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Interaction of a gibberellin-induced factor with the upstream region of an α-amylase gene in rice aleurone tissue

(*Oryza sativa*/gene regulation/DNA-binding factor/DNA–protein interaction/plant growth regulator)

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ABSTRACT **The interaction between the DNA sequences of an α-amylase (EC 3.2.1.1) gene and a tissue-specific factor induced in rice (*Oryza sativa* L.) aleurone tissue by gibberellin was studied. DNA mobility-shift during electrophoresis indicated that a 500-base-pair sequence (HS500) of a rice α-amylase genomic clone (OSamy-a) specifically interacted with a factor from gibberellin-induced rice aleurone tissue. The amount of complex formed between the HS500 DNA fragment and the gibberellin-induced factor increased in proportion to increasing amounts of aleurone extract and decreased with the addition of unlabeled HS500 DNA fragment. A specific segment of the HS500 fragment was protected from exonuclease III digestion by protein(s) in the aleurone extract, revealing the approximate position of the protein–DNA interaction. In the protected region, there is a direct repeat that overlaps with a potential stem–loop structure. Protein in extracts from leaves, roots, or deembryonated seeds incubated without gibberellin A_3 did not bind to the HS500 DNA fragment. However, the binding factor was induced in deembryonated seeds when exogenous gibberellin A_3 was added during seed imbibition. This suggests that activation of α-amylase synthesis by gibberellin may be mediated by the formation of a factor that interacts with a specific sequence of the α-amylase gene.**

Gibberellin is a class of plant growth regulators (hormones) that is essential for various stages of plant development (1, 2). During germination, the embryo secretes gibberellin into the endosperm. In response to gibberellin, α-amylase (EC 3.2.1.1) gene expression in aleurone tissue, which forms the outer layer of the endosperm, is substantially induced so that the enzyme constitutes about 40% of the *de novo* protein synthesis in barley grains (3). Studies have shown that in barley (*Hordeum vulgare* L.) (4, 5), oat (*Avena fatua* L.) (6), rice (*Oryza sativa* L.) (7, 8), and wheat (*Triticum aestivum* L.) (9, 10), gibberellin regulates α-amylase synthesis in aleurone tissue. Experiments have also indicated that this regulation is exerted at the transcription level (5, 9), a conclusion that has been confirmed by nuclear run-on studies (11, 12).

Initiation and regulation of transcription in eukaryotes studied to date depend on regulatory proteins (DNA-binding factors) in addition to RNA polymerase II. In plants, two DNA-binding factors for storage-protein genes (13, 14) and one light-inducible DNA-binding factor for a ribulose-bisphosphate carboxylase small-subunit (*rbcS*) gene (15) have been identified. Several DNA-binding factors have been isolated in animal systems (16, 17). However, we know of no report along similar lines on hormone-induced synthesis of DNA-binding factors in plants. We demonstrate here that gibberellin A_3 induces the production of a factor, presumably a protein, in rice aleurone tissue that binds to a specific region of the upstream (regulatory) sequence of a rice α-amylase gene.

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MATERIALS AND METHODS

Chemicals and Enzymes. Restriction endonucleases were purchased from New England Biolabs, Boehringer Mannheim, or Promega Biotec (Madison, WI). Radioactive isotopes were purchased from Amersham.

Rice Extracts and Fractionation. Aleurone tissue from rice (*O. sativa* cv. IR26) seeds was isolated according to Chrispeels and Varner (4) after 2–3 days of imbibition in water or 5 μM gibberellin A_3 at room temperature. The rice plants were grown in a greenhouse with a 14-hr light period at 25 ± 3°C. Supplemental light intensity was approximately 8500 lux at pot level. Crude cellular proteins were extracted from various tissues of rice plants by grinding in buffer and centrifugation as described by Jofuku *et al.* (14). After fractionation with ammonium sulfate at 30–60% saturation, the precipitated proteins were suspended, dialyzed, and used for binding assays. Protein concentration was determined by using the Bio-Rad protein assay reagent and bovine serum albumin as a standard.

DNA Mobility-Shift (Gel Retardation) Assays. Binding buffer contained 20 mM Tris·HCl (pH 8.0), 50 mM KCl, 3–5 μg of poly(dI-dC)·(dI-dC) (optimal for our system), 10% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, and 0.5 mM dithiothreitol. The amounts of DNA and protein in the reaction mixture were as described in *Results*. Reactions were carried out in a final volume of 15 μl at room temperature for 20 min. After incubation, 2 μl of loading buffer [10 mM Tris·HCl, pH 8.0/50 mM dithiothreitol/1% (wt/vol) bromophenol blue/1% (wt/vol) xylene cyanol/50% (vol/vol) glycerol] was added to stabilize the protein–DNA complex, and the samples were immediately electrophoresed in a 6% polyacrylamide or 1% agarose gel under nondenaturing conditions. Gels were prepared with, and run in, a low-ionic-strength buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA) at about 10 V/cm for 4–5 hr. After electrophoresis, the gel was dried and exposed to x-ray film with an intensifying screen at −20°C for 12–24 hr.

Exonuclease III Protection Assays. Probes for exonuclease III protection assays were 5′-end-labeled by bacteriophage T4 polynucleotide kinase (17) and cleaved by appropriate restriction enzymes to produce single-end-labeled probes. Experiments were carried out in a P1 facility according to the National Institutes of Health guidelines for recombinant DNA research. Purified single-end-labeled probe (5 ng of DNA; 10,000 cpm/ng) (17) was incubated in the absence or presence of aleurone extract (5 μg of protein). Exonuclease III (300 units) was added to a final volume of 20 μl and the reaction mixture was incubated at 30°C for 20 min. The protected DNA was phenol-extracted, ethanol-precipitated,

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dissolved in 5 μl of formamide/dye mixture, and subjected to electrophoresis in a 6% polyacrylamide sequencing gel.

RESULTS

A rice genomic library (IR26) was constructed in λ phage vector EMBL4 and screened with α-amylase cDNA clones from barley (18, 19). Twenty clones containing sequences that hybridized to the barley probes were obtained. By use of restriction enzymes and hybridization analysis, four different restriction patterns were characterized (OSamy-a, -b, -c, and d; Fig. 1), each containing several duplicate and/or partial gene clones. The coding region of clone OSamy-a was partially sequenced [about 800 base pairs (bp)], and the nucleotide sequence was found to be 77% identical to the coding region of the barley amy-2 genomic clone (20). Clones OSamy-a and OSamy-b showed similar restriction patterns at the 5′ region but differed at the 3′ region. Clones OSamy-c and OSamy-d had no common restriction sites with clones OSamy-a and OSamy-b.

DNA mobility-shift assays were performed to determine whether protein(s) in gibberellin-induced aleurone extract binds to any region of clone OSamy-a. Several restriction fragments (≤1 kb) of OSamy-a were end-labeled with [α-^{32}P]dATP by the Klenow fragment of DNA polymerase and were tested for binding activity. The results indicated that a 500-bp fragment (HS500) of OSamy-a upstream region bound to a factor in rice aleurone extract and was thereby retarded during electrophoresis. The α-amylase DNA-binding activity increased in proportion to increasing amounts of aleurone extract (Fig. 2). The DNA–protein complex was equally stable when electrophoresed at 4°C or at room temperature. In contrast, the complex was unstable at elevated temperatures; after 15 min of incubation at 75°C prior to electrophoresis there was significantly less DNA bound, and after 15 min at 100°C there was no binding. When competing DNA (unlabeled HS500) was incubated with aleurone extract and labeled HS500, the binding activity decreased in proportion to increasing amounts of the unlabeled α-amylase DNA (Fig. 2). There was no binding activity with bovine serum albumin, and no decrease in binding activity was observed upon addition of nonspecific DNA (pBR322 or pUC13) (data not shown).

To localize the binding segment of the HS500 fragment more precisely, exonuclease III protection experiments were performed. The HS500 fragment was single-end-labeled (17)

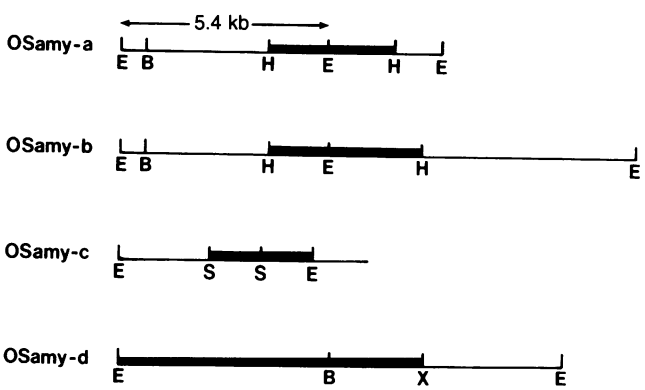

FIG. 1. Restriction map of rice α-amylase genomic clones. Clones containing sequences homologous to the barley α-amylase DNA probe were obtained by hybridization. Four different restriction patterns (OSamy-a, -b, -c and -d) with several duplicate and partial gene clones were identified by genomic blot analysis. Restriction sites are indicated (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal* I; X, *Xba* I). The regions of rice genomic clones that hybridized with barley α-amylase probe are represented by heavy lines. kb, Kilobases.

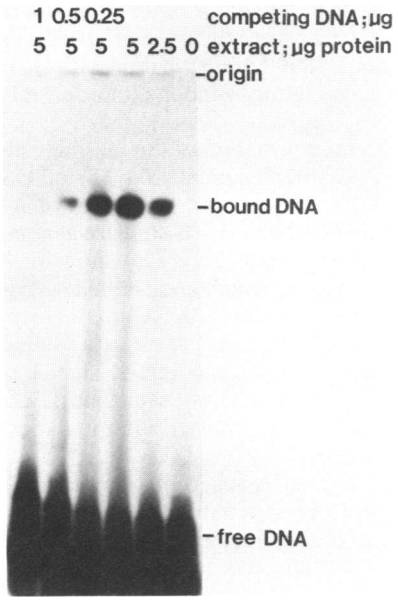

FIG. 2. α-Amylase DNA binding activity with rice aleurone protein. A labeled rice α-amylase DNA fragment (HS500; ≈10 ng, 10^6 cpm) from the upstream region of the OSamy-a genomic clone (details in Fig. 4) was incubated with various amounts of aleurone extract. The aleurone extract was prepared with rice aleurone tissue (plus scutella) after 2 days of gibberellin treatment. Interaction between α-amylase DNA (free DNA) and protein in aleurone extract retarded DNA migration so that the bound DNA moved slower during electrophoresis. In three samples, competing DNA (unlabeled HS500) was added to the binding mixture for specific DNA competition.

and incubated in the absence or presence of aleurone extract (5 μg of protein) as indicated (Fig. 3). An 80-bp fragment of OSamy-a upstream region was protected from exonuclease III digestion by the protein in aleurone extract. The 3′ end of this 80-bp fragment defines one boundary of the location

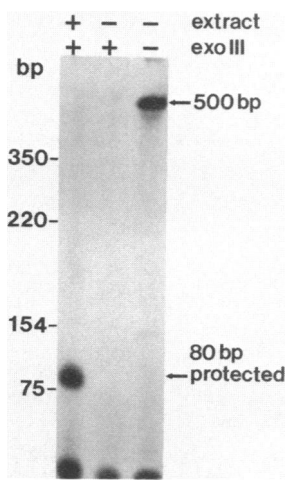

FIG. 3. Exonuclease III protection assay of DNA sequences that interact with aleurone protein. A rice α-amylase upstream fragment (HS500) was subcloned into pUC13, ^{32}P-labeled at the 5′ end, and cleaved with restriction enzyme to produce single-end-labeled probe. This purified DNA probe was incubated in the absence (−) or presence (+) of aleurone extract (5 μg of protein) as indicated. After reaction, the DNA was purified and electrophoresed in a 6% polyacrylamide sequencing gel. An 80-bp fragment of α-amylase DNA was protected from exonuclease III digestion by protein in the aleurone extract, indicating the position of the protein–DNA interaction. *Hin*fI-digested pBR322 DNA was used to provide size markers (lengths in bp at left). The corresponding protected region is shown in Fig. 4.

where the gibberellin-inducible protein binds to the upstream sequence of the rice α-amylase gene (Fig. 4). There was no DNA protection without preincubation with aleurone extract and no DNA degradation without exonuclease III treatment (Fig. 3).

A schematic representation of the plasmid subclone pOSamy-a-L, a 5′ *Eco*RI fragment (5.4 kb) of OSamy-a that contains the 500-bp fragment HS500, is shown in Fig. 4*a*. The upstream sequences of the rice α-amylase genomic clone and the barley amy-2 genomic clone (20) show distinct similarities. There are highly homologous regions around the transcription initiation site and "TATA box" (Fig. 4*b*). Within the 80-bp protected fragment, there is a tandem imperfect repeat that overlaps with a potential stem–loop structure (Fig. 4*b*). The protected DNA sequence is about 150 bp upstream of the putative transcription initiation site of the rice α-amylase gene.

Tissue extracts from leaves, roots, or seeds from which the embryos had been removed did not bind to HS500 (Fig. 5). However, the binding factor that interacted with the α-amylase gene was induced in deembryonated seeds by adding gibberellin A_3 during seed imbibition (Fig. 5, lane GA + D). The extra bands between free (F) and bound (B) DNA could be the result of an interaction with another binding protein.

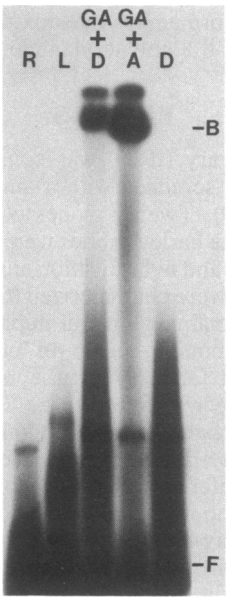

FIG. 5. Binding activity of α-amylase DNA in rice tissues. A rice α-amylase upstream fragment (HS500) was incubated under the same conditions as described for Fig. 2 except with various rice tissue extracts. Lane D, extract from deembryonated half-seeds with 2 days of imbibition without gibberellin A_3; lane GA + A, extract from rice aleurone tissue (plus scutella) with 2 days of gibberellin A_3 treatment; lane GA + D, extract from deembryonated half-seeds with 2 days of gibberellin A_3 treatment; lane L, extract from mature rice leaves; lane R, extract from rice roots. The top band, above the bound (B) form, is the labeled DNA remaining in the origin of the gel. Band F is free DNA.

DISCUSSION

Knowledge of the mechanisms of gene regulation has increased dramatically in recent years. In particular, progress in studies of steroid receptors (DNA-binding factors) has greatly advanced the understanding of gene regulation. By isolating, cloning, and analyzing the function of steroid-receptor genes, the specific region of the steroid receptor that binds to and activates the steroid-inducible genes has been delineated in animal systems (21–23). In plants, regulatory elements involved in gene expression have been identified by progessive deletion of 5′ upstream sequences, fusion with marker genes, and introduction into transgenic plants by the *Agrobacterium* tumor-inducing (Ti) plasmid (24–26). Studies of monocot gene expression have indicated that a DNA-binding factor interacts with 5′ upstream sequences of zein genes in maize (13). Deletion analysis of the zein gene by transient expression in carrot cell suspensions showed that

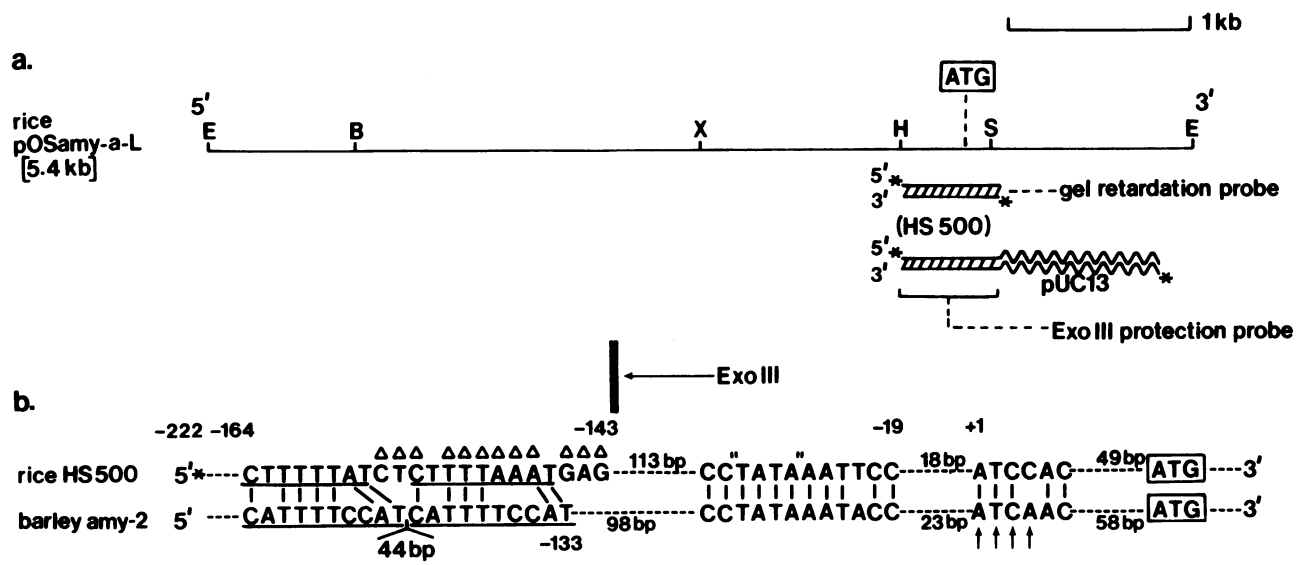

FIG. 4. Putative regulatory sequences of rice α-amylase gene. (*a*) Schematic representation of pOSamy-a-L, the 5′ *Eco*RI fragment (5.4 kb) from the rice α-amylase genomic clone OSamy-a. Restriction sites are indicated (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal* I; X, *Xba* I). ATG (also seen in b) represents the translation start site. Probes (hatched region) labeled at two ends and at one end were used for gel retardation and exonuclease III protein assays, respectively. Stars indicate the ^{32}P-labeled positions. (*b*) 5′ upstream sequences of rice α-amylase gene are compared with barley amylase-2 sequences (21). Sequence alignment has been maximized by introduction of gaps. Identical nucleotides are denoted with thin bars. Underlines indicate the repetitive sequences. Triangles indicate the sequences involved in a potential stem–loop structure. Horizontal arrow indicates the direction of exonuclease III digestion, and the heavy vertical bar above the sequences shows the boundary of the region in which exonuclease III digestion was blocked by DNA-binding protein. Numbers above rice or below barley sequences indicate the position relative to transcription initiation (+1). Quotation marks indicate a TATA box. Vertical arrows indicate barley transcription initiation region.

this protein-binding sequence was in the same region that was responsible for inducing expression *in vivo* (27). Analysis of DNase I-hypersensitive sites in the 5′ region of the maize alcohol dehydrogenase gene *Adh1* (28) was also correlated with the results from deletion and linker-scanning analysis in homologous transient expression *in vivo* (29). To our knowledge, DNA-binding experiments have not been reported in studies of hormone-induced gene regulation in plants. In studies of hormone regulation, it is particularly important to identify and then to isolate the intermediate(s) between the hormone and the target sites (induced genes). Therefore, we initiated the present investigation in analyzing the steps between gibberellin induction and α-amylase gene expression (induced gene).

In this report, we describe the isolation of four different rice α-amylase genomic clones and the identification of a tissue-specific DNA-binding factor after gibberellin induction. DNA mobility-shift (Figs. 2 and 5) and exonuclease III protection (Fig. 3) assays indicated that this binding factor interacted with the 5′ upstream region of a rice α-amylase gene. The DNA mobility-shift assay has become a very powerful tool with which to visualize the interaction between DNA molecules and proteins; for example, it has been used to identify the sequence-specific DNA-binding protein(s) that mediate the processes of gene regulation. This technique has advanced our understanding in several research areas (13, 17, 30) but has not been commonly used in plant research.

Exonuclease III protection assays were performed to better define the important region involved in DNA–protein interaction. Since exonuclease III digestion took place from the 3′ end, the functional boundary of the protein binding site corresponds to the 3′ end of the 80-bp protected fragment. However, the labeled 5′ end of HS500 corresponds to a restriction site and not to the functional boundary. In the 80-bp protected region, we found an imperfect repeat, CTTTTaAaT and CTTTTtAT, that overlapped with a potential stem–loop structure, CTCTTTTAAATGAG. This imperfect repeat begins 143 bp from the transcription initiation site in rice. In barley, the similar, but perfect, repeat CATTTTCCAT occurs 133 bp from the transcription initiation site. Although there are 44 bp between the repeating segments in barley, it is possible that this repeat interacts with DNA-binding protein by "looping-out" the DNA sequence (31). A comparison of sequences in the 5′ noncoding region of rice and barley α-amylase (Fig. 4*b*) indicates that there is high homology in those regions required for sequence-specific recognition, such as the translation initiation site, the transcription initiation site, the TATA box, and a repeat within the exonuclease III-protected region. We suggest that this repeat interacts with a regulatory protein. The potential stem–loop structure of rice also may have a regulatory role. Although the repeat and the stem–loop sequences overlap, it is possible that more than one binding factor is involved, as found in virus studies (32). Consistent with this possibility, each rice tissue examined had a faint band above the free DNA in mobility-shift gels (Fig. 5).

Although the results presented here have certain parallels with those obtained with steroid receptors in animal systems, we are of the opinion that the DNA-binding factor probably does not act directly as a hormone receptor. In preliminary studies, incubation of radioactive gibberellin with protein that had interacted with HS500 DNA did not reveal gibberellin–protein binding activity (data not shown). Also, the long lag time between gibberellin induction and α-amylase expression (12–24 hr; ref. 1) indicates that several steps may occur between gibberellin application and response. We cannot rule out the possibility that a preexisting factor was activated by gibberellin or that a weak interaction between radioactive gibberellin and DNA-binding protein occurred but was below our detection limit.

Our demonstration that a gibberellin-induced DNA-binding factor interacted with the 5′ upstream region of a rice α-amylase gene is a step toward understanding gibberellin action at the molecular level in the cereal aleurone system.

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1. Jacobsen, J. V. & Chandler, P. M. (1987) in *Plant Hormones and Their Role in Plant Growth and Development*, ed. Davies, P. J. (Nijhoff, The Hague, The Netherlands), pp. 164–193.
2. Graebe, J. E. (1987) *Annu. Rev. Plant Physiol.* **38**, 419–465.
3. Varner, J. E. & Ho, D. T.-H. (1976) in *The Molecular Biology of Hormone Action*, ed. Papaconstantinou, J. (Academic, New York), pp. 173–194.
4. Chrispeels, M. J. & Varner, J. E. (1967) *Plant Physiol.* **42**, 398–406.
5. Higgins, T. J. V., Jacobsen, J. V. & Zwar, J. A. (1982) *Plant Mol. Biol.* **1**, 191–215.
6. Hooley, R. (1982) *Planta* **154**, 29–40.
7. Tanaka, Y., Ito, T. & Akazawa, T. (1970) *Plant Physiol.* **46**, 650–654.
8. Akazawa, T. & Mitsui, T. (1985) in *New Approaches to Research on Cereal Carbohydrates*, eds. Hill, R. D. & Muner, L. (Elsevier, Amsterdam), pp. 129–137.
9. Baulcombe, D. C. & Buffard, D. (1983) *Planta* **157**, 493–501.
10. Baulcombe, D. C., Lazarus, C. & Martienssen, R. (1984) *J. Embryol. Exp. Morphol.* **83**, 119–135.
11. Jacobsen, J. V. & Beach, L. R. (1985) *Nature (London)* **316**, 275–277.
12. Zwar, J. A. & Hooley, R. (1986) *Plant Physiol.* **80**, 459–463.
13. Maier, U.-G., Brown, J. W. S., Toloczyki, C. & Feix, G. (1987) *EMBO J.* **6**, 17–22.
14. Jofuku, K. D., Okamuro, J. K. & Goldberg, R. B. (1987) *Nature (London)* **328**, 734–737.
15. Green, P. J., Kay, S. A. & Chua, N.-H. (1987) *EMBO J.* **6**, 2543–2549.
16. Guha, A., Bach, R., Konigsberg, W. & Nemerson, Y. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 299–302.
17. Wu, C., Wilson, S., Walker, B., Dawid, I., Paisley, T., Zimarino, V. & Ueda, H. (1987) *Science* **238**, 1247–1253.
18. Huang, J.-K., Swegle, M., Dandekar, A. M. & Muthukrishnan, S. (1984) *J. Mol. Appl. Genet.* **2**, 579–588.
19. Rogers, J. C. (1985) *J. Biol. Chem.* **260**, 3731–3738.
20. Knox, C. A. P., Sonthayanon, B., Chandra, G. R. & Muthukrishnan, S. (1987) *Plant Mol. Biol.* **9**, 3–17.
21. Yamamoto, K. R. (1985) *Annu. Rev. Genet.* **19**, 209–252.
22. Rusconi, S. & Yamamoto, K. R. (1987) *EMBO J.* **6**, 1309–1315.
23. Reiser, H., Coligan, J. Palmer, E., Benacerraf, B. & Rock, K. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2255–2259.
24. Chen, Z.-L., Schuler, M. A. & Beachy, R. N. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8560–8564.
25. Colot, V., Robert, L. S., Kavanagh, T. A., Bevan, M. W. & Thompson, R. D. (1987) *EMBO J.* **6**, 3559–3564.
26. Stockhaus, J., Eckes, P., Rocha-Sosa, M., Schell, J. & Willmitzer, L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7943–7947.
27. Roussell, D. L., Boston, R. S., Goldsbrough, P. B. & Larkins, B. A. (1988) *Mol. Gen. Genet.* **211**, 202–209.
28. Paul, A.-L., Vasil, V., Vasil, I. K. & Ferl, R. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 799–803.
29. Walker, J. C., Howard, E. A., Dennis, E. S. & Peacock, W. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6624–6628.
30. Carey, J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 975–979.
31. Dynan, W. S. & Tjian, R. (1985) *Nature (London)* **316**, 774–778.
32. Jones, N. C., Rigby, P. W. J. & Ziff, E. B. (1988) *Genes Dev.* **2**, 267–281.