, only a single form of calmodulin is produced by a calmodulin multigene family (8, 9). However, it has not been determined whether plant calmodulin isoforms have the same biochemical properties such as calcium-binding abilities and activation of calmodulin-dependent enzymes. Also the biological role of multiple calmodulin isoforms in vivo is completely unknown.

To understand Ca\textsuperscript{2+}/calmodulin-mediated signal transduction mechanisms in plants, first we cloned calmodulin cDNAs from soybean. From these cDNA clones, we have identified two novel divergent calmodulin isoforms. One of the calmodulin isoforms showed several distinct characteristics to other highly conserved plant calmodulin isoforms. Here we describe the structural and functional differences between the novel divergent calmodulin isoform SCaM-4 and the highly conserved calmodulin SCaM-1.

MATERIALS AND METHODS

Isolation of Calmodulin cDNAs from Soybean—A cDNA library was constructed in a ZAPII (Stratagene) from half-apical and half-elongating regions of hypocotyls (0.3–1.3 cm section below cotyledon tissues) of 4-day-old etiolated soybean Glycinemax L. cv. Williams seedlings. The library was screened by using the ECL gene detection system (Amersham) with a rice calmodulin genomic clone, cam-2, (10) as a probe. Nucleotide sequences were determined from both strands of cDNAs using a Taq dye primer cycling sequencing kit on a 373A automatic DNA sequencer (Applied Biosystems Inc.). Nucleotide and deduced amino acid sequences were analyzed using the GCG sequence analysis program (11). For phylogenetic analysis of calmodulin, amino acid sequences of calmodulins were aligned with CLUSTAL V (12) and by eye with LINEUP of the GCG sequence analysis program. Those positions not occupied in all OTUs were excluded from the alignment (available upon request) leaving 130 sites for phylogenetic inference. Distance between sequences was measured as number of amino acid substitutions/site using a $g$ distribution for the variability of substitution rate across positions (13). For this, a neighbor-joining tree (14) was constructed using the proportion of amino acid differences between sequences, from which the $g$ parameters were estimated. The value thus determined (2.162) was used to estimate numbers of substitutions/site.
between sequences using the γ correction. The resulting distance matrix yielded the final neighbor joining tree. The reliability of branches was estimated by bootstrapping using the same γ parameter.

Production of Calmodulin Proteins in E. coli—A T7 expression vector, pET-3d, was used for production of SCaM-1 and SCaM-4 protein in Escherichia coli BL21(plySIDE3) (15). The start codon of SCaM-1 cDNA was modified to introduce an Ncol site by PCR with a mutant oligomer (5′-CAATTACAGTGGCGAGAATATCCAT-GCCAG-3′) and the T3 oligomer (5′-ATAACCTCTCAATAGG-3′) as primers. The PCR product digested with Ncol and BamHI was subcloned into the Ncol and BamHI site of the pET-3d vector. Integrity of the SCaM-pET-3d construct was verified by nucleotide sequencing.

SCaM-4 cDNA had an Ncol site at the start codon and was processed as described for SCaM-1 without a modification. Calmodulin proteins expressed in E. coli were purified to homogeneity by Ca2+-dependent phenyl-Sepharose (Pharmacia) column chromatography as described (16, 17). Protein concentration was determined using a Protein assay kit (Bio-Rad) with bovine serum albumin and bovine brain calmodulin (Sigma) as standards.

Preparation of Antibodies and Immunoblotting—Polyclonal antibodies against two SCaM isoforms, SCaM-1 and SCaM-4, were prepared by immunizing goats subcutaneously with 10 mg of each purified SCaM protein in the Freund’s complete adjuvant. Subsequent boosting injections were done at 3-week intervals with 1 mg of protein in the Freund’s incomplete adjuvant. To determine cross-reactivities among SCaM-1, -4, and bovine brain calmodulin, unpurified antisera were used in immunoblot experiments. Anti-bovine brain calmodulin polyclonal antibody and bovine brain calmodulin, unpurified antisera were used in incomplete adjuvant. To determine cross-reactivities among SCaM-1, -4, and bovine brain calmodulin, the antibody was incubated with the antibody was incubated with the antibody was incubated in the antibody was incubated with the antibody was incubated with

**RESULTS**

Comparison of the DEDUCED AMINO ACID SEQUENCES OF SCaMs with Other Calmodulins—SCaM-1, -2, and -3, encoded calmodulins which were very similar to other plant calmodulins such as alfalfa, barley, and Arabidopsis calmodulins (4). As shown in Fig. 2, both SCaM-1 and SCaM-3 encoded the same calmodulin isoform, SCaM-1, and have an identical amino acid

---

1 The abbreviations used are: PCR, polymerase chain reaction; CNBr, cyanogen bromide; PDE, 3,5-cyclic nucleotide phosphodiesterase; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription-coupled polymerase chain reaction; SCaMs, soybean calmodulins; bp, base pair(s).
sequence to that of alfalfa calmodulin (32). SCaM-2 differed by only two amino acid residues from SCaM-1 and was identical to barley calmodulin (33). However, SCaM-4 and -5 encoded novel divergent calmodulins, and the two isoforms were different from SCaM-1 by 32 amino acid residues out of 149 amino acid residues in both cases. Among them, at least 10 substitutions were non-conservative exchanges which had not been found in any of plant and animal calmodulins. The extent of amino acid substitutions found in SCaM-4 and -5 was very surprising since potato calmodulin (34), the most divergent calmodulin isoform isolated in plants, has 10 amino acid substitutions from SCaM-1. Thus these results indicate that SCaM-4 and -5 are the most divergent calmodulins identified so far in both animals and plants. To see the primary sequence relationship of the two divergent SCaM isoforms with other known calmodulins, a phylogenetic analysis was performed using amino acid sequences of 33 known calmodulins by the neighbor-joining method (see "Materials and Methods"). The neighbor-joining tree, Fig. 3, showed a closer relationship of SCaM-1 and SCaM-2 to most of plant calmodulins as expected. Potato, tomato, and one petunia calmodulin isoform constitute a separate group, which reflects primary structural diversities of them. However, two novel SCaM-4 and -5 did not belong to either of the two plant calmodulin groups and instead constituted a new independent group. Furthermore, the long branch length for SCaM-4 and -5 suggests a release of functional constraint and attainment of new functions for the two soybean calmodulin isoforms.

Characteristics of SCaM-1 and SCaM-4 Proteins Expressed in E. coli—To investigate significance of multiple amino acid substitutions at the protein level, two representative calmodulin isoforms, SCaM-1 and SCaM-4 proteins, were produced in E. coli (see "Materials and Methods") and purified to homogeneity by Ca$^{2+}$-dependent hydrophobic interaction chromatography (17). During the purification, the two proteins behaved similarly with respect to heat stability and elution profiles on a phenyl-Sepharose column. First, we investigated electrophoretic mobility shift of the two calmodulin isoforms upon Ca$^{2+}$ binding which is a typical characteristic of calmodulins (35, 36). Both SCaM-1 and SCaM-4 proteins showed Ca$^{2+}$-dependent electrophoretic mobility shifts. However, the extent of shift was different among calmodulin isoforms (Fig. 4). SCaM-4 showed the greatest degree of mobility shift and the extent of shift was approximately 2-fold greater than that of SCaM-1. This is very interesting because SCaM-1 and -4 have nearly identical calculated molecular weights of 16,862 and 16,819 and isoelectric points of 4.26 and 4.36, respectively. These results suggest that SCaM-4 may become a more compact structure than SCaM-1 and bovine calmodulin upon Ca$^{2+}$ binding. Also, we investigated antigenic differences among the two isoforms and bovine calmodulin. Unpurified antisera raised against SCaM-1 and SCaM-4 in goats were examined for cross-reactivity by immunoblot analysis. As shown in Fig. 5, anti-SCaM-4 antiserum recognized SCaM-4 as expected but not SCaM-1 and bovine calmodulin. In contrast, anti-SCaM-1 antiserum recognized SCaM-1 and bovine calmodulin but not SCaM-4, suggesting that SCaM-1 has a more closely related structure to bovine calmodulin than SCaM-4. However, after overexposure of immunoblots, faint cross-reacting bands were observed in both blots, indicating that the two isoforms may have common but very weak antigenic epitopes (data not shown). Thus the two calmodulin isoforms have different major antigenic determinants despite 70% of amino acid identity. Control experiments with anti-bovine brain calmodulin antibody showed a poor cross-reaction with SCaM-4 in contrast to...
a strong cross-reaction with SCaM-1 as effective as bovine brain calmodulin (data not shown). These data strongly suggest that the multiple amino acid substitutions may confer significant differences on the protein structure of SCaM-4.

**Differential Activation of Phosphodiesterase and NAD Kinase by SCaM-1 and SCaM-4—**

To assess the ability of the divergent calmodulin, SCaM-4, to activate calmodulin-dependent enzymes, phosphodiesterase and NAD kinase assays were performed. For these assays, bovine heart calmodulin-deficient cyclic nucleotide phosphodiesterase was purchased from a commercial source, and NAD kinase was partially purified from pea seedlings as described (see "Materials and Methods"). SCaM-1 activated NAD kinase approximately 5-fold higher than bovine brain calmodulin (data not shown). This is most likely due to the absence of post-translational modification of SCaM-1 protein prepared in *E. coli* because trimethylation of calmodulin has been shown to decrease NAD kinase activation by 4-fold (37). In the presence of EGTA, both enzymes did not show any activity regardless of the presence of activator calmodulins. Thus, the activation of both enzymes by calmodulin is a calcium-dependent process.

**Differential Expression of SCaM-1 and SCaM-4 in Various Tissues—**

To examine the expression pattern of the five calmodulin genes, we carried out Northern blot analyses using total RNA. RNA gel blots prepared from total RNA of various tissues and organs were hybridized with each gene-specific probe prepared from the 3' untranslated region of each cDNA (see "Materials and Methods"). We first examined total calmodulin gene expression with a probe made from the coding region of SCaM-1 since this probe cross-hybridized with all other calmodulin cDNAs isolated from Arabidopsis (5–7). The expression pattern of SCaM-4 is a bona fide functional calmodulin isoform despite of its primary structural diversity. However, surprisingly, when examined for the activation of pea seedling NAD kinase, the divergent SCaM-4 did not activate NAD kinase at all even at 500-fold higher concentration than that of SCaM-1 for a maximal activation of NAD kinase (Fig. 6B). SCaM-1 activated NAD kinase approximately 5-fold higher than bovine brain calmodulin (data not shown). This is most likely due to the absence of post-translational modification of SCaM-1 protein prepared in *E. coli* because trimethylation of calmodulin has been shown to decrease NAD kinase activation by 4-fold (37). In the presence of EGTA, both enzymes did not show any activity regardless of the presence of activator calmodulins. Thus, the activation of both enzymes by calmodulin is a calcium-dependent process.

**Differential Expression of SCaM-1 and SCaM-4 in Various Tissues—**

To examine the expression pattern of the five calmodulin genes, we carried out Northern blot analyses using total RNA. RNA gel blots prepared from total RNA of various tissues and organs were hybridized with each gene-specific probe prepared from the 3' untranslated region of each cDNA (see "Materials and Methods"). We first examined total calmodulin gene expression with a probe made from the coding region of SCaM-1 since this probe cross-hybridized with all other calmodulin cDNAs under hybridization conditions used. As shown in Fig. 6A, both of the two calmodulin isoforms activated phosphodiesterase equally well and the half-maximal activation values of the two isoforms were 7.63 and 6.17 nM for SCaM-1 and SCaM-4, respectively. In addition, maximal activation values of the two SCaM isoforms for PDE were not significantly different from each other. These results clearly indicate that SCaM-4 is a bona fide functional calmodulin isoform despite of its primary structural diversity. However, surprisingly, when examined for the activation of pea seedling NAD kinase, the divergent SCaM-4 did not activate NAD kinase at all even at 500-fold higher concentration than that of SCaM-1 for a maximal activation of NAD kinase (Fig. 6B). SCaM-1 activated NAD kinase approximately 5-fold higher than bovine brain calmodulin (data not shown). This is most likely due to the absence of post-translational modification of SCaM-1 protein prepared in E. coli because trimethylation of calmodulin has been shown to decrease NAD kinase activation by 4-fold (37). In the presence of EGTA, both enzymes did not show any activity regardless of the presence of activator calmodulins. Thus, the activation of both enzymes by calmodulin is a calcium-dependent process.
expression of SCaM-4 was barely detected with total RNA gel blots probably due to a low expression level of the gene. However, when poly(A) RNA was used for Northern blot analysis, the expression of SCaM-4 was clearly visible in apical and elongating regions of hypocotyls of soybean seedlings (Fig. 7, panel C). To quantitate the relative expression level of SCaM-1 and SCaM-4, semi-quantitive RT-PCR analysis was performed. The primer set used for the amplification of SCaM-1 mRNA was designed to co-amplify SCaM-1 and SCaM-3 transcripts because both genes encode the same protein, SCaM-1. The specificity of these primers for SCaM-1, -2, and -3 were shown using SCaM-1, -2, and -3 cDNA clones (data not shown). As shown in Fig. 7D, the result of RT-PCR assay for SCaM-1 and SCaM-4 was consistent with that of Northern analysis. The two isoform genes were actively expressed in apical and elongating regions of hypocotyls although the level of SCaM-4 mRNA was at least 5-fold lower than that of SCaM-1. In mature hypocotyls, both genes were expressed at very low but similar levels so that bands were detected only after prolonged exposure of the blot (data not shown). Interestingly, in roots SCaM-4 mRNA was almost undetectable whereas expression level of SCaM-1 was as abundant as for that of apical and elongating regions of hypocotyls. The differential expression patterns of SCaM-1 and SCaM-4 suggest that these genes may be subject to completely different transcriptional regulations.

Presence of SCaM-4 Homologs in Other Plants—The finding of the functionally different calmodulin isoform in soybean prompted us to investigate the presence of SCaM-4 homologs in various plants at the protein level. A Western blot analysis with SCaM-4-specific antibody (see "Materials and Methods" for preparation) was employed to study this possibility using protein extracts from six different plant species including five dicot and one monocot plants. As shown in Fig. 8A, soybean extract and all other plant extracts contained protein species recognized by the anti-SCaM-4-specific antibody, suggesting that SCaM-4 homologs or closely related proteins may be expressed in other plants. The multiple bands migrated between 14.4 and 21.5 kDa may represent one group of proteins closely related to SCaM-4 which have different electrophoretic mobilities, such as SCaM-5, or post-translationally modified SCaM-4 homologs. The multiple bands migrated between 14.4 and 21.5 kDa may represent one group of proteins closely related to SCaM-4 which have different electrophoretic mobilities, such as SCaM-5, or post-translationally modified SCaM-4 homologs.

Presence of SCaM-4 Homologs in Other Plants—The finding of the functionally different calmodulin isoform in soybean prompted us to investigate the presence of SCaM-4 homologs in various plants at the protein level. A Western blot analysis with SCaM-4-specific antibody (see "Materials and Methods" for preparation) was employed to study this possibility using protein extracts from six different plant species including five dicot and one monocot plants. As shown in Fig. 8A, soybean extract and all other plant extracts contained protein species recognized by the anti-SCaM-4-specific antibody, suggesting that SCaM-4 homologs or closely related proteins may be expressed in other plants. The multiple bands migrated between 14.4 and 21.5 kDa may represent either a group of proteins closely related to SCaM-4 which have different electrophoretic mobilities, such as SCaM-5, or post-translationally modified forms of SCaM-4 homologs (38). When the same blot was reprobed with anti-SCaM-1-specific antibody to examine cross-reactivity, as shown in Fig. 8B, a different immunostaining pattern was obtained, which further ensured no detectable cross-reactivity of antibodies used in this experiment. Interestingly, the protein level of SCaM-4 is relatively lower than that of SCaM-1. These results indicate that other plants may also have both forms of calmodulin isoforms, SCaM-1 and SCaM-4 homologs.
Calmodulin is one of the most highly conserved proteins in higher eukaryotes. The essential role of calmodulin in a variety of cellular processes may be the reason for strict conservation of the primary structure of calmodulin during evolution. However, recent studies in a plant system revealed the presence of multiple calmodulin isoforms in a single organism (4–7). This is very interesting because there exists only a single form of calmodulin in animal systems although calmodulin is encoded by multiple genes (8, 9). This implicates that the plant system may have a unique feature although the overall Ca$^{2+}$-dependent electrophoretic shift, antigenicity, and, most importantly, the ability to activate calmodulin-dependent enzymes. To our surprise, SCaM-4 did not activate NAD kinase at all although its ability to activate phosphodiesterase was as good as SCaM-1. In addition, SCaM-4 did not inhibit the activation of NAD kinase by SCaM-1 even in the presence of 100-fold molar excess of SCaM-4 to that of SCaM-1, suggesting that SCaM-4 may not bind NAD kinase at the assay condition used (data not shown). The reason for this difference may be due to structural difference between SCaM-1 and SCaM-4. Previous studies on the relationship between structure and function of calmodulin by site-directed mutagenesis or isolation of mutants showed that only single critical amino acid substitution could change the ability of calmodulin to activate target enzymes (39–41). Thus the failure of SCaM-4 to activate NAD kinase is most likely due to the divergent primary structure of SCaM-4. However, we cannot exclude the possibility that plant phosphodiesterase is differently regulated by these isoforms.

It is very intriguing to have such divergent calmodulin isoforms in plants compared to animal systems. Although, currently there is no definitive answers to the role(s) of the divergent isoforms, our studies with SCaM-1 and SCaM-4 provide important clues. The differential activation of calmodulin-dependent enzymes by different calmodulin isoforms suggests that each isoform may have its own target enzymes although some of them may be shared. This implicates that, in plant, there may exist calmodulin isoform-specific processes. However, this notion needs further studies on the identification of more calmodulin-dependent enzymes. Currently a very limited number of calmodulin-dependent enzymes have been identified or cloned in plants, which makes these studies difficult. Another possible function of plant calmodulin isoforms is signal-, tissue-, and/or developmental stage-specific roles. Consistent with this proposal, differential expression of two mungbean calmodulin isoforms in response to various stimuli have been reported recently (42). Interestingly, the two mungbean calmodulin isoforms MBCaM-1 and MBCaM-2 have identical amino acid sequences to SCaM-1 and SCaM-2, respectively. Thus it is plausible that SCaM-1 and -2 may respond similarly to these various stimuli although SCaM-1,-2, and -3 showed the same expression patterns in various tissues we examined. Also the different expression level and patterns of SCaM-4 compared to other calmodulin genes support the notion of the tissue- and/or organ-specific functions of calmodulin isoforms. Further studies on the response of each calmodulin gene to various stimuli will clarify these possibilities.

Currently we are in the process of generating transgenic tobacco plants with sense and antisense constructs of SCaM-1 and -4 to investigate the biological function of each isoform in vivo. However, until now we were not very successful in obtaining transgenic plants. One of reasons may be due to the essential role(s) of calmodulin in various processes in cells, thus plants with perturbation of calmodulin expression level may not be able to survive.

Acknowledgments—We thank Dr. Y. J. Choi for providing the rice calmodulin genomic clone cam-2. We greatly appreciate Dr. W. Martin for his kind help in the phylogenetic analysis and Dr. S. G. Rhee for critical reading of the manuscript. We also appreciate all members of PMBRRC for their kind help and discussions.

REFERENCES

1. Cheung, W. Y. (1980) Sience, 207, 19–27
2. Roberts, D. M., and Harmon, A. C. (1992) Plant Mol. Biol. 20, 1705–1706
3. Penniston, J. T., Carafoli, E., and Strehler, E. (1988) J. Biol. Chem. 263, 17055–17062
4. Higgins, D. H., and Sharp, P. M. (1988) Gene (Amst.) 73, 237–244
5. Ota, T., and Nei, M. (1994) J. Mol. Evol. 39, 442–445
6. Saito, N., and Nei, M. (1987) J. Mol. Biol. 202, 387–395

Fig. 8. Detection of SCaM-1 and -4 in soybean and other plants by immunoblotting. Protein extracts from six different plant species were prepared as described under "Materials and Methods." Sixty μg of total protein extracts in sample buffer containing 5 mM CaCl$_2$ were subjected to a 13.5% SDS-polyacrylamide gel electrophoresis. Immunoblotting was done using affinity-purified anti-SCaM-1 or -4-specific antibodies (for preparation of isofom-specific antibodies, see "Materials and Methods"). Panel A, anti-SCaM-4 immunoblot. Panel B, anti-SCaM-1 immunoblot. Size markers are indicated in the left of panels A and B in kilodaltons. Lanes 1–6 contain soybean, Arabidopsis, Chinese cabbage, rice, tobacco, and tomato extracts, respectively. Lanes 7 and 8 contain 10 ng of purified SCaM-1 and SCaM-4, respectively.
Novel Plant Calmodulin Isoforms

21812

15. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89
16. Fromm, H., and Chua, N.-H. (1992) Plant Mol. Biol. Rep. 10, 199–206
17. Gopalakrishna, R., and Anderson, W. B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836
18. Laemmli, U. K. (1970) Nature 227, 680–685
19. Chafouleas, J. G., Riser, M. E., Lagace, L., and Means, A. R. (1983) Methods Enzymol. 102, 104–110
20. Ling, V., and Assmann, S. M. (1992) Plant Physiol. 100, 970–978
21. Schachté, C., and Marme, D. (1988) in Calcium-binding proteins (Thompson, M., ed) Vol. I, pp. 83–96, CRC Press, Boca Raton, FL
22. Muto, S., and Miyachi, S. (1977) Plant Physiol. 59, 55–60
23. Harmon, A. C., Jarrett, H. W., and Cormier, M. J. (1984) Anal. Biochem. 141, 168–178
24. Leatherbarrow, R. J. (1992) GraFit Version 3.0, Erithacus Software Ltd., Staines, United Kingdom
25. VanBerkum, M. F. A., and Means, A. R. (1991) J. Biol. Chem. 266, 21488–21495
26. Deliaporta, S., Wood, J., and Hicks, J. (1983) Plant Mol. Biol. Rep. 1, 19–21
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
29. Hong, J. C., Nagao, R. T., and Key, J. L. (1989) Plant Cell 1, 937–943
30. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
31. Galea, E., and Feinstein, D. L. (1992) PCR Methods Appli. 2, 66–69
32. Barnett, M. J., and Long, S. R. (1990) Nucléac Acids Res. 18, 3395
33. Ling, V., and Zielsinski, R. E. (1989) Plant Physiol. 90, 714–719
34. Jena, P.K., Reddy, A. S. N., and Poovaiah, B. W. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3644–3648
35. Burgess, W. H., Jemiolo, D. K., and Kretsinger, R. H. (1980) Biochim. Biophys. Acta 623, 257–270
36. Klee, C. B., Crouch, T. H., and Krinks, M. H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6270–6273
37. Roberts, D. M., Rowe, P. M., Siegel, F. L., Lukas, T. J., and Watterson, D. M. (1986) J. Biol. Chem. 261, 1491–1494
38. Jablonsky, P. P., Grollig, F., Perkin, J. L., and Williamson, R. E. (1991) Plant Sci. 76, 175–184
39. Kink, J. A., Maley, M. E., Preston, R. R., Ling, K., Wallen-Friedman, M. A., Saini, Y., and Kung, C. (1990) Cell 62, 165–174
40. Ohy, Y., and Bobstlein, D. (1994) Science 263, 963–966
41. Gao, Z. H., Krebs, J., VanBerkum, M. F. A., Tang, W.-J., Maune, J. F., Means, A. R., Stull, J. T., and Beckingham, K. (1993) J. Biol. Chem. 268, 20096–20104
42. Botella, J. R., and Arteca, R. N. (1994) Plant Mol. Biol. 24, 757–766
43. Watterson, D. M., Sharief, F., and Vanaman, T. C. (1980) J. Biol. Chem. 255, 962–975
44. Strynadka, N. C. J., and James, M. N. G. (1989) Annu. Rev. Biochem. 58, 951–998