

Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree)

(latex/rubber biosynthesis/ethylene/plant defense genes/cell specificity)

ANIL KUSH*[†], ELISABETH GOYVAERTS*[†], MEE-LEN CHYE*, AND NAM-HAI CHUA[†]

*Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511, Republic of Singapore; and [†]Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399

Communicated by James Bonner, December 26, 1989

ABSTRACT Natural rubber, *cis*-1,4-polyisoprene, is obtained from a colloidal fluid called latex, which represents the cytoplasmic content of the laticifers of the rubber tree (*Hevea brasiliensis*). We have developed a method of extracting translatable mRNA from freshly tapped latex. Analysis of *in vitro* translation products of latex mRNA showed that the encoded polypeptides are very different from those of leaf mRNA and these differences are visible in the protein profiles of latex and leaf as well. Northern blot analysis demonstrated that laticifer RNA is 20- to 100-fold enriched in transcripts encoding enzymes involved in rubber biosynthesis. Plant defense genes encoding chitinases, pathogenesis-related protein, phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, cinnamyl alcohol dehydrogenase, and 5-enolpyruvylshikimate-3-phosphate synthase show a 10- to 50-fold higher expression in laticifers than in leaves, indicating the probable response of rubber trees to tapping and ethylene treatment. Photosynthetic genes encoding ribulose-bisphosphate carboxylase small subunit and chlorophyll *a/b*-binding protein are not expressed at a detectable level in laticifers. In contrast, genes encoding two hydrolytic enzymes, cellulase and polygalacturonase, are more highly expressed in laticifers than in leaves. Transcripts for the cytoplasmic form of glutamine synthase are preferentially expressed in laticifers, whereas those for the chloroplastic form of the same enzyme are present mainly in leaves. Control experiments demonstrated that β -ATPase, actin, and ubiquitin are equally expressed in laticifers and leaves. Therefore, the differences in specific transcript abundance between laticifers and leaves are due to differential expression of the genes for these transcripts in the laticifers.

Hevea brasiliensis is a perennial tropical plant that belongs to the family *Euphorbiaceae* (1). This plant is widely cultivated in Southeast Asia for its ability to produce natural rubber, which is *cis*-1,4-polyisoprene (2). In 1988, the worldwide production of natural rubber was 5 million tons, accounting for 32% of the total rubber consumption in the world.

The economic importance of natural rubber has prompted active investigations on the biochemical and cell biological aspects of rubber biosynthesis. Through the efforts of several laboratories, it is now known that *cis*-1,4-polyisoprene is synthesized on particles suspended in the cytosol of laticifers (2–4). The latter are specialized vessels that are arranged as concentric sheaths in the phloem (5, 6). The cells in these mantles are anastomosed, forming a true paracirculatory system. After tapping, the severed laticiferous mantles expel a fraction of their contents in the form of latex. It has been estimated that latex contains 30–50% (wt/wt) of *cis*-1,4-polyisoprene (7).

A unique feature of the laticifers is the absence of cytoplasmic connections or plasmodesmata between these cells

and their neighboring cells (8). Therefore, the exuded latex should represent only the cytoplasmic contents of the laticifers, uncontaminated by those of other cells. Since latex can be readily obtained in large quantities, it provides an opportunity to investigate the biochemical properties of a single, specialized cell type.

We are interested in the regulation of gene expression in the laticifers of *H. brasiliensis*. Although Tupy (9) has previously demonstrated the presence of ribosomes and polysomes in latex, the isolation of translatable mRNA has not been reported thus far. This is presumably due to the presence in latex of active RNase, which is a constituent of the luteoid particles (10). In this paper, we have modified the procedure of Prescott and Martin (11) to prepare RNA from latex. Latex poly(A) RNAs are active in an *in vitro* translation system and direct the synthesis of polypeptides distinct from those programmed with leaf mRNA. Northern blot analysis demonstrated that transcript levels of genes involved in rubber biosynthesis and genes induced by wounding and ethylene treatment are higher in laticifers than in leaves.

MATERIALS AND METHODS

Plant Material. Thirty-year-old rubber plants (*H. brasiliensis*), grown in the Sebang region of Singapore, were used for regular tapping and collection of latex and other plant materials. One week before tapping, the plants were treated with 0.1% ethephon as recommended by the manufacturer. Materials from these plants were used for RNA extraction and Northern blot analyses. Experiments on *in vitro* translation were performed with materials collected from untreated 2-year-old plants (RRIM 600) grown in a greenhouse.

Protein Analysis. Leaves from *H. brasiliensis* (RRIM 600) were frozen in liquid nitrogen and ground to a powder with a mortar and pestle. The powder was suspended in a buffer containing 10% (vol/vol) glycerol, 50 mM Tris-HCl (pH 6.8), 14 mM 2-mercaptoethanol, 2% NaDodSO₄, and 0.8 mM phenylmethylsulfonyl fluoride, and the suspension was incubated at 100°C for 5 min. Insoluble debris was pelleted by centrifugation at 10,000 × *g* for 10 min. Latex was mixed 1:1 (vol/vol) with the loading buffer containing 62 mM Tris-HCl (pH 6.8), 3% NaDodSO₄, 10% glycerol, 6 M 2-mercaptoethanol, and the sample was incubated at 100°C for 5 min. Protein concentrations were determined according to Bradford (12). Before loading, *in vitro* translation products were treated with 1 μg of RNase for 15 min on ice and precipitated with an equal volume of saturated ammonium sulfate. The protein pellets were washed with 80% (vol/vol) acetone and dried under vacuum. Two-dimensional gel electrophoresis using nonequilibrium pH gradient gel electrophoresis and NaDodSO₄/PAGE was essentially according to Tingey *et al.* (13).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HMGS, hydroxymethylglutaryl CoA synthase; HMGR, hydroxymethylglutaryl CoA reductase.

Table 1. cDNA probes used for Northern blot analysis.

Probe	Source (ref.)
HMGS	<i>H. brasiliensis</i> *
HMGR	<i>H. brasiliensis</i> *
rbcS	Tobacco (15)
Cab	Pea (16)
Pathogenesis-related protein	Tobacco (17)
Chitinase	Bean (18)
Chalcone synthase	Parsley (19)
Chalcone isomerase	<i>Antirrhinum majus</i> (E. Vrijlandt and C. Martin) [†]
Phenylalanine ammonia-lyase	Bean (20)
5-Enolpyruvylshikimate-3-phosphate synthase	Petunia (21)
Cinnamyl alcohol dehydrogenase	Bean (22)
Glutamine synthase, cytosolic	Pea (23)
Glutamine synthase, chloroplastic	Pea (23)
Polygalacturonase	Tomato (24)
Cellulase	Avocado (25)
Actin	Soybean (26)
Ubiquitin	Yeast (27)
β subunit of mitochondrial ATPase	Tobacco (28)

*Homologous cDNA clones were isolated from *Hevea* cDNA libraries and used as probes.

[†]Personal communication.

RNA Extraction and Analysis. Total RNA was extracted from *Hevea* leaves using guanidine thiocyanate (14) and from the latex by a modification of the method of Prescott and Martin (11). The pH of the extraction buffer was increased to 9.5 from 9.0. All precautions were taken to minimize possible contamination by RNases. For RNA extraction from latex, *Hevea* trees were tapped by giving inclined semicircular incisions ≈ 1 cm deep, using a tapping knife. The first few drops of latex, which contained mostly debris from the plant were discarded. A 50-ml Falcon tube containing ≈ 20.0 ml of 2 $\times$ RNA extraction buffer (0.1 M Tris-HCl/0.3 M LiCl/0.01 M EDTA/10% NaDodSO₄, pH 9.5) was tied to a tree in such a way that the fresh latex flowed directly into the tube. A stirrer was used to mix the incoming latex continuously with the buffer until ≈ 20.0 ml of latex was collected in each Falcon tube. The tubes were immediately frozen in liquid nitrogen and brought back to the laboratory for RNA extraction.

Frozen latex could be stored at -70°C without any adverse effects on RNA extraction.

After thawing, the latex was spun at $8000 \times g$ for 10 min to sediment debris, vacuolar membranes, and Frey-Wyssling particles. The $8000 \times g$ supernatant fraction was blended in the cold two times for 10 sec each with a Polytron. After blending, the supernatant fraction was processed essentially by the method of Prescott and Martin (11). RNA was precipitated twice with LiCl.

For Northern blot analysis, 10 μg of total RNAs, unless stated otherwise, was separated in formaldehyde gels and then blotted onto Zeta-Probes (Bio-Rad). The conditions of blotting, prehybridization, and hybridization, were as recommended by the manufacturer. For heterologous probes, the temperature of prehybridization and hybridization was lowered to 55°C . The list of probes along with their sources is given in Table 1.

RESULTS

Hevea latex is known to contain a high concentration of protein (7) and has been shown to be metabolically very active. Therefore, as a first step toward understanding the expression of various gene products in the laticifers, we compared the polypeptide profiles of leaf and latex by two-dimensional gel electrophoresis. Fig. 1 shows that the major protein constituents of leaf and latex are very different, indicating differences in their patterns of gene expression. To determine whether the differences in the polypeptide profile are reflected at the level of translatable mRNAs, we prepared poly(A) RNAs from leaf and latex. The method of Chirgwin *et al.* (14) using guanidine thiocyanate as a protein denaturant gave good quality RNA from *Hevea* leaves. On the other hand, this method, as well as Parish's method (15), which used urea as a denaturant, were unsuitable for the extraction of RNA from latex. However, a modification of the procedure described by Prescott and Martin (11) gave high quality RNA and good yield (30–50 μg per ml of latex).

Poly(A) RNAs from leaf and latex were found to be good templates in the rabbit reticulocyte *in vitro* translation system. Fig. 2 shows that the *in vitro* translation products of leaf mRNA are distinct from those of latex mRNA, indicating major differences in the translatable mRNA population.

To explore further the molecular basis of differential gene expression in leaf and laticifers, we carried out Northern blot analyses. Homologous and heterologous cDNAs encoding

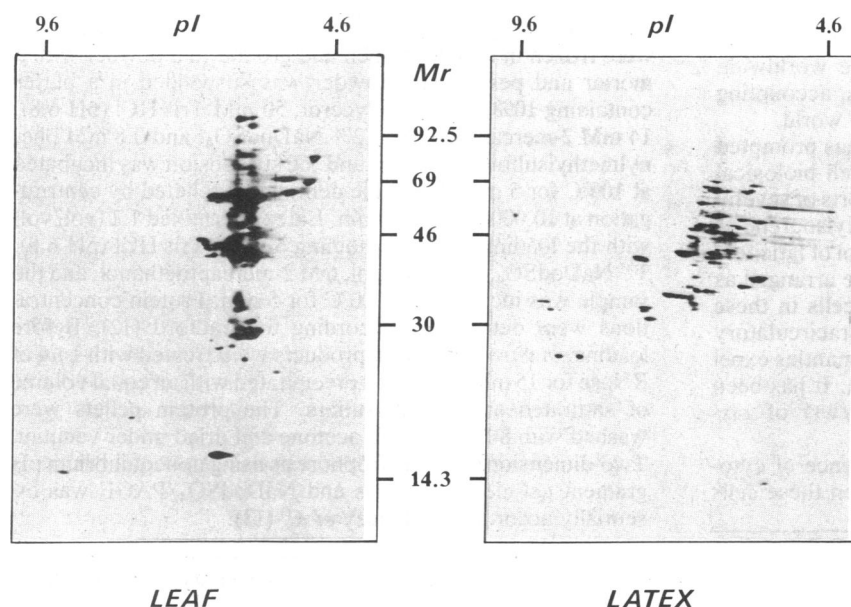


FIG. 1. Analysis of soluble polypeptides of leaf and laticifers by two-dimensional gel electrophoresis. Each sample contained 50 μg of total protein. Proteins were resolved by isoelectric focusing in the first dimension and NaDodSO₄/polyacrylamide gel electrophoresis in the second dimension. The polypeptides were visualized by silver staining according to Wray *et al.* (29). The pH gradient was from 10 to 3. Migration positions of the molecular weight markers in the second dimension are shown.

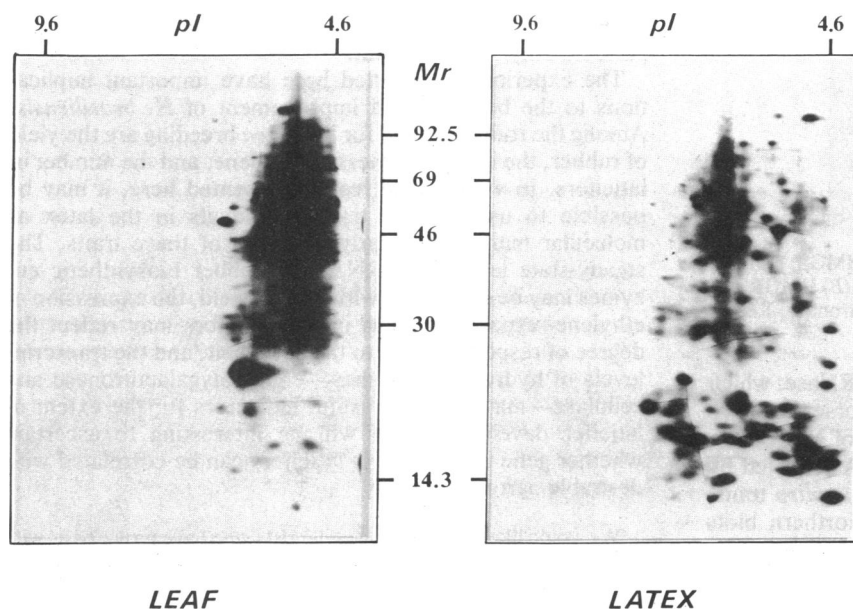


FIG. 2. Analysis of L-[^{35} S]methionine-labeled *in vitro* translation products of leaf and laticifer RNA by two-dimensional gel electrophoresis. Poly(A) RNAs were translated in rabbit reticulocyte lysate (Promega) following the instructions of the manufacturer. The gels were treated with Enhance (DuPont) followed by autoradiography. The translation products were resolved by two-dimensional gel electrophoresis according to Tingey *et al.* (13). For other details, see legend to Fig. 1.

known protein products were used as probes. As controls, we first hybridized the blots to cDNAs encoding actin, ubiquitin or the β subunit of the mitochondrial ATPase. Genes for these proteins are known to be constitutively expressed (25–27). We found that their transcripts are present in about equal amounts in both leaves and laticifers (Fig. 3).

Among the genes that have been shown to be highly expressed in green tissues of various higher plants (16) are *rbcS* and *Cab* encoding the small subunit of ribulose-1,5-bisphosphate carboxylase and the major chlorophyll *a/b*-binding protein, respectively. The Northern blot analysis in Fig. 4 shows clearly that these two photosynthetic genes are highly expressed in leaves but not in laticifers. The same expression pattern also prevails for the gene encoding the chloroplastic form of glutamine synthase (22). However, the gene encoding the cytosolic glutamine synthase displays the opposite expression pattern—i.e., high in laticifers and low in leaves (Fig. 4).

Since laticifers are the site of rubber biosynthesis, we investigated the expression of genes encoding enzymes of the rubber biosynthetic pathway. Hydroxymethylglutaryl CoA synthase (HMGS) and hydroxymethylglutaryl CoA reductase (HMGR) have been shown to be involved in early steps of rubber biosynthesis (30) and are regulatory enzymes in isoprenoid biosynthesis (31). We used *Hevea* cDNA clones encoding HMGS and HMGR as probes. Fig. 5 shows that transcripts for these two proteins are highly expressed in laticifers as compared to leaves. By serial dilution experiments, we estimated that these genes were expressed 20- to 100-fold higher in laticifers.

In our experiments, the latex was collected from *H. brasiliensis* by making semicircular incisions of ≈ 1 cm on the bark of the tree trunks. The incisions severed the laticifers, which then discharged their milky cytoplasmic contents. To

stimulate latex flow, we followed a common practice of rubber estates and treated the tapping panels with ethephon 1 week prior to tapping. Ethephon, or 2-chloroethylphosphonic acid, can be easily converted to ethylene, which is the stimulant (32). In other plant systems, wounding and ethylene are known to induce the expression of genes involved in plant defense (33–35). To determine whether defense genes are induced specifically in *Hevea* laticifers, we carried out Northern blot analysis with a number of heterologous cDNA probes (Table 1). Fig. 6 shows that the expression levels of chalcone synthase, chalcone isomerase, phenylalanine ammonia-lyase, cinnamyl alcohol dehydrogenase, pathogenesis-regulated protein, chitinase, and 5-enolpyruvylshikimate-3-phosphate synthase are indeed higher in laticifers than in leaves; the expression ratio is approximately 5–20 times, depending on the gene in question.

Since the activities of several hydrolytic enzymes are implicated in laticifer biogenesis (31), we compared the expression of genes encoding cellulase and polygalacturonase in laticifers and leaves. Fig. 5 documents that mRNAs for these two enzymes are about 5- to 10-fold more abundant in laticifers than in leaves.

DISCUSSION

Hevea plantations occupy >700 million hectares of land in the tropical regions of Asia, Africa, and South America and are the only source of natural rubber that is being used in the world. Natural rubber is extracted from latex, which is the exuded cytoplasmic content of laticifers. In an attempt to understand the regulation of gene expression in this particular cell type, we report here the successful isolation of translatable RNA from latex of *H. brasiliensis*. Previous failure to isolate active RNA could be attributed to two problems: the

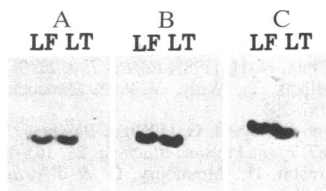


FIG. 3. Transcripts for actin, ubiquitin, and the β subunit of ATPase are equally expressed in leaf and laticifer. (A) Actin, 1.2 kilobases (kb). (B) Ubiquitin, 1.1 kb. (C) β -ATPase, 2.1 kb. LF, leaf; LT, laticifer.

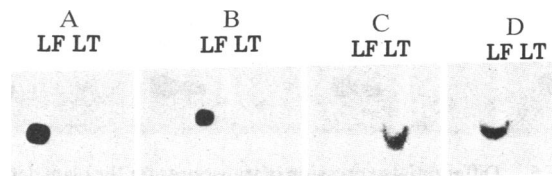


FIG. 4. Differential expression of transcripts encoding photosynthetic enzymes and enzymes for amino acid biosynthesis. (A) *rbcS*, 0.7 kb. (B) *Cab*, 1.2 kb. (C) Glutamine synthase, cytosolic, 1.3 kb. (D) Glutamine synthase, chloroplastic, 1.5 kb. LF, leaf; LT, laticifer.

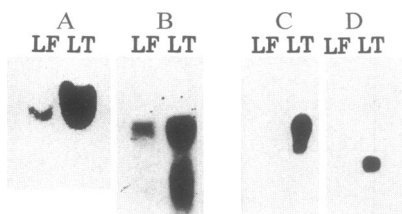


FIG. 5. Differential expression of transcripts for HMGS, HMGR, cellulase, and polygalacturonase. (A) HMGS, 3.4 kb. (B) HMGR, 2.4 kb and 1.4 kb. (C) Cellulase, 2.0 kb. (D) Polygalacturonase, 1.6 kb. LF, leaf; LT, laticifer.

coagulation of latex and the presence of active RNase, which compromises the RNA quality. We have overcome these two problems with the use of high concentrations of NaDodSO₄ and alkaline pH in the extraction buffer. RNAs isolated by our method serve as efficient templates in an *in vitro* translation system and give discrete signals in Northern blots indicating that they are not degraded.

The ability to isolate undegraded RNAs from latex has allowed us to compare the gene expression pattern in laticifers and leaves. It must be emphasized that although the leaf material used contains predominantly mesophyll cells, other cell types, including laticifers, are also present in low amounts. From the results obtained with *in vitro* translation and Northern blot analyses, we conclude that gene expression in laticifers is regulated at least at the level of steady-state mRNA. For example, *rbcs* and *Cab* mRNAs, which are major transcripts of leaf cells are not detectable in laticifers (Fig. 3). These results are entirely consistent with the observation that laticifers do not contain chloroplasts and are not photosynthetic. In contrast to photosynthetic genes, transcripts of genes involved in rubber biosynthesis are 20–100 times more abundant in laticifers than in leaves.

An interesting situation was seen with the genes encoding the chloroplastic and cytoplasmic form of glutamine synthase (23). Transcripts for the former are detected mainly in leaves, whereas those for the latter are restricted to laticifers, which are phloem derived. This differential expression suggests that the cytoplasmic form of glutamine synthase plays a special role in the amino acid metabolism of the laticifers.

Stimulation of latex production by the application of an ethylene generator (chloroethylphosphonic acid) to the bark is a very widely used practice. The increase in latex quantities obtained from ethephon-treated trees is due to reduced coagulation and an increased rate of laticifer regeneration *in situ*. We have observed that genes induced by wounding and ethylene are also highly expressed in laticifers. This is perhaps not surprising since ethephon was used as a stimulant and the trees were tapped by shaving the bark with a tapping knife. In the future it would be interesting to investigate the effects of ethephon alone on gene expression. Since most of the metabolic modifications caused by ethylene treatment are transient, it would also be interesting to ex-

amine the possible regulation of these genes in response to periodic ethylene treatment.

The experiments reported here have important implications to the breeding and improvement of *H. brasiliensis*. Among the traits selected for in *Hevea* breeding are the yield of rubber, the responsiveness to ethylene, and the number of laticifers. In view of the results presented here, it may be possible to use specific transcript levels in the latex as molecular markers for early selection of these traits. The steady-state levels of mRNAs for rubber biosynthetic enzymes may be correlated with rubber yield, the expression of ethylene-responsive genes in the laticifers may reflect the degree of responsiveness to the stimulant, and the transcript levels of hydrolytic enzymes—e.g., polygalacturonase and cellulase—may serve as useful indicators for the extent of laticifer development. It will be interesting to ascertain whether gene expression in laticifers can be correlated with desirable agronomic traits.

We would like to thank all the scientists who have provided us with the cDNA probes (Table 1) used in this work, and Low Kit Yi and Wendy Roine for typing the manuscript. We are grateful to Kalwant Singh for his help in tapping rubber trees.

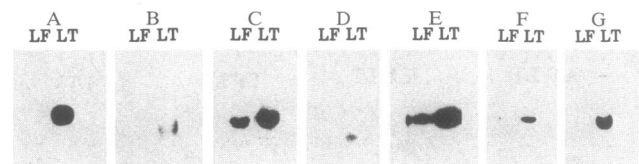


FIG. 6. Differential expression of transcripts for the plant defense genes. (A) Chitinase, 1.1 kb. (B) Pathogenesis-related protein, 1.0 kb. (C) Chalcone synthase, 1.2 kb. (D) Chalcone isomerase, 0.7 kb. (E) Phenylalanine ammonia-lyase, 1.5 kb. (F) Cinnamyl alcohol dehydrogenase, 1.7 kb. (G) 5-Enolpyruvylshikimate-3-phosphate synthase, 1.8 kb. LF, leaf; LT, laticifer.

1. Archer, B. L. & Audley, B. G. (1987) *Bot. J. Linn. Soc.* **94**, 181–186.
2. Backhaus, R. A. (1985) *Isr. J. Bot.* **34**, 283–293.
3. Barnard, D. (1965) in *Proceedings of the National Rubber Products Research Association* (MacLaren & Sons, London), p. 89.
4. Hepper, C. M. & Audley, B. G. (1969) *Biochem. J.* **114**, 379–386.
5. Dickenson, P. B. (1969) *J. Rubber Res. Inst. Malays.* **21**, 543–549.
6. Hebant, C. & de Fay, E. (1980) *Z. Pflanzenphysiol.* **97**, 391–398.
7. Kekwick, R. G. O. (1989) in *Physiology of Rubber Tree Latex*, eds. d'Auzac, J., Jacob, J. L. & Chrestin, H. (CRC, Boca Raton, FL), pp. 145–164.
8. Hebant, C. (1981) *Can. J. Bot.* **59**, 974–985.
9. Tupy, J. (1988) *Plant Sci.* **55**, 137–144.
10. Pujarniscle, S. (1968) *Physiol. Veg.* **6**, 27–46.
11. Prescott, A. & Martin, C. (1987) *Plant Mol. Biol. Reporter* **4**, 219–224.
12. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254.
13. Tingey, S. V., Walker, E. L. & Coruzzi, G. M. (1987) *EMBO J.* **6**, 1–9.
14. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
15. Parish, J. H. (1972) *Principles and Practice of Experiments With Nucleic Acids* (Halstead, New York), pp. 1–511.
16. Kuhlemeier, C., Green, P. J. & Chua, N.-H. (1987) *Annu. Rev. Plant Physiol.* **38**, 221–257.
17. Matsuoka, M., Yamamoto, N., Kano-Murakami, Y., Tanaka, Y., Ozeki, Y., Hirano, H., Oshima, M. & Ohashi, Y. (1987) *Plant Physiol.* **85**, 942–946.
18. Broglie, K. E., Gaynor, J. J. & Broglie, R. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6820–6824.
19. Reimold, U., Kroger, M., Kreuzaler, F. & Hahlbrock, K. (1983) *EMBO J.* **2**, 1801–1805.
20. Edward, K., Crama, C. L., Bolwell, G. P., Dixon, R. A., Schuch, W. & Lamb, C. J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6731–6735.
21. Gasser, C. S., Winter, J. A., Hironaka, C. M. & Shah, D. M. (1988) *J. Biol. Chem.* **263**, 4280–4289.
22. Walter, M. H., Grima-Pettenati, J., Grand, C., Boudier, A. M. & Lamb, C. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5546–5550.
23. Tingey, S., Tsai, F. Y., Edwards, J. W., Walker, E. L. & Coruzzi, G. M. (1988) *J. Biol. Chem.* **263**, 9651–9667.
24. DellaPenna, D., Alexander, D. C. & Bennett, A. B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6420–6424.
25. Christoffersen, R. E., Tucker, M. L. & Laties, G. G. (1987) *Plant Mol. Biol.* **3**, 385–391.
26. Shah, D. M., Hightower, R. C. & Meagher, R. B. (1983) *J. Mol. Appl. Gen.* **2**, 111–126.
27. Ozkaynak, E., Finley, D. & Varshavsky, A. (1988) *Nature (London)* **312**, 663–666.
28. Boutry, M. & Chua, N.-H. (1985) *EMBO J.* **4**, 2159–2165.
29. Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981) *Anal. Biochem.* **118**, 197–203.
30. Hepper, C. M. & Audley, B. G. (1969) *J. Biochem.* **114**, 379–386.
31. Bach, T. J. (1987) *Plant Physiol. Biochem.* **25**, 163–167.
32. Gidrol, X., Chrestin, H., Mounoury, G. & d'Auzac, J. (1988) *Plant Physiol.* **86**, 899–903.
33. Corbin, D. R., Sauer, N. M. & Lamb, C. J. (1987) *Mol. Cell. Biol.* **7**, 4334–4337.
34. Ecker, J. R. & Davis, R. W. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5202–5206.
35. Lawton, M. A. & Lamb, C. J. (1987) *Mol. Cell. Biol.* **7**, 335–341.