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The *cis*-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene *rab*28

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Abstract

The maize gene *rab*28 has been identified as ABA-inducible in embryos and vegetative tissues. It is also induced by water stress in young leaves. The proximal promoter region contains the conserved *cis*-acting element CCACGTGG (ABRE) reported for ABA induction in other plant genes. Transient expression assays in rice protoplasts indicate that a 134 bp fragment (−194 to −60 containing the ABRE) fused to a truncated cauliflower mosaic virus promoter (35S) is sufficient to confer ABA-responsiveness upon the GUS reporter gene. Gel retardation experiments indicate that nuclear proteins from tissues in which the *rab*28 gene is expressed can interact specifically with this 134 bp DNA fragment. Nuclear protein extracts from embryo and water-stressed leaves generate specific complexes of different electrophoretic mobility which are stable in the presence of detergent and high salt. However, by DMS footprinting the same guanine-specific contacts with the ABRE in both the embryo and leaf binding activities were detected. These results indicate that the *rab*28 promoter sequence CCACGTGG is a functional ABA-responsive element, and suggest that distinct regulatory factors with apparent similar affinity for the ABRE sequence may be involved in the hormone action during embryo development and in vegetative tissues subjected to osmotic stress.

Introduction

The plant hormone abscisic acid (ABA) mediates a number of important developmental and physiological processes in plants including embryo maturation and the response of vegetative tissues to osmotic stress [5, 22]. It has been shown that abscisic acid modulates mRNA levels of specific genes in different plant tissues [25]. However, the molecular mechanism by which the hormone affects specific gene expression remains poorly understood.

Promoter deletion analyses of ABA responsive genes from different plants show that a conserved sequence motif (CCACGTGG) functions as an ABA-responsive element (ABRE) to modulate

reporter gene induction in transient expression systems [12, 13, 18, 28]. Moreover cDNA encoding leucine zipper proteins have recently been cloned, from wheat and tobacco, which specifically bind this ABRE sequence [9, 19]. These data strongly suggest that transcriptional controls are involved in ABA regulation. But the role of the ABRE sequence is complicated by the fact that, in addition to the ABA-responsive genes, the promoters of other plant genes, which are not known to be modulated by ABA, contain the CCACGTGG or similar DNA sequences which in many cases have been demonstrated to be functionally important [14, 26].

We have previously shown that in the vegetative tissues of maize, ABA and water stress induce the expression of specific genes (*rab*: responsive to ABA) which are normally expressed during late embryogenesis [8, 20, 27]. A large number of plant genes showing similar behaviour have been so far identified [2, 14, 17]. However, previous studies in ABA-deficient *viviparous* mutants in maize showed that developmental cues other than ABA may affect *rab* gene expression, and a differential regulation of *rab* genes in embryo and vegetative tissues was described [20, 21]. Furthermore, ABA-responsive genes which are not inducible in vegetative tissues by ABA or water stress have also been reported [6, 10].

Because of the apparent complexity in the regulation of these genes it is of special interest to define *cis*-acting elements of the *rab* genes involved in ABA and water stress regulation in embryo and vegetative tissues.

Recently we reported the cloning and characterization of the *rab*28 gene from maize [21]. Transcriptional regulation was investigated and indicated that *rab*28 gene expression is developmentally regulated during embryogenesis and it is induced in young embryos and in vegetative tissues by ABA treatment and water stress.

Here using transient assays of chimeric promoter constructs we present evidence to show that the *rab*28 gene is transcriptionally regulated by ABA. Furthermore we provide *in vitro* data indicating that the same conserved sequence motif CCACGTGG in the *rab*28 promoter specifically binds nuclear protein factors from mature embryos and water-stressed leaves.

Materials and methods

Construction of chimeric genes

Plasmid pBM173 contains the *Escherichia coli uidA* gene flanked 5′ by a fragment of the CaMV 35S gene (−90 to +3). The *uidA* gene encodes a glucuronidase enzyme (GUS) and is used as an assayable reporter gene product [13]. For transient expression assays, the following promoter constructs were prepared. Plasmid pMP130: *rab*28 5′ upstream fragments (−393, −60) (*Hin*d II/*Xho* I) blunt-ended, was ligated into pBM173. Plasmids pMP982 and pMP984: *rab*28 5′ fragment −194 to −60 (*Msp* I/*Xho* I) blunt-ended was ligated into pBM173 which had been cut with *Hin*d III and the ends rendered blunt. Orientation of the insert was determined by sequencing and two isolates were retained, one in each orientation.

Plasmid pBM173 containing the CaMV 35S promoter (−90, +3) and pBM113 Kp containing 650 bp from the wheat Em promoter region [13] were used as controls.

Transient expression assays

Rice suspension cells were maintained with subculturing at one-week intervals as described [12]. PEG-mediated transfection was used to introduce the constructions into rice protoplasts as described [12, 13]. Samples after transfection were incubated at 25 °C in the dark, either containing no ABA or in 10 μM ABA, harvested 24 h later, and assayed for GUS activity as described by Jefferson *et al.* [11].

Nuclear extracts and gel retardation assays

Nuclear proteins were extracted from maize mature embryos and water stressed leaves according

to Carballo *et al.* [3] and from wheat as described [13]. End-labelled and competitor DNA fragments were isolated on 4% polyacrylamide gels followed by isotachophoresis. Binding reactions (10 μl) contained 10 to 20 fmol end-labelled DNA probe (5000–20000 cpm), 1–5 μg of poly(dIdC), poly(dAdT), or salmon sperm DNA, 35 mM KCl, 7.5 mM $MgCl_2$, 13 mM Tris-HCl pH 7.9, 0.17 mM EDTA, 1.1 mM DTT, 0.6 mM PMSF, 0.5 mM 2-mercaptoethanol, 14% v/v glycerol and 25- to 100-fold molar competitor DNA [16]. Reactions were started by the addition of 2 to 6 μg nuclear protein per 10 μl reaction, followed by incubation at room temperature for 20 min. Samples were subjected to electrophoresis on 4% polyacrylamid 0.7% agarose gels with constant buffer recirculation.

Methylation interference analysis

DNA probes were end-labelled with Klenow enzyme and ^{32}P-dA/CTP *in vitro* dimethyl sulfate (DMS) footprinting was carried out as described [1]. Probes were partially methylated by DMS and bound to protein extracts from embryo and stressed leaves. After electrophoresis, labelled DNA was purified from free and bound bands [16]. For embryo extracts the two major type of complexes were isolated together. DNA was then cleaved with piperidine, washed and lyophilized three times and analyzed on 6% sequencing gels.

Results

Abscisic acid-responsive DNA fragment of the rab*28 promoter*

The *rab*28 gene promoter contains the sequence motif CCACGTGG, which appears to function as the ABA responsive element in the wheat Em gene [13], the *rab*16 gene from rice [18] and the *rab*17 gene from maize [28]. To test whether this sequence located at −146 in the *rab*28 gene promoter is an ABA-responsive DNA element, chimeric promoters consisting of the *rab*28 −393/−60 (construct 1) and −194/−60 (construct 2, and in reverse orientation construct 3) regions were fused to the 35S TATAA region (−90/+35), and the GUS reporter gene and were assayed for their ability to confer ABA-responsive expression on the bacterial GUS reporter gene in rice protoplasts. These fusion gene constructs were introduced into rice protoplasts using PEG-mediated transfection and cell extracts were assayed for GUS activity after incubation in 10 μM ABA for 24 h.

Figure 1 shows that constructs 1 and 2 confer a low level of GUS activity in protoplasts incu-

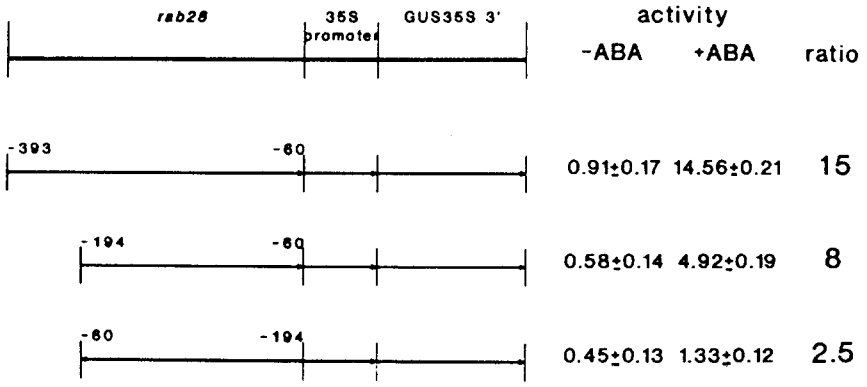

Fig. 1. Transient expression of GUS gene fusions in rice protoplasts. GUS assays of rice protoplasts transfected with constructs 1 to 3 (see text) and incubated without or with 10 μM ABA as marked. Numbers represent the GUS activity measured in three different experiments. The ratio is the GUS activity measured in the presence of ABA/GUS activity measured in the absence of ABA.

bated without ABA; however, they resulted in 15-fold and 8-fold induction, respectively, of GUS activity in ABA-treated protoplasts. Furthermore, construct 3 (the −194 to −60 DNA fragment in the reverse orientation) results in twofold to threefold induction of GUS activity when incubated with ABA. The control construct containing only the 35S TATAA region (construct 4), was not responsive to ABA. Therefore, sequences between −194 and −60 of the maize *rab*28 gene modulate transcription of the gene by ABA.

Nuclear protein binding to the rab*28 promoter*

Northern analysis of the gene *rab*28 has indicated that transcripts not only accumulate in mature embryos but also in vegetative tissues when incubated in ABA or under conditions of water stress. The transcript was not detected in young embryos and well watered vegetative tissues [21].

To initially identify *trans*-acting factors involved in the regulation of *rab*28, nuclear protein extracts from tissues in which the gene is expressed were assayed for their ability to bind to the *rab*28 promoter region.

Binding experiments were performed with nuclear proteins extracted from mature embryos and leaves from water stressed 7-day-old plants using both the −393/−60 and the −194/−60 DNA fragments. Binding experiments were conducted in the presence of non-specific competitors to demonstrate selective binding with excess heterologous carrier DNA.

By using embryo protein extracts in the binding assay, specific complexes were detected in both the −393 to −60 and the −194 to −60 DNA probes indicating that embryonic nuclei contain protein(s) which bind this DNA fragment. However, binding reactions with water stressed leaf extracts exhibited protein-DNA complexes with a slightly increased migration rate relative to embryo forms (see Fig. 2).

The specificity of DNA/protein complex formation was shown by competition experiments with various DNA fragments. While the coding *rab*28 DNA, salmon sperm DNA (not shown) and non-specific (poly-dI-dC, and poly-dA-dT)

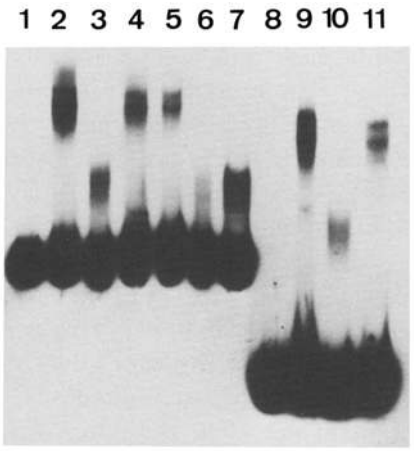

Fig. 2. Binding activities of nuclear protein extracts from different maize tissues using both the −393/−60 and the −194/−60 DNA fragments. Reaction with the −393/−60 DNA fragment plus poly(dIdC) and embryo extracts 6 μg (lane 2) 3 μg (lane 4) and 2 μg (lane 5); and water stressed leaf extracts 6 μg (lane 7), 3 μg (lane 3) and 2 μg (lane 6); control reaction without proteins (lane 1). Reaction with the −194/−60 DNA fragment plus poly(dAdT) with 6 μg (lane 9) and 3 μg (lane 11) of embryo extracts; and 4 μg of water-stressed leaf extracts (lane 10); control reaction without proteins (lane 8).

competitors had no effect on the binding, unlabelled probe fragments −393/−60 and −194/−60 (Fig. 3, lanes 6 to 14) as well as a 50 bp oligonucleotide from the Em, containing the ABRE element (not shown), totally eliminated binding of nuclear protein(s) to the labelled probe.

Interaction of embryo and water-stressed leaf nuclear factors with the ABRE element in the rab*28 promoter*

The *in vitro* competition analysis demonstrates the specificity of a protein factor binding the DNA fragment containing the CCACGTGG motif with both embryo and water-stressed leaf nuclear protein extracts. However, the different band-shift pattern raises the question of whether the same protein factor or different protein factors are responsible for the specific binding in the different tissues, and it does not indicate if a difference exists between the specific DNA elements recognized by the proteins in these two tissues.

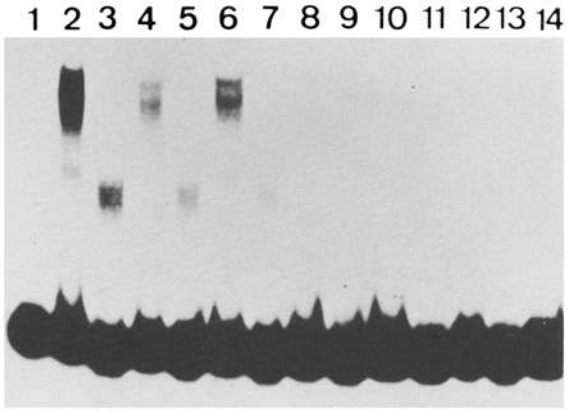

Fig. 3. Competition analyses of labelled 134 bp DNA fragment with unlabelled homologous sequences. Control reaction without proteins (lane 1); reaction with 6 μg (lane 2) and 2 μg (lane 4) of embryo extracts; reaction with 6 μg (lane 3) and 2 μg (lane 5) of water-stressed leaf extracts. Reactions in the presence of unlabelled 134 bp homologous fragment at 5-, 20- and 100-fold molar ratios with 6 μg of embryo extracts (lanes 6, 8 and 10 respectively), and 6 μg of leaf extracts (lanes 7, 9 and 11). Reaction of 6 μg embryo extracts with 20- and 100-fold molar ratio of unlabelled 333 bp fragment (lanes 12 and 14 respectively), 6 μg leaf extract and 100-fold molar ratio of unlabelled 333 bp fragment (lane 13).

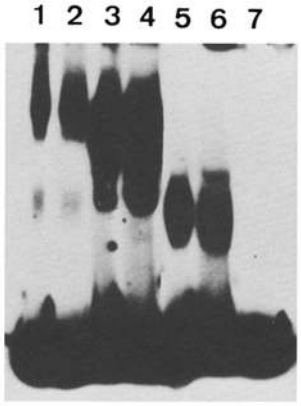

Fig. 4. Binding reactions in the presence of 0.5% Triton X-100. Protein extracts were from: lysogen extract from the GC19 clone (lanes 1 and 2), embryo (lanes 3 and 4), and water-stressed leaves (lanes 5 and 6). Lane 7 is the control reaction without proteins.

To further characterize these binding activities different binding conditions were tested. The specific binding pattern with protein extracts from embryos and water-stressed leaves was maintained when the amount of the protein was varied in the presence of a constant high level of non-specific competitor. Increasing protein/DNA ratios resulted in the same complexes which indicates that the mobility shift does not reflect specific addition of protein(s) to the complex (Fig. 2, lanes 1 to 4; and Fig. 3, lanes 2 to 5).

Binding reactions were performed in the presence of 0.5% Triton X-100. Results obtained using embryo extracts, lysogen extract from the GC19 clone and water-stressed leaf extracts are shown in Fig. 4. The GC19 clone encodes the wheat embryo leucine zipper protein EmBP-1 [13]. In all cases the resulting complexes comigrate with the resulting DNA protein complexes obtained from nuclear extracts without Triton addition. Moreover, pretreatment of the protein extracts with heat (55 °C for 15 min) had no effect on binding activity (not shown).

Specific competition of the protein factors present in embryos and water-stressed leaves was also studied by mixing the two extracts in equal or different proportions prior to binding assays. Equivalent quantities (μg of protein) of the two extracts were mixed and used for binding reactions in the presence of increasing amounts of KCl (up to 0.4 M). Figure 5 shows that none of the complexes disappear even at 0.4 M KCl. When the two extracts were mixed in different

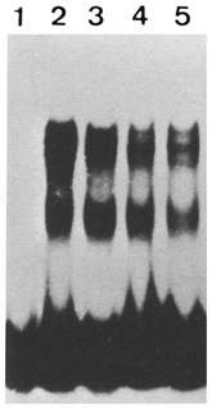

Fig. 5. Binding reactions with equivalent quantities (μg of protein) of the embryo and leaf extracts mixed together and the 134 bp DNA fragment in the presence of increasing amounts of KCl. Lane 1, control reaction without proteins; lane 2, no KCl addition; reactions in the presence of 0.07 M (lane 3), 0.2 M (lane 4) and 0.4 M (lane 5) KCl.

proportions the relative intensities of the complexes due to embryo and to water-stressed leaf protein factor(s) showed a close correlation with the relative proportions of the embryo and water-stressed leaf extracts present in the binding reaction (not shown). These results also indicate that the appearance of only one complex in leaf extracts is not due to endogenous proteolytic activity in this extract. All these data are consistent with the possibility that different proteins with apparent similar affinity to the ABRE sequence are present in these two tissues.

In vitro *DMS footprinting of the ABRE with embryo and water-stressed leaf nuclear protein extracts*

To precisely define the nucleotides within the −194/−60 DNA fragment important for binding the nuclear factors in the maize embryo and leaf extracts, methylation interference footprinting was performed. Figure 6 shows the *in vitro* Dimethyl Sulphate (DMS) footprint of the ABRE box from embryo and water stressed leaves. Methylation of guanine residues only within the ABRE sequence interfered with binding of nuclear proteins from both types of extracts. No other residues within the DNA probe showed detectable methylation interference (not shown). Interactions that occur in embryos are found at identical residues using water stressed extracts and are summarized in Fig. 6C.

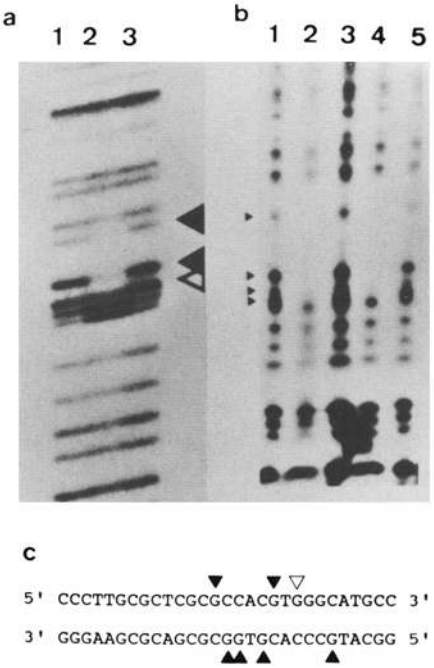

Fig. 6. In vitro DMS footprinting of the maize *rab*28 ABRE box from embryo and water-stressed leaf nuclear extracts. The 134 bp DNA fragment was partially methylated and bound to either wheat (A), maize embryo extracts (B, lanes 1 and 2) or water-stressed leaf extracts (B, lanes 3 to 5). After preparative electrophoresis ^{32}P-labelled DNA (A, coding strand; B, non-coding strand) was purified from free (A; lanes 1 and 3; B, lanes 1, 3 and 5) and bound (A, lane 2; B, lanes 2 and 4) bands, cleaved and resolved on sequencing gels. The positions of methylated guanines that interfered with binding are indicated with triangles (open for partially interfering bases, closed for more completely interfering bases). A summary of these results is depicted below (C).

Discussion

The maize gene *rab*28 has been previously identified as ABA-inducible in embryo and vegetative tissues. It is also induced by water stress in leaves [21]. The proximal promoter region of *rab*28 contains the conserved ABA-responsive element CCACGTGG reported in other plant genes as responsible for their ABA induction [23].

Here we show that a 134 bp DNA fragment containing the CCACGTGG element from *rab*28 is able to modulate GUS enzyme activity by ABA on the −90/+35 promoter derived from the 35S CaMV promoter in an orientation independent manner. Therefore sequences between −194 and −60 of the maize *rab*28 gene modulate transcription of the gene by ABA.

It has been reported that transcriptional regulation of gene expression in other ABA-responsive genes involves the interaction of DNA-binding proteins with this ABRE motif [9]. Gel retardation experiments indicate that maize nuclear proteins from embryo and water-stressed leaves bind to the *rab*28 134 bp DNA fragment. The specificity of binding was confirmed by adding specific and non-specific competitors to the binding reactions.

Previous studies on the pattern of expression of the *rab*28 and *rab*17 genes in ABA-deficient *viviparous* mutants indicated a differential regulation in embryo and vegetative tissues, suggesting that distinct regulatory factors may be involved in the hormone action during embryo development and in vegetative organs subjected to osmotic stress.

Comparison of the discrete mobility shifts in embryos and water stressed leaves reveals significant differences between these two types of extracts. The same complexes were observed when we increase the protein/DNA ratios in the binding assays which indicates that the mobility shift does not reflect the specific addition of protein(s) to the complex. Moreover, when we increase the length of the probe, including flanking sequences that may bind other protein factors the pattern of specific binding was not altered suggesting that the differences observed are not the result of adjacent protein/DNA interactions. This is in agreement with the methylation interference data which indicate that no other residues in the ABRE-flanking region showed detectable interference.

We have found a relative insensitivity of this binding activity to thermal denaturation, Triton X-100, and increasing concentrations of KCl. Furthermore, the ABRE does not compete for protein binding when embryo and leaf extracts are mixed. However, although the leaf binding activity forms a complex of different electrophoretic mobility it has the same guanine-specific contacts with the ABRE box as does the embryo binding activity. All these data seem to indicate that differences in electrophoretic mobility are due to different protein binding factors involved with the CACGTGG motif.

Lysogen extract from the GC19 clone which encodes a wheat embryo leucine zipper protein EmBP-1 [9] formed complexes similar to those obtained with embryo protein extracts suggesting close similarities between the embryo protein and EmBP-1. In both cases the same major type of complexes were observed. This may reflect that the different bands result from an interaction of protein/DNA with each other.

EmBP-1 interacts specifically with an 8 bp sequence CACGTGGC in the wheat Em gene [9]. This ABA-responsive element (ABRE) is highly conserved among ABA-responsive genes [23]. In addition, the CACGTG or closely related DNA motifs are essential for the expression of other genes, none of them appearing to be regulated by ABA [7, 14, 15, 26].

Several possibilities exist to explain our results: (1) more than one CCACGTGG-binding protein, (2) the same CCACGTGG-binding protein differentially modified, or (3) additional tissue-specific proteins acting in concert with the CCACGTGG-binding protein mediate the expression of the *rab*28 gene promoter in embryo and vegetative tissues. It is not known whether there are multiple binding factors that possess overlapping sequence specificities in the different tissues of the plant, nor if the expression of the *rab* genes is mediated by one or more CCACGTGG-binding proteins. However, several bZIP proteins have been so far identified possessing both distinct DNA-binding properties and expression characteristics [23, 24]. Moreover, genomic analysis of the EmBP-1 gene revealed a gene family composed of at least 7 genes in wheat. It has been suggested that the various bZIP proteins may individually mediate distinct classes of expression assigned to the CACGTG motif [24]. Further characterization of the protein members involved in the regulation of *rab* genes in embryo and vegetative tissues as well as further elucidation of the level of specificity involved in the interaction of bZIP proteins with their cognate DNA sequence may help understand the observed specificities of *rab*28 gene activation.

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