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Abscisic acid and water-stress induce the expression of a novel rice gene

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We have identified a novel rice gene, called RAB 21, which is induced when plants are subject to water-stress. This gene encodes a basic, glycine-rich protein (mol. wt 16 529) which has a duplicated domain structure. Immunoblots probed with antibodies raised against β-galactosidase/RAB 21 fusion protein detect RAB 21 protein only in cytosolic cell fractions. RAB 21 mRNA and protein accumulate in rice embryos, leaves, roots and callus-derived suspension cells upon treatment with NaCl (200 mM) and/or the plant hormone abscisic acid (10 μM ABA). The effects of NaCl and ABA are not cumulative, suggesting that these two inducers share a common response pathway. Induction of RAB 21 mRNA accumulation by ABA is rapid (< 15 min in suspension cells) and does not require protein synthesis, indicating that preformed nuclear and/or cytosolic factors mediate the response to this hormone. We have characterized the RAB 21 gene by determining the complete nucleotide sequence of a nearly full-length cDNA and corresponding genomic copy, and by mapping the start site of its major transcript. The proximal promoter region contains various GC-rich repeats.

Key words: genomic sequence/cDNA sequence/cycloheximide/osmotic regulation/water deficit

Introduction

The hormone abscisic acid (ABA) mediates a number of important physiological processes in plants (King, 1976; Jones *et al.*, 1987). Developmental studies have shown that ABA induces the accumulation of specific mRNAs and proteins late during embryogenesis in seeds of diverse species (Galau *et al.*, 1986; Finklestein *et al.*, 1985; Litts *et al.*, 1987). At this time, the level of endogenous ABA increases, the seeds desiccate and the embryos of some species become dormant (King, 1976; Suzuki *et al.*, 1981). In cereal seeds, some of the 'late' developmental mRNAs are long-lived in mature, dry grains, but are rapidly degraded during seed germination. However, exogenously applied ABA causes the precocious accumulation of these mRNAs in immature embryos and their reappearance in germinating seeds (Finklestein *et al.*, 1985; Mundy *et al.*, 1986). Little is known about the functions of these ABA-inducible proteins or about their intracellular localization. Some of them are storage polypeptides (Finklestein *et al.*, 1985; Bray and Beachy, 1985), while others, such as lectins (Raikel and Wilkins, 1987) or an enzyme inhibitor (Mundy *et al.*, 1986) may be involved in seed protection and/or the maintenance of dormancy.

Physiological studies have shown that endogenous ABA levels increase in plant tissues subjected to water-stress by high osmoticum, NaCl, or drying (Henson, 1984; Jones *et al.*, 1987). Under these conditions, specific mRNAs and proteins accumulate which could affect intra-cellular osmolarity or have other protective functions (Finklestein and Crouch, 1986; Ramagopal, 1987). One such salt-inducible protein whose accumulation is increased by ABA has been isolated from tobacco and shown to be homologous to members of a group of proteinase inhibitors (Singh *et al.*, 1987; Richardson *et al.*, 1987). These results suggest that some of the ABA-inducible mRNAs and proteins which accumulate during seed desiccation are part of a general response by the plant to water deficit. If so, then part of this response involves the synthesis of enzyme inhibitors and lectins which may protect plant tissues from degradation by pathogens during periods of arrested growth and development.

Both the developmental studies on seeds and the physiological studies on water-stress indicate that ABA controls the accumulation of specific mRNAs and proteins. However, it is unclear whether ABA acts at the transcriptional or post-transcriptional level, or both (Jacobsen and Beach, 1986; Mozer, 1980). The mode of action of the hormone via receptors and/or transducing pathways also remains obscure (Hornberg and Weiler, 1984) and it is not known whether transduction of the ABA response signals requires *de novo* protein synthesis. To date, no genomic sequences have been reported for plant genes strongly induced by ABA. Characterization of promoter sequences of such genes will provide a tool with which to dissect the mechanism by which ABA regulates specific gene expression.

We are interested in determining how gene expression is regulated by plant hormones. We have chosen to study the effect of ABA on gene expression in rice because, as we demonstrate here, this hormone plays a central role in seed development and in the response of rice plants to water-stress, two important agronomic traits (Chang *et al.*, 1986; Seshu and Sorrells, 1986). Knowledge of the structure and function of ABA-responsive proteins will aid our understanding of the physiology of seed maturation and of drought tolerance in cereals. As a first step, we have isolated several cDNA clones whose expression is induced by ABA and water-stress. One of these clones, called RAB 21 (for Responsive to ABA), was fully characterized. We present here the sequence of this novel rice gene, and a characterization of the RAB 21 protein product. We show that the induction of the RAB 21 by ABA and water-stress is rapid and independent of *de novo* protein synthesis.

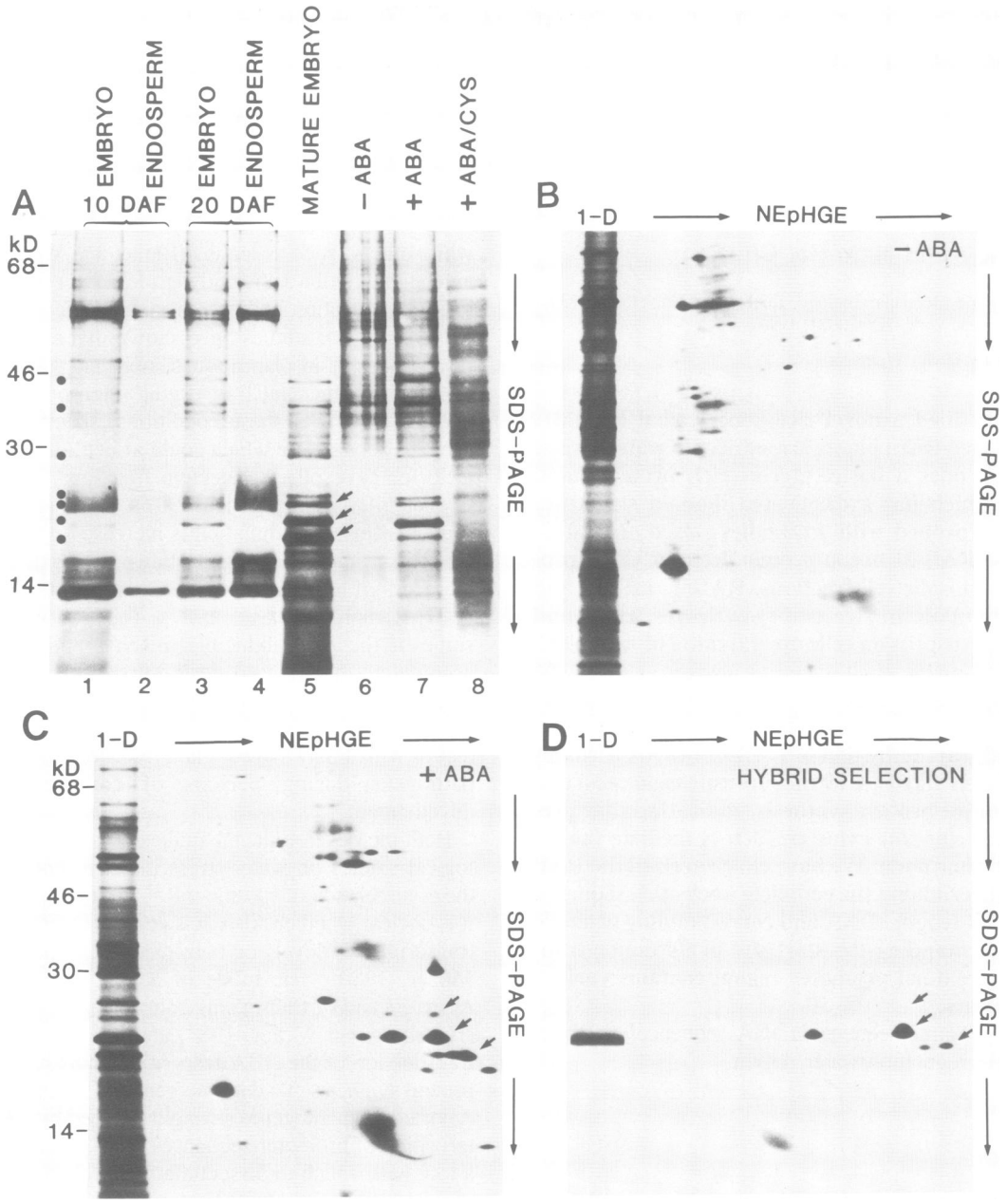

Fig. 1. Translation products of developing and germinating seed mRNAs. (**A**) One-dimensional SDS−PAGE of *in vitro* products of mRNAs from embryo and endosperm half-seeds harvested 10 days after flowering (DAF) (**lanes 1** and **2**), embryo and endosperm at 20 DAF (**lanes 3** and **4**), mature embryo at 30 DAF (**lane 5**), mature embryo germinated 3 days and then incubated 12 h without (**lane 6**) and with 10 μM ABA (**lanes 7** and **8**). ABA-responsive products are marked on the far left with dots; 23-, 21- and 20-kd polypeptides, referred to in **panels C** and **D**, are marked with arrows (**lane 5**). Products in **lanes 1−7** were labelled with [^{35}S]methionine, those in **lane 8** with [^{35}S]cysteine. (**B**) Two-dimensional NepHGE/SDS−PAGE of [^{35}S]methionine-labelled translation products of mRNAS from germinated embryo half-seeds incubated without ABA (as in A, lane 6). One-dimensional gel also shown at left. (**C**) NEpHGE/SDS−PAGE of translation products of mRNAs from germinated embryo half-seeds incubated with 10 μM ABA (as in A, lane 7). (**D**) NEpHGE/SDS−PAGE of translation products of poly(A) RNA selected by hybridization to RAB 21 cDNA. Mol. wt markers are indicated at the left in kd.

Results

Isolation of an ABA-inducible cDNA encoding a 21-kd polypeptide

As a first step toward isolating genes from rice whose expression is affected by ABA, mRNA populations from developing rice seeds were analyzed by *in vitro* translation and SDS−PAGE of the protein products. This experiment identified mRNAs encoding prominent polypeptides of mol. wts 45, 39, 30, 25, 23, 21 and 20 kd which accumulate late during rice embryogenesis (Figure 1A, lanes 1−5, see dots). These mRNAs are long-lived in mature grain harvested 30 days after flowering (DAF) (Figure 1A, lane 5) and disappear completely during normal germination (Figure 1A, lane 6). However,their accumulation can be recapitulated during germination by a 12-h incubation with ABA (Figure 1A, lane 7). These results indicate that the levels of mRNA encoding these polypeptides are modulated by ABA.

Two-dimensional separation of these translation products revealed that the 23-, 21- and 20-kd polypeptides are com-

A

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         10        ┌β-gal fusion →    30                            50
N-MEHQGQHGHV  TSRVDEYGNP  VGTGAGHGQM  GTAGMGTHGT  AGTGRQFQPM  REEHKTGGVL
          70                      90                      110
QRSGSSSSSS  SEDDGMGGRR  KKGIKEKIKE  KLPGGNKGEQ  QHAMGGTGTG  TGTGTGTGGA
         130                     150          163
YGQQGHGTGM  TTGTTGAHGT  TTTDTGEKKG  IMDKIKEKLP  GQH-C
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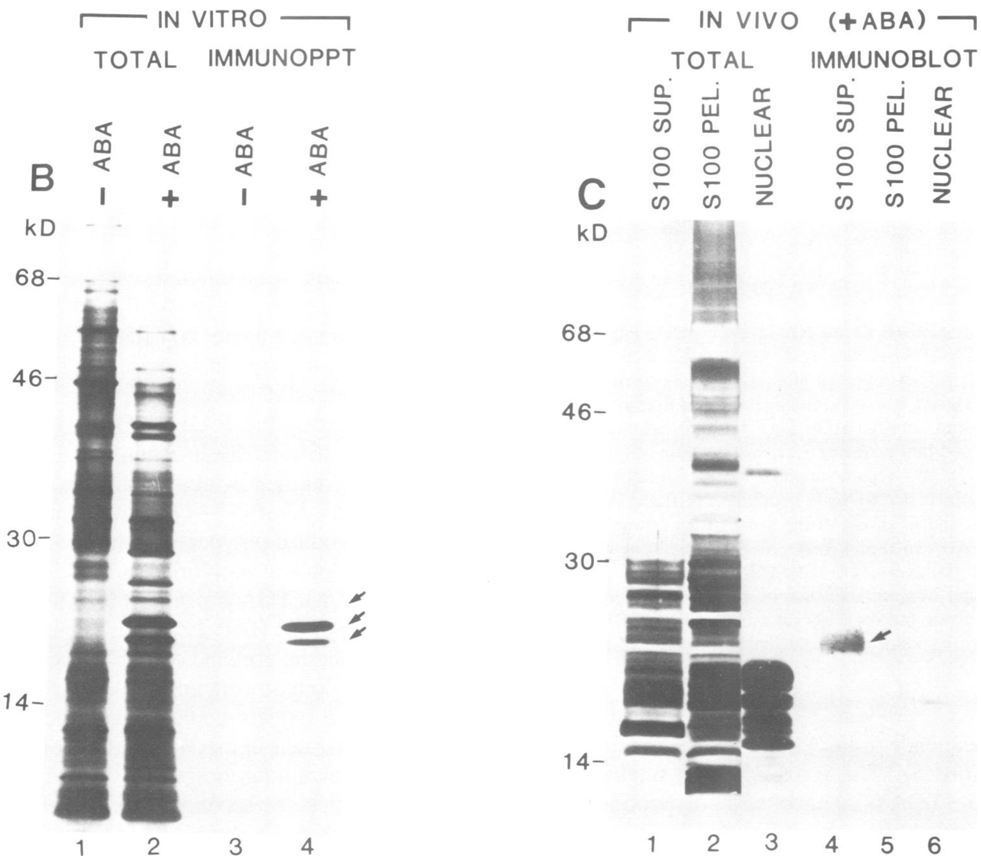


Fig. 2. Structure and expression of the RAB 21 protein. (**A**) Amino acid sequence of the RAB 21 protein deduced from the ORF of the cDNA (see Figure 5). The A- and B-type sequence repeats are underlined with solid or dashed lines respectively. The splice site used in constructing the β-galactosidase fusion proteins is marked by an arrow. (**B**) Immunoprecipitation of *in vitro* synthesized RAB 21 polypeptide. Total translation products of mRNAs from embryo half-seeds germinated 3 days and then incubated without (**lane 1**) or with 10 μM ABA (**lane 2**); immunoprecipitates of these products with antibodies raised against 'in frame' β-galactosidase/RAB 21 fusion protein (**lanes 3** and **4**). (**C**) Immunodetection of RAB 21 protein synthesized *in vivo* in leaves sprayed with aqueous 100 μM ABA solutions. Total proteins of cytosolic, organellar and nuclear leaf cell fractions were silver-stained (**lanes 1–3**), or electroblotted onto nitrocellulose and the RAB 21 protein was then detected with antibodies raised against the β-galactosidase/RAB 21 fusion protein (**lanes 4–6**). Mol. wt markers are indicated at the left in kd.

prised of multiple isoelectric forms (Figure 1C, see arrows). The even spacing of spots suggests that some of this heterogeneity may be due to serial charge differences, a common electrophoretic artefact. Alternatively, some of the different isoforms may be products of different genes (see Discussion). Differential labelling experiments suggest that the 23-, 21- and 20-kd polypeptides are rich in methionine but lacking in cysteine (Figure 1A, lanes 7 and 8).

A cDNA library was constructed using the ABA-treated seed mRNA as template. ABA-responsive cDNA clones were isolated by differential screening and subsequent Northern blot analysis. One clone, called pRAB 21, was chosen for further characterization. Figure 1D shows that pRAB 21 hybridizes to mRNA(s) that encode the prominent polypeptide(s) of apparent mol. wt 21 000. At the low hybridization stringency shown here, mRNAs encoding the 23- and 20-kd polypeptides are also hybrid-selected (the 23-kd group is very faint in this exposure). At higher stringencies of hybrid-selection, only the 21-kd polypeptides are seen (not shown). This cross-hybridization, together with several lines of evidence discussed later, indicate that the mRNAs for the 23-, 21- and 20-kd polypeptides are homologous.

RAB 21 is a basic, glycine-rich protein that accumulates in the cytosol of ABA-treated cells

The amino acid sequence of RAB 21 was determined from

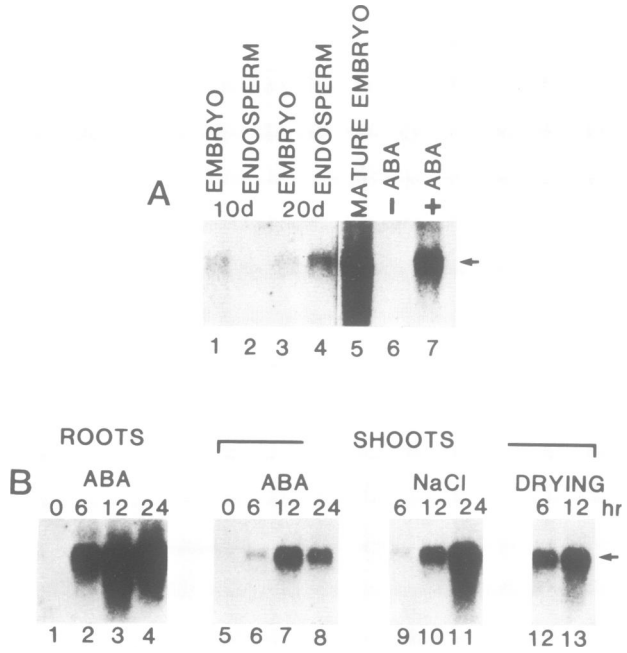


Fig. 3. (**A**) Steady-state RAB 21 mRNA levels in rice seeds, leaves and roots. 15 μg of total RNA was used per lane. Northern blot analysis of RAB 21 mRNA levels in embryo and endosperm half-seeds harvested 10 DAF (**lanes 1** and **2**), embryo and endosperm at 20 DAF (**lanes 3** and **4**), mature embryo at 30 DAF (**lane 5**), mature embryo germinated 3 days and the incubated 12 h without (**lane 6**) and with 10 μM ABA (**lane 7**). (**B**) Accumulation of RAB 21 mRNA in roots of hydroponically grown rice following addition of 10 μM ABA to media (**lanes 1–4**), and in shoots of the same plants following addition of 10 μM ABA to media (**lanes 5–8**), addition of NaCl (200 mM) to media (**lanes 9–11**), and air-drying the whole plants (**lanes 12,13**). Drying was accomplished by removing plants from growth media and air-drying on a laboratory bench at 27°C for the indicated times. Arrows mark the single major transcript of 850 bases.

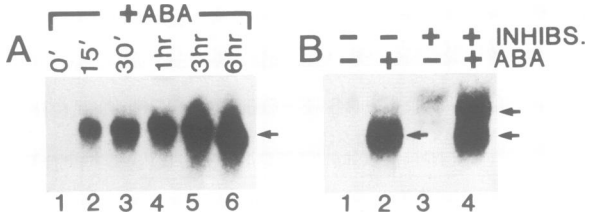
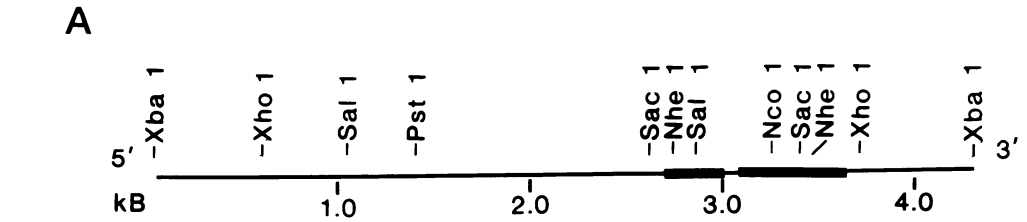


Fig. 4. Steady-state RAB 21 mRNA levels in suspension cells. (**A**) Time course of accumulation of RAB 21 mRNA in suspension cells incubated for 0 min, 15 min, 30 min, 1 h, 3 h and 6 h with 10 μM ABA (**lanes 1–6**). (**B**) The effect of pre-incubation with inhibitors of protein synthesis. RAB 21 mRNA levels after 6 h incubations without (**lane 1**) and with 10 μM ABA (**lane 2**). **Lanes 3** and **4** show the same incubations following a 1.5-h pre-incubation with inhibitors of protein synthesis [cycloheximide (10 μM), anisomycin (60 μM) and chloramphenicol (100 μM)]. The arrows mark the two different transcripts which accumulate. The size of the smaller transcript is 850 bases.

the nucleotide sequence of the RAB 21 cDNA and genomic clones (see Figure 5). The RAB 21 open reading frame (ORF) (ATG 93–TGA 665) encodes a polypeptide of mol. wt 16 529 (Figure 2A), roughly 4500 smaller than the mol. wt predicted from the mobility of the RAB 21 protein on SDS–PAGE. However, several lines of evidence suggest that this is the correct sequence of the RAB 21 protein. First, the ORF encodes eight methionines and no cysteines, consistent with the results of *in vitro* translation experiments with labelled cysteine or methionine (Figure 1A). Second, the ORF-encoded polypeptide is basic (approximate pI = 9.4), in keeping with mobility of the RAB 21 polypeptide(s) in NEpHGE (Figure 1D). Third, the GC content of the ORF is high (70%) while that of the 5′- and 3′-untranslated regions is low (45%). This GC-codon bias has been noted in the coding regions of other seed protein genes (Rogers *et al.*, 1985). To obtain direct confirmatory evidence, antibodies were raised against portions of the RAB 21 protein. Figure 2B and C show that antibodies against a β-galactosidase/RAB 21 fusion protein (RAB 21 residues 15–163) specifically recognize *in vitro* and *in vivo* synthesized polypeptides which correspond to RAB 21. Furthermore, fusion proteins with the RAB 21 cDNA in all three reading frames were produced in *Escherichia coli* and analyzed on Western blots with polyclonal antibodies raised against an extract of total soluble proteins from mature rice seed. Only the 'in frame' fusion was immunoreactive (results not shown), demonstrating conclusively that the other two possible reading frames are not translated *in vivo*.

In vitro transcription/translation of RAB 21 cDNA constructs was used to show that the predicted stop codon (TGA, 665) terminates the RAB 21 ORF. In these experiments, ptz19U vectors (USBC) containing either the *Nhe*I–*Sac*I (57–670) fragment which terminates at the expected TGA, or the *Nhe*I—*Nhe*I (57–741) fragment, were used as templates to produce polypeptides *in vitro*. These two polypeptides had identical mobilities in SDS–PAGE (not shown), confirming that TGA at residue 665 is used as the stop codon.

Analysis of the RAB 21 protein sequence using Harr plots revealed that the protein contains a duplicated domain structure. Each domain contains an A and B repeat (Figure 2A). The A and B repeats are adjacent in the C-terminal domain, but are separated in the N-terminal domain by a portion of the ORF containing the gene's single intron. An algorithm which predicts the secondary structure of RAB 21 suggests that most of the polypeptide, including the A repeats, is composed of random turns due to the irregular spacing of glycine residues. The B-repeats are very basic and contain the conserved sequence KIKEKLPG which may be part of a helix bounded by turns. Further computer analysis showed weak homology between the entire protein (or the A repeats alone) to regions of several viral nuclear proteins. The highest homology occurred with the glycine–alanine copolymer domain of the major nuclear antigen encoded by the Epstein–Barr virus (Hennessy and Kieff, 1983). Not surprisingly, the very basic, lysine-rich B-repeats are weakly homologous to various DNA-binding proteins such as histones. These results suggest that the RAB 21 polypeptide may be a nuclear protein. To examine this possibility, RAB 21 antibodies were used to probe soluble, organellar, and nuclear fractions prepared from leaves of ABA-treated rice plants. These experiments show that the RAB 21 protein is found in the soluble fraction (Figure 2C). Since the protein has not been detected in crude organellar, chloroplastic, mitochondrial or nuclear extracts, we suggest that RAB 21 is a cytosolic protein. However, definitive proof of its cellular location remains to be established by immuno-electron microscopy.



B

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                                                                                                    CTGCAG -1501
AGAGGATGACCCTTGTCACCACCGTCATGTACGAGGCTGCTTCACCACTGCCTCACTGCCACCAGCGTCTCCCGCCGCGTGCAATACAAGAAGAAACATC -1401
GAACGGTCATATAAGGTAAGACCCACTACCGATTTAACCTATCATTCCCACAATCTAATCCACTTATTTCTCTTCCCATGATCTTATCCTCTCATTTCTC -1301
CTCACTACTTTTGCATTTGTAGGAAACACAATGACACCGTCGAAGAAAGCTGGTGGAGCACCGTAGCCAGCAATCACCAAAACACAGAGGGGAGGAGGTC -1201
GGCAGCGGCCATGCGGACGGCGATGAGACAACGCGACGCAAAGAGGGAGGAGGACGTTGGCGATCATGCTGGTGTTGGCGGAGGAGGTCACTGGCCATGC -1101
GAATGACAGCGGGGCAGCGCAACACAAAAAGGGGGGAGGATGCCGGCGACCACGCTAGTACCATGAAGCAAGATGATGTGAAAGGGAGGACCGGACGAGG -1001
GTTGGACCTCTGCCGCCGACGTGAAGAGCGTGATGTGTAGAAGGAGATGTTAGACCAGATGCCGACGCAACTTAGCCCTGCAAGTCACCCGACTGCATAT -901
CGCTGCTTGCCCTCGTCCTCATGTACACAATCAGCTTGCTTATCTCTCCATACTTGTCGTTTGTTTCCCGTGGCCGAAATAGAAGAAGACAGAGGTGGGT -801
TTTGTTGGAGAGTTTTAGTGGTATTGTAGGCCTATTTGTAATTTTGTTGTACTTTATTGTATTAATCAATAAAGGTGTTTCATTCTATTTTGACTCAATG -701
TTGAATCCATTGATCTCTTGGTGTTGCACTCAGTATGTTAGAATATTCATTCCGTTGAAACAATCTTGGTTAAGGGTTGGAACATTTTTATCTGTTCGGT -601
GAAACATCCGTAATATTTTCGTTGAAACAATTTTTATCCGACAGCACCGTCCAACAATTTACACCAATTTGGACGTGTGATACATAGCAGTCCCCAAGTG -501
AAACTGACCACCAGTTGAAAGGTATACAAAGTGAACTTATTCATCTAAAAGACCGCAGAGATGGGCCGTGGCCGTGGCTGCGAAACGACAGCGTTCAGGC -401
CCATGAGCCATTTATTTTTTAAAAAAATATTTCAACAAAAAAGAGAACGGATAAAATCCATCGAAAAAAAAAAAACTTTCCTACGCATCCTCTCCTATCTC -301
CATCCACGGCGAGCACTCATCCAAACCGTCCATCCACGCGCACAGTACACACACATAGTTATCGTCTCTCCCCCCGATGAGTCACCACCCGTGTCTTCGA -201
GAAACGCCCTCGCCCGACACCGTACGTGCGCCACCGCCGCGCCTGCCGCCTGGACACGTCCGGCTCCTCTCCCGCCGCGCTGGCCACCGTCCACCGGCTCC -101
CGCACACGTCTCCCTGTCTCCCTCCACCCATGCCGTGGCAATCGAGCTCATCTCCTCGCCTCCTCCGGCTTATAAATGGCGGCCACCACCTTCACCTGCT -1

TGCACACCACAGCAAGAGCTAAGTGAGCTAGCCACTGATCAGAAGAACACCTCGATCTCTGAGAGTGTTTTTTCAGCTTTAGCTTAAGCAGGATGCGAGCA  100
CCAGGGGCAGCACGGCCACGTGACCAGCCGCGTCGACGAGTACGGCAACCCGGTCGGCCACCGGCGCCCGGACACGCGCAGATGGGCACCGCCGGCATGGGG  200
ACGCACGGCACCGCCGGCACCGGCCGCCAGTTCCAGCCGATGAGGGAGGAGCACAAGACCGGCGGCGCTCCTGCAACGCTCCGGCAGCTCCAGCTCAAGCT  300
CGgtacaacattttgaccccaattctttaccccccactaaaaccttgcgtacaattcgttgaaaattttaatgtcttgtgacagTCTGAGGATGATGGA  400
ATGGGAGGGAGGAGGAAGAAGGGGATCAAGGAGAAGATCAAGGAGAAGCTCCCCGGCGGCAACAAGGCGCGAGCAGCAGCATGCCATGGGCGGCACCGGCA  500
CCGGCACCGGCACCGGCACCGGAACCGGCGGCGCCCTACGGGCAGCAGGGCCACGGCACCGGGATGACCACCGGCACCACCGGCGCACACGGCACCACCAC  600
CACCGACACCGGCGAGAAGAAGGGCATCATGGACAAGATCAAGGAGAAGCTGCCCGGCCAGCACTGAGCTCGACACACCACCACACACCATGTGTGCCTGCG  700
CCGGACGGCCGCCACGTCACCTTCCTGAATAATAAGATGAGCTAGCCGAGCGCAATAAAAGGAAAAAAAAATGTTACTGTCGTGTGATGAGTGTGAATGT  800
GTGATGGCGTTCTCCAGTACGGACCGTGTCTGCGTGTTGTTTGTACTGTGAAAGTACGCTGTGTATGTACGTCGTATGTGTACAATTCCGCTTATACTGT  900
                    * poly A+                                                                  * poly A+
TGTTGAAATACGTATAAATATGTGTACATTATACGGTGTATATCTCATCGTGCATATGTACACAACGTTTTGGTGATCGTTATAATGTTCATTTTTTTCC 1000
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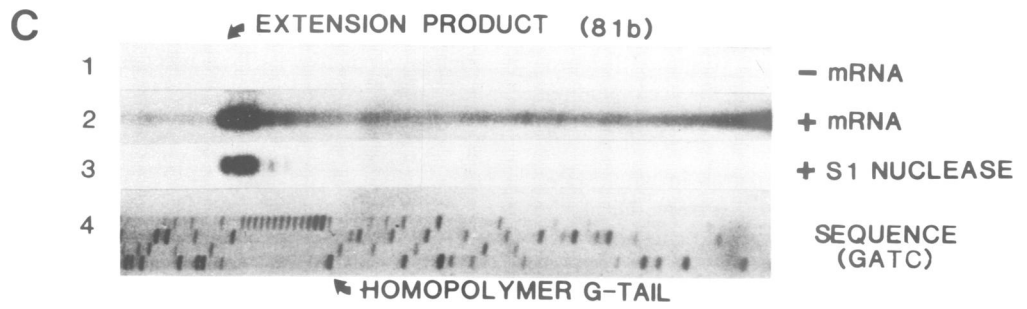

Fig. 5. Structure of the RAB 21 cDNA and gene. (**A**) Restriction map of the 4.3-kb *Bam*HI genomic fragment containing the RAB 21 gene. The map was generated by multiple enzyme restriction analysis. The two *Sac*I sites are at −52, downstream of CAAT, and at +670, downstream of the TGA terminating the ORF (see Figure 2B). (**B**) Nucleotide sequence of the RAB 21 gene. The 2.4-kb *Pst*I−*Xho*I genomic fragment (see A) and the cDNA were sequenced as described in Materials and methods. The ORF is in bold-faced type, the single intron is in lower case. Putative transcription and the start and stop codons of the ORF are underlined. Polyadenylation sites found in the cDNAs are marked with an asterisk. Various GC-rich repeats in the proximal promoter region, including five sequences which resemble the consensus binding sites of the SP1 animal transcription factor (Briggs *et al.*, 1986) are discussed in Results. (**C**) Primer extension using a synthetic oligonucleotide (+84 to +108). The end-labelled oligo was used as a primer to reverse transcribe without RNA (**panel 1**) and with 1 μg of poly(A)$^+$ RNA from ABA-treated half-seeds without (**panel 2**) and with subsequent incubation with S1 nuclease (**panel 3**). **Panel 4** shows the 5′ sequence (GATC top to bottom) of the pRAB 21 cDNA obtained with the same oligo as primer.

Accumulation of RAB 21 mRNA is induced in different tissues by ABA and water-stress

Northern blot analysis was used to analyze the steady-state levels of RAB 21 mRNA in various rice tissues during different treatments. Experiments with total RNA from developing seed tissues (Figure 3A) confirmed the patterns of RAB 21 mRNA accumulation as assayed by *in vitro* translation (Figure 1A). During seed development, low levels of RAB 21 mRNA are found in both endosperm and embryo seed halves (Figure 3A, lanes 1–4). High levels of this mRNA accumulate in embryos between 20 DAF and maturity (30 DAF) and survive as long-lived mRNAs in the resting grain (Figure 3A, lane 5). Homologous signals were also seen among long-lived RNAs extracted from mature seeds of maize, barley, wheat and millet (not shown), suggesting that proteins homologous to RAB 21 are functionally conserved among the Graminae. As was shown for barley (Mundy *et al.*, 1986), mature rice endosperm does not contain detectable mRNA levels. Therefore RAB 21 mRNA was not assayed in this tissue.

The long-lived RAB 21 mRNA in rice embryos is rapidly turned over at the onset of germination (Figure 3A, lane 6). However, accumulation of this mRNA can be recapitulated by incubating the germinating embryos in 10 μM ABA (Figure 3A, lane 7). This indicates that ABA affects the steady-state level of RAB 21 mRNA.

RAB 21 is not present at detectable levels in the roots and shoots of hydroponically-grown, 1-month-old rice plants (Figure 3B, lanes 1 and 5). However, upon addition of ABA to the solution bathing the roots, the mRNA accumulates in both organs and reaches a steady-state level after 12 h (Figure 3B, lanes 2–4, 6–8). At this time the level of RAB 21 mRNA is induced at least 20-fold over the control. Therefore the increase of the RAB 21 mRNA levels in the presence of ABA is not restricted to seed tissues.

To determine if the expression of RAB 21 could also be induced by water-stress, NaCl was added to the hydroponic solution to a final concentration of 200 mM. Figure 3B (lanes 9–11) indicates that RAB 21 mRNA levels increase in response to prolonged growth in solutions containing elevated salt concentrations. The time course of induction in response to NaCl is similar to that obtained with ABA; the maximal level of accumulation (20- to 30-fold induction) being attained after 12–24 h. The pattern of RAB 21 mRNA accumulation was unchanged when both ABA and salt were added to the hydroponic solution. RAB 21 mRNA levels are also induced by desiccation of rice plants (Figure 3B, lanes 12, 13). These results strongly suggest that the accumulation of RAB 21 mRNA is regulated by the water status of the plant.

Detectable RAB 21 mRNA accumulation in whole rice plants generally requires 3–4 h of ABA treatment. This long response time may reflect a low rate of exogenous hormone uptake by roots and translocation to the leaves. To examine this possibility, RAB 21 mRNA accumulation was measured in cultured suspension cells derived from embryogenic calli. In contrast to whole plants, the increased RAB 21 mRNA levels are easily detected in suspension culture cells after only 15 min of ABA treatment (Figure 4, lanes 1 and 2). During prolonged incubation with ABA, RAB 21 mRNA levels increase steadily to a maximum of ~20 times the control level at 3–6 h (Figure 4, lanes 3–6).

The rapidity of the ABA response in cell cultures suggests that protein synthesis is not required for RAB 21 gene expression. To obtain direct evidence on this point, cells were treated with a combination of protein synthesis inhibitors affecting 70S and 80S ribosomes which reduced total protein synthesis by >90%. The protein synthesis inhibitors neither induce the expression of RAB 21 (Figure 4, lanes 1 and 3) nor block its induction by ABA (lanes 2 and 4). A longer transcript (1000–1200 bases) that hybridizes to the RAB 21 cDNA probe accumulates in the presence of the inhibitors. These results have also been seen in leaf tissues treated with ABA plus inhibitors (results not shown). This longer transcript has not yet been characterized but it may correspond to the primary transcript of a homologous gene which contains an intron of 300–400 bp.

Structure of RAB 21 gene

The nucleotide sequence of the pRAB 21 cDNA was used to deduce the primary structure of the encoded polypeptide. The clone was also used to isolate a corresponding genomic clone, gRAB 211. Analysis of sequences upstream of the RAB 21 gene's coding region identified putative regulatory elements. The restriction map and nucleotide sequence of the cDNA and the corresponding region of the genomic clone (2.5-kb restriction fragment) are presented in Figure 5A and B. The major ORF of the transcribed region is 489 bp (93–665) encoding the 162 amino acids of RAB 21 (Figure 2A). Flanking the ORF is a 92-bp 5′ leader containing three stop codons and a 261-bp 3′ tail containing the putative polyadenylation sequence ATAAA 12 bp upstream of the site of poly(A) addition. The 3′-non-coding regions of six homologous cDNAs were sequenced. Five of them were identical to pRAB 21, being polyadenylated at position +926. This correlates well with the size of the RAB 21 mRNA (800 nucleotides). The sequence of the sixth clone was identical to that of the genomic DNA but contained a poly(A)$^+$ tail farther downstream at position +991. The original 824-bp cDNA was shown by primer extension to lack only 18 nucleotides from the 5′-untranslated leader (Figure 5C). The transcription start site is shown in Figure 2B (nucleotide +1).

In the genomic DNA, the ORF is interrupted by a 83-bp, AT-rich intron flanked by the consensus border sequences GT and AG. The sequence of gRAB 211 was identical to the cDNA RAB 21, indicating that this gene is transcribed *in vivo*. The proximal GC-rich promoter (−200 to −1) contains a putative TATAA box (−30) and a putative CAAT box (−62). This region contains the following four types of GC-rich repeats (see Figure 5B): (1) TGCGCCACCG at −175 and −121; (2) CGCCGCGC at −167 and −129; (3) TCCGGCTCC at −143, −108 and −37; (4) GTCTCCCT at −93 and −85.

This region also contains five other GC-rich repeats at −200, −195, −166, −133 and −48 whose opposite strand sequence shows 80% homology to the decanucleotide (G/TG/AGGCGG/TG/AG/AC/T) binding site of the SP1 transcription factor (Briggs *et al.*, 1986).

Discussion

We are interested in studying the molecular mechanism of action of plant hormones on gene expression. To begin this work we isolated a cDNA encoding a major transcript that

is inducible in rice tissues by the plant hormone ABA. This cDNA was shown by hybridization and immunoassay to encode a prominent member of a group of basic polypeptides of mol. wt 23−20 kd. We call this protein RAB 21. The different RAB polypeptides may be post-translational modifications of a single gene product or products of closely related genes. The rice genome contains at least three closely-linked (within 30 bp) genes homologous to RAB 21 (K.Yamaguchi-Shinozaki, unpublished results). These genes encode proteins of slightly different amino acid sequence that may account for the different groups of polypeptides immunoreactive to RAB 21 antibodies.

Northern blot hybridizations show that RAB 21 gene expression is not tissue specific, as shown by the accumulation of its transcript in seeds, roots, leaves and in undifferentiated suspension cells. Since there at least three other rice genes closely related to RAB 21, gene-specific probes are needed to ascertain whether these genes are differentially expressed. Physiological experiments show that RAB 21 mRNA accumulates not only in ABA-treated tissues but also in leaves, roots and suspension cells under conditions of water deficit. These results suggest that RAB 21 gene expression is dependent upon the water status of plants and that ABA may act as a signal in this response. The pathway of this response is different to that mediating the more general heat shock response (Heikkila *et al.*, 1984), because RAB 21 mRNA is undetectable in rice tissues after heat shock for various periods of time (not shown).

Experiments with cultured cells show that accumulation of RAB 21 mRNA following ABA treatment is very rapid and that it is insensitive to inhibitors of protein synthesis. These data strongly suggest that ABA-induced gene expression does not require protein synthesis but probably involves modification of pre-existing factors, as is the case for the heat shock response (Zimarino and Wu, 1987). NaCl also induces RAB 21 mRNA rapidly in cultured cells (not shown). This response is not additive to that attributed to ABA: addition of NaCl and hormone together does not 'superinduce' mRNA accumulation at any point in the time course. These results corroborate the findings of physiological (Jones *et al.*, 1986) and genetic (Chandler *et al.*, 1988) studies which show that the response of plants to water-stress is mediated by ABA at the level of specific gene expression.

To initiate studies on the molecular mechanism of ABA action, the gene encoding RAB 21 was isolated and its nucleotide sequence determined. This is the first published genomic sequence of a strongly ABA-responsive gene. The proximal promoter region is GC-rich and contains numerous repeats detailed in Results. Another group of repeats is closely related to to the GC element found in various cellular and viral genes in mammalian cells. This *cis*-acting element promotes the expression of genes by binding the *trans*-acting protein factor SP1 (Briggs *et al.*, 1986). Similar sequences have been noted in the promoter of the α-subunit of β-conglycinin (Chen *et al.*, 1986) and in that of oat phytochrome (Hershey *et al.*, 1987), plant genes which are not known to be responsive to ABA treatment. The regulatory roles of these different GC-rich repeats remain to be established by functional assays. These experiments, now in progress in our laboratory, may elucidate the molecular mechanism by which ABA regulates gene expression and mediates the adaptation of plants to water-stress.

Materials and methods

Plant materials

Seeds of rice (*Oryza sativa*, var. Indica, cv. IR 36) were obtained from the International Rice Research Institute, Philippines. Plants for developmental studies were grown in soil at 27°C and a day length of 11 h. Plants flowered 10−11 weeks after planting. Seeds were then collected after 10 days (milk stage), 20 days (starchy, green pericarp) and 30 days (dry, brown pericarp, mature). Plants were also grown hydroponically in Hoagland's solution supplemented with 20 mM NH_4NO_3. Preparation and treatment of embryo-containing half-seeds at 27°C was performed as described previously for barley (Mundy *et al.*, 1986). Suspension cells derived from embryonic callus of IR 36 were grown at 25°C in Kao's medium (Kao, 1977) containing 2.5 mg/l 2-4 D and 0.2 mg/l kinetin with subculturing at 1-week intervals. Cells for mRNA isolation were subcultured 5−7 days prior to harvest.

Protein analysis

Proteins were analyzed by NEpHGE, SDS−PAGE and Western blots according to Tingey *et al.* (1987). Isolation of mRNA, *in vitro* translation using reticulocyte lysate and immunoprecipitation with Protein A Sepharose 4B were as described previously (Mundy *et al.*, 1986). Leaf cell fractions were prepared from 7-day-old plantlets sprayed three times with 100 μM aqueous ABA solutions during the 24 h prior to harvest. Chloroplasts and mitochondria were prepared from leaf tissue after Boutry and Chua (1985) while nuclei were prepared after Green *et al.* (1987). The National Biomedical Research Foundation Protein Sequence Databank carried in the Rockefeller University 7000/40 computer was screened for sequences related to the RAB 21 ORF with the SEARCH program of Dayhoff *et al.* (1983). Detailed comparisons of protein sequences thought to be related to the RAB 21 sequence were made with ALIGN, utilizing the mutation data matrix [250 PAMs = 6, and a gap penalty of 8 (Dayhoff *et al.*, 1983)].

Isolation of cDNA and genomic clones

Double-stranded cDNA synthesized by the RNase H method (Gubler and Hoffman, 1983) was size-fractionated on a column of Bio-gel A50m (Bio-Rad Laboratories). Molecules of 450−4500 bp were then cloned by homopolymer GC-tailing into pEMBL 12 plasmid (Dente *et al.*, 1983). Six thousand recombinant clones, replica-transferred onto nitrocellulose filters from 96-well microtiter plates, were screened with single-stranded cDNA probes synthesized from mRNA isolated from control and ABA-treated half-seed mRNAs. Hybridizations were performed in 50% formamide, 6 × SSC, 1 × Denhardt's, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA at 42°C with denatured ^{32}P-labelled DNA probes (sp. act. 1 × 10^8 c.p.m./μmg DNA, final concentration 1 × 10^6 c.p.m./ml). Sixty ABA-responsive clones were then tested on Northern blots of the same RNAs. Hybridization selection after Tingey *et al.* (1987) was used to identify polypeptides encoded by specific clones. Standard protocols and conditions were used for agarose electrophoresis, hybridization in 50% formamide, DNA fragment isolation with DE-81 paper, and plasmid DNA preparation (Maniatis *et al.*, 1982).

Genomic DNA was isolated from 10-day-old etiolated leaves by CsCl centrifugation (Maniatis *et al.*, 1982). Southern hybridization of restricted DNA revealed a 4.3-kb *Xba*I fragment which hybridized strongly to the selected RAB 21 cDNA. This genomic fragment was partially purified by size fractionation in agarose gels and then cloned by insertion into *Xba*I-digested lambda ZAP (Stratagene). A total of 75 000 recombinant plaques were screened on duplicate filters and three clones containing identical 4.3-kb *Xba*I inserts were identified by hybridization to pRAB 21. Excision and recircularization of pBLUESCRIPT SK(m13) plasmid following superinfection of lambda ZAP infected cells with IR 408 helper phage (Russel *et al.*, 1986) was performed according to the manufacturer's instructions (Stratagene).

DNA sequencing and primer extension

The pRAB 21 cDNA insert and a 2.5-kb *Pst*I−*Xho*I genomic fragment taining the RAB 21 sequences were sequenced from overlapping deletions created by *Bal*31 exonuclease (Misra, 1985). Single-stranded templates were prepared by superinfecting pEMBL containing recombinants with the IR 408 helper phage (Russel *et al.*, 1986). Sequencing reactions were performed according to Biggin *et al.* (1983) and the products separated on 6% polyacrylamide/urea gels. Inosine was used to resolve GC-compressions. More than 90% of the sequence was obtained for both strands of DNA. At least two overlapping clones were used when only one strand was sequenced.

A 24-base oligonucleotide corresponding to 5′ sequences of pRAB 21 was synthesized on an Applied Biosystems model 380A DNA synthesizer

after the manufacturer's instructions. The gel-purified oligonucleotide was used for primer extension according to Shelness and Williams (1984).

Fusion protein and antibody production

The *SalI* fragment of RAB 21 was ligated into the *SalI* site of expression vectors pUR 288 (in frame) and pUR 278 and 289 (out of frame controls, Ruther and Muller-Hill, 1983). The pUR 288 fusion plasmid encodes amino acids 15–163 of the RAB 21 ORF fused to the C terminus of *E.coli* β-galactosidase. Fusion proteins purified from cell extracts on anti-β-galactosidase–Sepharose columns according to the manufacturer's instructions (Promega Biotech) were used for immunization of rabbits.

RNA blot analysis

Total RNA was prepared by a miniprep procedure (Nagy *et al.*, 1988). RNAs were separated in formaldehyde gels, blotted onto nitrocellulose and hybridized to random-primed cDNA probes after standard protocols (Maniatis *et al.*, 1982). Replicate gels were stained with ethidium bromide to ensure that samples contained approximately equal amounts of rRNA. A commercial RNA ladder was used as size marker (BRL).

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Note added in proof

These sequence data will appear in the EMBL/GenBank/DDBJ Sequence Databases under the accession number Y00842.