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Transcriptional regulation of a seed-specific carrot gene, DC8

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Abstract

Many late embryogenesis abundant (Lea) protein genes in plants are regulated by abscisic acid (ABA). The RNA level of a carrot gene, DC8, increases in response to ABA in developing seeds. However, DC8 cannot be induced by ABA in adult tissues. We used chimeric genes made of various DC8 promoter fragments fused to β-glucuronidase (GUS) to analyze the transcriptional regulation of DC8. DC8:GUS expression was measured in electroporated carrot protoplasts and in stably transformed carrots. The region of the DC8 promoter from −170 to −51 contained ABA-responsive sequences that required a 5′ upstream region for high levels of expression in embryogenic callus protoplasts. 505 bp of the DC8 promoter conferred GUS expression in stably transformed somatic and zygotic embryos. DC8:GUS was expressed only in tissues formed in the seed. This includes cells in the embryo, the endosperm and the germinating seedlings. Gel retardation and competition experiments were performed to analyze the embryo nuclear protein-DNA binding activities *in vitro*. No binding activity was detected on the putative ABA-responsive region; however the 5′ upstream regions located between −505 and −301 interacted with embryo nuclear factors. An additional site of DNA-protein interaction was located between positions −32 and + 178. The nuclear proteins that bind these sequences were found in the embryo nuclei only, not in the nuclei from leaves or roots.

Introduction

Many proteins accumulate during seed maturation and desiccation [11]. Some are seed storage proteins and proteins involved in the synthesis and accumulation of lipid and carbohydrate [14]. The others are believed to play a role in seed desiccation, and are often referred to as the late embryogenesis abundant (Lea) proteins or genes [22]. The Lea genes share common sequences in their structural and regulatory regions [8]. Because they are regulated by abscisic acid, analysis of Lea genes has been directed toward understanding the molecular mechanism of hormonal regulation [27, 30].

The Lea genes of monocots, i.e. Rab gene from rice, Em gene from wheat, glycine-rich protein gene from maize [15, 28, 29], can be induced by

ABA in embryos and young plants. However, a dicot Lea gene, DC8 [10], is primarily a seed-specific gene, i.e. the gene is expressed in all cells formed in the seed including the endosperm, the developing embryo and the germinating embryo cells. New cells formed from the meristem during seedling and adult development lose the ability to express DC8 even in the presence of ABA [19]. Similarly, a rice Lea promoter and GUS fusion gene, Rab-16B:GUS, was introduced into tobacco and shown to be expressed only in the embryos. It cannot be induced in any vegetative tissue of the transgenic tobacco [35]. To investigate the seed-specific and ABA-regulated transcription of DC8, we analyzed the function and nuclear protein binding activity of various DC8 promoter fragments. This paper reports our finding that two regions in the 5′ upstream sequence of the DC8 gene were simultaneously needed for transcription. Finally, based on a consideration of ontogenetic differences, we contend that monocot Lea genes, like their dicot homologues, might also be seed-specific.

Materials and methods

Plant materials and tissue culture

Carrot (*Daucus carota* cv. Juwarot) plants were grown under standard greenhouse conditions. Leaves and roots were collected from flowering plants, frozen in liquid nitrogen and stored at −80 °C until use. The carrot cell lines used in this study are W001C [33] and a highly embryogenic cell line derived from a plant called Juwa 2 from *Daucus carota* cv. Juwarot. W001C suspension cultures were maintained in Murashige and Skoog medium [31] in the presence of 0.1 μg/ml 2,4-dichlorophenoxyacetic acid (2,4-D). The Juwa 2 cell line was maintained in Gamborg (B5) medium [12] supplemented with 1 μg/ml 2,4-D. These suspensions were subcultured at 8×10^5 cells/ml. Somatic embryos were produced from 10–15-day old suspension cultures as previously described [10].

Transient expression in carrot protoplasts

Protoplasts were isolated as follows. W001C suspension cells were transferred into Murashige and Skoog medium containing 0.1 μg/ml 2,4-D and grown for 4 days. Cells were pelleted at $300 \times g$ for 5 min, resuspended in 5 ml of 1% cellulase, 0.1% pectolyase and 0.1% driselase in 0.2 M mannitol, 50 mM $CaCl_2$ and 10 mM Sodium acetate (pH 5.8) per ml of packed cells, and incubated for 3 h at 26 °C in the dark. Cells were washed 3 times in the same buffer to eliminate the enzymes, and protoplasts were isolated by filtration through 50 μm mesh. Protoplasts were pelleted and resuspended in 10 mM HEPES pH 7.2, 150 mM NaCl, 5 mM $CaCl_2$, 0.2 M mannitol to 5×10^6 cells/ml. Cell viability was determined by staining with 2 mg/ml fluorescein diacetate under a fluorescence microscope. Protoplast cells (900 μl) were electroporated with or without (control) plasmid DNA (50–80 μg) at 250 V, 1200 μF (capacitance) for 50 ms, and immediately put on ice. In each experiment protoplasts electroporated with pUC DNA were analyzed as control. The electroporated protoplasts were then diluted with 5 ml of Murashige and Skoog medium containing 0.3 M mannitol with or without 0.01 mM abscisic acid at 26 °C overnight. Protoplasts were collected by centrifugation and lysed by the addition of 0.5 ml GUS extraction buffer (50 mM NaH_2PO_4 pH 7.0, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 10 mM 2-mercaptoethanol). After removing insoluble materials by centrifugation, a portion of the supernatant was used to determine the protein content according to Bradford [2]. The remainder of the supernatant was assayed for GUS activity using a fluorogenic assay [24].

Carrot transformation and regeneration of transgenic plants

Transgenic plants were generated by both callus and petiole co-cultivation experiments using the binary vector described in Fig. 1A. Juwa 2 petiole segments or callus were transformed with *Agro-*

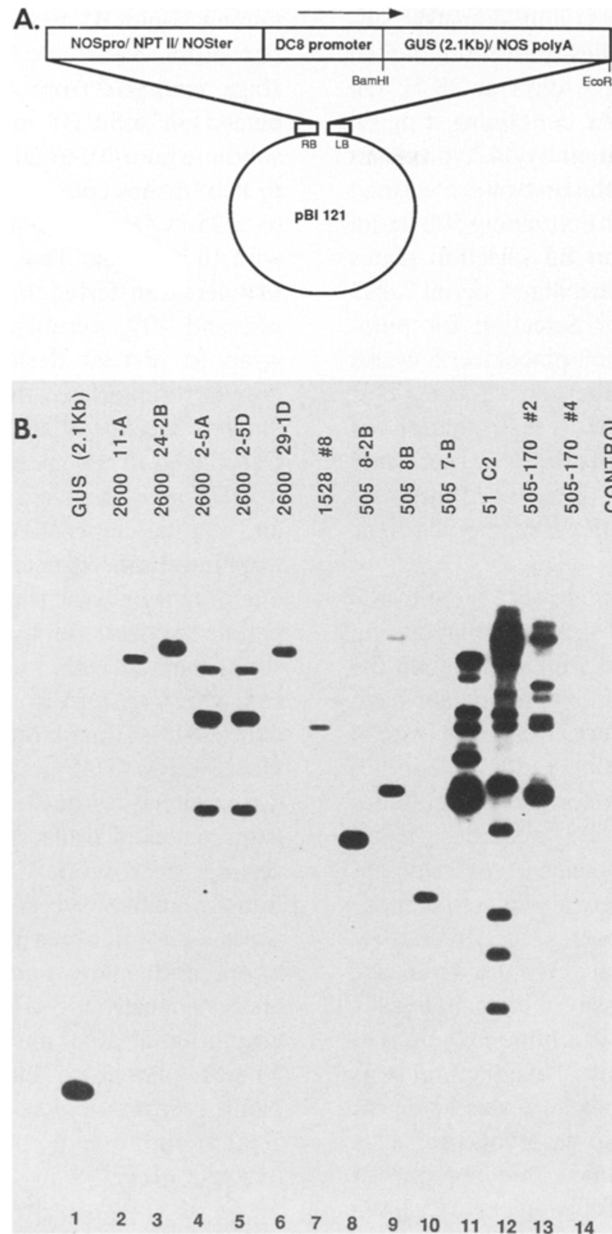


Fig. 1. DNA analysis of transgenic plants. A. Structure of the binary vector containing DC8 promoter:GUS chimeric gene. The different DC8 promoter fragments were inserted upstream of the GUS coding region. RB and LB: right and left border sequences of T-DNA. NPT II: neomycin phosphotransferase II gene. NOSpro-NOSter: nopaline synthase promoter and terminator. B. Hybridization of plant DNA with GUS probe. Genomic DNA of carrot leaves were extracted, digested with *Bam* HI and, after gel separation and blotting to nitrocellulose filters, hybridized with the *Eco* RI/*Bam* HI fragment containing the GUS coding region. Lane 1: 2.1 kb GUS/NOS-poly(A) probe. Lanes 2–6: individual plants regenerated from Juwa 2 petioles co-cultivated with *Agrobacterium* containing the DC8 (2600):GUS construct. Lanes 7–13: transgenic plants derived from callus co-cultivated with *Agrobacterium* containing chimeric genes, 1528:GUS, 505:GUS, 51:GUS, 505–170:GUS. Lane 14: control, untransformed Juwa 2 plant.

bacterium harboring plasmids containing the different chimeric genes. Petioles were co-cultivated with *Agrobacterium* for three days at 28 °C on Gamborg's B5 solid media containing 1 μg/ml 2,4-D and 100 μg/ml 3,5 dimethoxy-4-hydroxyacetophenone. After 3 days, the disks were washed 3 times in liquid B5 medium containing 500 μg/ml carbenecillin and placed on B5 selection plates containing 500 μg/ml carbenecillin, 1 μg/ml 2,4-D and 100 μg/ml kanamycin. Selection for putatively transformed callus took place over 5 weeks in a 25 °C constant temperature room. This method was used for the transformation of DC8(2600):GUS, into carrots. The remaining DC8:GUS constructs were introduced into carrot via the callus co-cultivation method (see below).

Carrot calli initiated from Juwa 2 were transformed with the constructs shown in Fig. 1 using the method of Wurtele and Bulka [34] with the following modifications. Embryogenic cells from 14-day-old suspension cultures were used instead of callus from agar plates. G418 (20 or 30 μg/ml) was used to select for transformed cell colonies on agar plates containing B5 medium, 1 μg/ml 2,4-D and 500 μg/ml carbenecillin. *Agrobacterium*-co-cultivated carrot cells were also plated on non-selective B5 media as controls. Twenty days after co-cultivation, 200–300 cell colonies from the nonselective media, were assayed histochemically for GUS activity [24] to determine if the transformations were successful. This method was used when the promoter fragment was sufficient to drive GUS expression in embryogenic callus cells. After it was determined that a group of transformation experiments was successful, tissue from 15–20 individual G418 resistant colonies from the selective plates was assayed. GUS-positive colonies were regenerated to produce transgenic plants. For constructs which could not produce high levels of GUS activity we relied on G418 resistance as the means for identifying potential transformants, which were later confirmed by the presence of DC8: GUS DNA inserts in the plant genome.

G418^r cell colonies from callus transformation experiments were transferred to Petri dishes containing liquid B5 medium and no plant growth regulators. Ten days later, 50 to 100 torpedo-stage embryos from the liquid medium were placed on solid B5 medium. After 10 days on solid medium, 10 to 20 plantlets were transferred to Petri dishes containing B5 medium and moved to a 25 °C growth chamber set for a 24 h cycle with 16 h of light. Two to four weeks later, plantlets were transferred to pots containing 60% loam soil and 40% vermiculite, covered with plastic wrap to prevent desiccation and moved to a growth room or growth chamber for acclimation. After 2 weeks of acclimation the plants were transferred to the greenhouse.

GUS activities were determined in the somatic and zygotic embryos of the same primary transformant. Embryogenic calli were initiated from our primary carrot transformants by cultivating petiole segments for 4 weeks on B5 solid medium supplemented with 1 μg/ml 2,4-D. The initiated calli were transferred onto B5 solid medium without 2,4-D. Globular-stage embryos developed after 7 days. GUS activities were assayed in endosperm tissues and zygotic embryos dissected from ovules of transformed carrots under a dissecting microscope. Seedlings of 2600:GUS transformants were germinated by soaking the seeds with water on a Whatman filter paper. Embryos, endosperm and seedlings were assayed histochemically for GUS activity. Tissues were vacuum-infiltrated and incubated overnight in 50 mM phosphate buffer (pH 7.0) containing 1 mM 5-brom-4-chloro-3-indolyl glucuronide (X-gluc) at room temperature, as described by Jefferson *et al.* [24].

Molecular techniques

Genomic DNA was isolated from carrot leaves regenerated from transformed callus cells using the CTAB method described by Rogers and Bendich [32] followed by phenol-chloroform extractions. DNA blot filters were hybridized with the 2.1 kb *Eco* RI/*Bam* HI fragment of GUS gene (see Fig. 1A). The hybridization and washings were performed at 65 °C according to Church

and Gilbert [6]. Plasmid isolation, digestion, nick translation, end labelling and all molecular techniques used in this study were performed according to Maniatis *et al.* [26]. Plasmids for electroporation were constructed as follows: the pBI 221 vector (Clontech), containing the CaMV 35S promoter, the GUS coding sequence and the NOS terminator, was used as primary vector for the transient expression experiment. From this the *Hin*d III-*Bam* HI fragment, containing the CaMV 35S promoter, was removed and replaced by the different DC8 promoter fragments. For the generation of transgenic plants the equivalent constructs were made in the vector pBI 121 (Clontech; see Fig. 1A).

The DC8 promoter deletions were produced as follows. The coding sequence and part of the leader sequence of DC8 (allele 8/6 [10]) were removed by *Bal* 31 digestion starting at the *Pst* I site in exon 1. Therefore, all promoter fragments used here end 3′ at position +55. At the 5′ end appropriate restriction sites were used to generate

Table 1. DC8:GUS activities in protoplasts. Protoplasts were electroporated with chimeric genes as described in Materials and methods. GUS activity is expressed in pmol of MU per hour per mg of protein. The first three rows represent the GUS activity measured in three different experiments. The last bold row represents the average. The ratio is the GUS activity measured in the presence of ABA/GUS activity measured in the absence of ABA. pUC19 DNA was used as a control.

CONSTRUCTS	TRANSIENT EXPRESSION PROTOPLAST ELECTROPORATION	- ABA	+ ABA	RATIO
-1528 — +55 GUS	expt. 1	274	1905	6.9
	expt. 2	213	1363	6.4
	expt. 3	234	1167	5.0
	avg.	**240**	**1478**	**6.1**
-505 — +55 GUS	expt. 1	417	1413	3.4
	expt. 2	63	182	2.9
	expt. 3	134	804	6.0
	avg.	**205**	**800**	**4.1**
-170 — +55 GUS	expt. 1	130	174	1.3
	expt. 2	53	59	1.1
	expt. 3	95	216	2.3
	avg.	**93**	**150**	**1.6**
-51 — +55 GUS	expt. 1	57	50	0.9
	expt. 2	48	44	0.9
	expt. 3	46	50	1.1
	avg.	**50**	**48**	**1.0**
-2600 — -505, -170 — +55 GUS	expt. 1	191	1500	7.9
	expt. 2	159	598	3.8
	expt. 3	167	583	3.5
	avg.	**172**	**894**	**5.1**
-2600 — -505, -51 — +55 GUS	expt. 1	65	65	1.0
	expt. 2	50	45	0.9
	expt. 3	107	98	0.9
	avg.	**74**	**69**	**0.9**
pUC DNA	expt. 1	54	48	0.9
	expt. 2	48	53	1.1
	expt. 3	52	56	1.1
	avg.	**51**	**52**	**1.0**

the respective deletions (see Table 1) and the numbers refer to the 5′-most base pair.

Nuclear extract preparation and gel retardation experiments

Nuclear extracts were isolated from somatic embryos and mature tissues, i.e. leaves and roots, according to Green *et al.* [17]. DC8 promoter fragments described in the previous section were subcloned in pUC 19. For gel retardation experiments they were isolated from the vector via restriction digest and low-melting-point agarose gel electrophoresis, end-labelled for binding with nuclear proteins. Unlabelled fragments were used in competition experiments. Binding reactions (10–25 μl) containing 0.1–0.2 ng of end labelled DNA fragments, 5 μg of poly(dIdC)-poly(dIdC) abbreviated as IC, 45 mM KCl, 25 mM HEPES pH 7.6, 1.1 mM EDTA, 0.5 mM DTT and 5% glycerol, were started by the addition of nuclear extracts (1.75 μg of protein per 10 μl reaction for embryos) and followed by incubation at room temperature for 10–20 min. Competitor DNA, in molar quantities, was used as indicated in the figures. The samples were separated by electrophores on 1% agarose gels [20]. Pronase 1(10 μg), RNase A (10 μg) and DNase 1(1 μg) were added to the reactions at the end of incubation and allowed to react for 10 min at room temperature. Heat treatment consisted of incubating completed reactions for additional 10 min at 65 or 85 °C.

Results

Transient expression of the DC8:GUS constructs in protoplasts

We used a transient expression assay of chimeric DC8:GUS genes in carrot protoplasts to determine the 5′ promoter regions required for DC8 transcription. Because protoplasts do not synthesize ABA [36], ABA-stimulated promoter activity could be studied by manipulating ABA concentrations in the medium.

Table 1 shows the activity of the various DC8 promoter:GUS chimeric genes electroporated into carrot protoplasts. The 1528 bp of sequence upstream from the transcription initiation site of DC8 gene were sufficient for DC8 activity in protoplasts and produced 6-fold higher activity in response to ABA. The −505 fragment had similar activity. However, DC8 activity was drastically reduced for the −170 and the −51 fragments. These fragments hardly responded to ABA stimulation. These results are evidence that the 505 bp upstream from the start site are sufficient for high levels of DC8 promoter activity and for ABA-stimulated transcription.

To determine the effects of the sequences within the −505 bp promoter, we made two internal deletions of the 2600:GUS construct, one deleted between positions −505 and −51 (505-51:GUS construct), and one between −505 and −170 (505-170:GUS construct), and assayed for DC8 promoter activity. The 505-170:GUS construct behaved much like the −1528 and −505 fragments in terms of basal level activity and ability to respond to ABA. These data indicated that an upstream located between positions −2600 and 505 could substitute for the sequence located between positions −505 and −170. However, the 505-51:GUS construct behaved like the −51 fragment; it lost its basal activity and did not respond to ABA. These results demonstrated the uniqueness of the sequence between positions −170 and −51 bp. No upstream sequence could replace its role in conferring GUS activity. The 2600:GUS construct produced the same amount of activity in protoplasts as the 1528:GUS construct.

Recent studies have identified conserved motifs in ABA-responsive regulatory elements [28, 30]. In the DC8 promoter, three of these ABA motifs are located between positions −170 and −51, at −132 (ACGTGG), at −113 (AC GTGT), and at −81 (ACGTGT). They are close to the TATA box as is the ABA motif in the Rab promoter, −290 to 51, and in the Em promoter, −168 to −106. The DC8 sequence between −170 and −51 is also required for ABA-stimulated activity. Although the −170 fragment

produced very low GUS activity in protoplasts, a comparison between the two internal deletions showed that it is nevertheless essential for both basal and ABA-stimulated activities. To achieve high level of transcription, the ABA motif-containing element of DC8 requires the presence of an upstream element located between positions −2600 and −505 or positions −505 and −170.

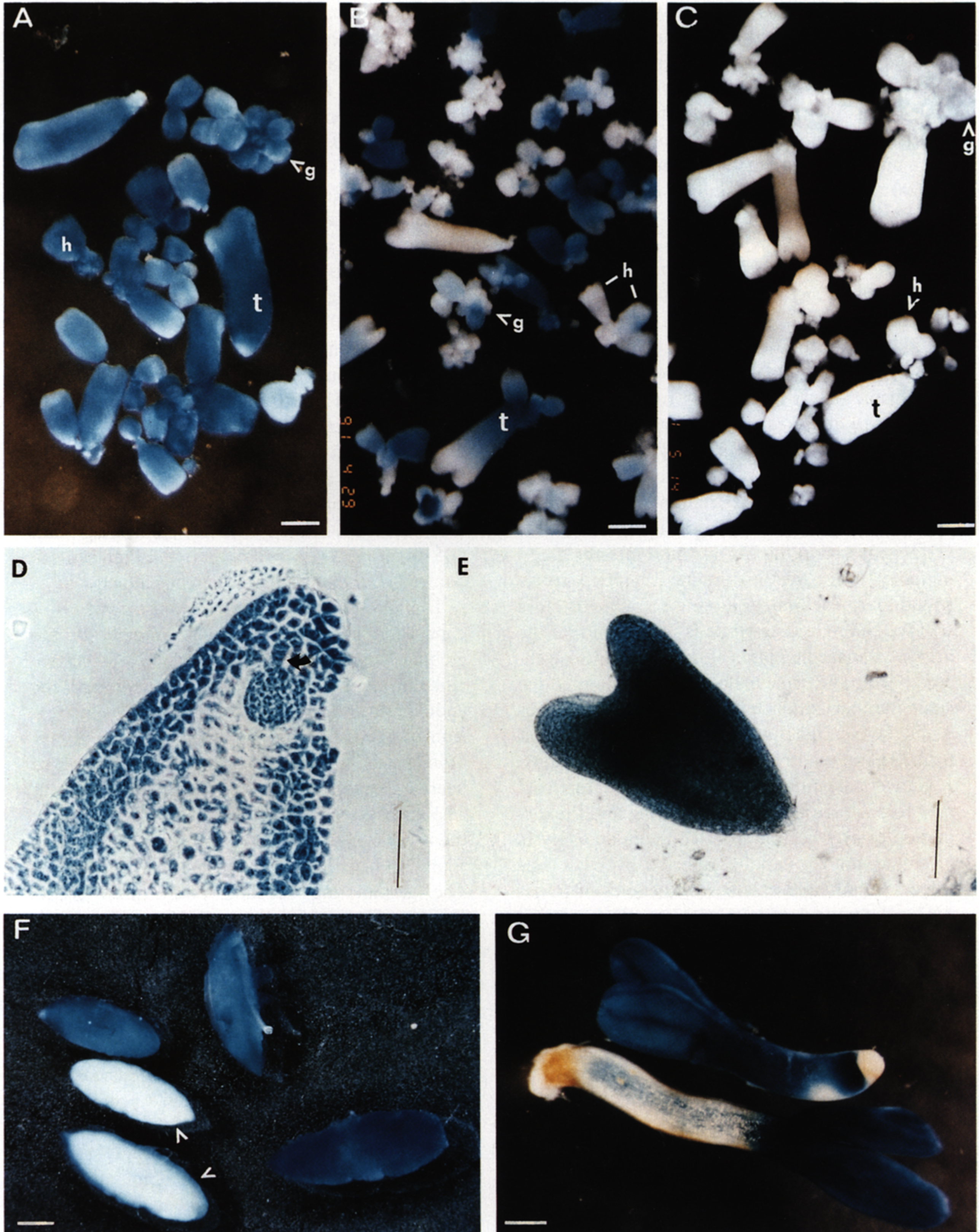
Expression of the DC8:GUS chimeric genes in somatic embryos and selfed seeds of transgenic plants

Various DC8 promoter deletion:GUS constructs (Fig. 1A) were introduced into carrot callus. Transgenic plants regenerated from transformed callus were analysed by Southern blot to detect the integration of DC8:GUS chimeric genes into the genome (Fig. 1B). The genomic DNA was digested with the restriction endonuclease *Bam* HI which cuts at the 5′ upstream of the GUS gene within the T-DNA and into the flanking plant DNA, yielding unique fragments corresponding to each integration event. One or more GUS DNA hybridizing bands were found for the various transgenic plants (Fig. 1B). Plants regenerated from the same callus colony contained the same number and size of inserts (Fig. 1B, Lanes 4 and 5). No signal was detected in the untransformed plant (control) under the same conditions.

Carrot is biennial. Vernalization of mature carrot roots is required before flowering and the formation of zygotic embryos. To facilitate analysis of embryonic regulation conferred by DC8 promoter, we initiated callus from petioles of transgenic plants and regenerated somatic embryos from 3–4-week old callus. At least two independent transgenic plants were used for each construct. The ability of the various promoter fragments to confer GUS expression in somatic embryos of transformed carrot was similar to that of transient expression in callus protoplasts, although the level of expression in the embryos was not quantified. Somatic embryos of all stages – globular, heart and torpedo stages – were GUS-positive when initiated from transgenic carrots containing constructs with both the ABA element and the upstream element located between positions −505 to −170 such as the 2600:GUS (Fig. 2A), 1528:GUS and 505:GUS constructs. Only 50% of the somatic embryos regenerated from plants containing the 2600 bp deleted of the −505 to −170 bp were GUS-positive (Fig. 2B). The multiple T-DNA insertions (Fig. 1B) in these transgenic carrots may have resulted in the methylation and inactivation of the transgenes in some embryos [21].

Embryo cells containing constructs missing either the upstream sequence or the ABA element (170:GUS, 505-51:GUS), or both sequences (51:GUS) did not express GUS activity (Fig. 2C). The expression of DC8:GUS was also analyzed in selfed F1 seeds and vegetative tissues of the primary transformants. Nine independent 2600:GUS and four independent 505:GUS transformants were analyzed. The pattern of GUS expression was similar between the 2600:GUS and the 505:GUS transformants confirming that 505 bp of 5′ DC8 promoter were sufficient to confer GUS expression in carrot seeds.

The 505 bp DC8 promoter could induce GUS expression in zygotic embryos as early as the globular stage. Figure 2D shows a GUS-positive globular-stage embryo. The suspensor and the endosperm were also GUS-positive. The expression of GUS activity continued throughout embryogenesis. Figure 2E shows a GUS-positive heart stage embryo dissected from the endosperm. The mature seed displayed GUS activity only at the outer cell layer of the endosperm (Fig. 2F), probably because the inner cells have died [9]. The germinating seedlings were also GUS-positive. Figure 2G shows GUS-positive seedlings four days after germination. As the seedlings grow, cells in the cotyledons and hypocotyl enlarge and elongate and slowly lose GUS activity. Leaf and root cells grown from the meristems after germination do not express GUS in the presence or absence of ABA or after desiccation. The kinetics of appearance of DC8 protein corresponds with that of DC8:GUS activity in germinating carrot seedlings (data not shown). The presence of DC8 protein and GUS activity in the seedlings may result from new gene transcription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after germination, or may be protein and GUS enzyme produced in the embryo which are stable enough to be detected even after desiccation and germination.

Interaction of nuclear extracts from somatic embryos with 5′ flanking sequences of DC8 gene

In order to identify DC8 promoter sequences involved in transcriptional regulation, we incubated embryo nuclear extracts with fragments of the DC8 promoter (Fig. 3A) and assayed for binding activity. Nuclear extracts from 10–15-day old somatic embryos were incubated with labelled DNA fragments from −1528 bp to +178. Figure 3B shows that nuclear factors reacted with and retarded the mobility of almost all the fragments tested. Each of the four non-overlapping fragments (301E/S, 213S/H, 178S/Sp and 333H/P) showed a single complex with the nuclear extracts (Fig. 3B). The other two non-contiguous fragments (356H/H and 350Sp/H) showed two complexes with mobility different from that observed with the first four fragments (Fig. 3B).

We investigated further the interactions of 350Sp/H and 333H/P fragments with nuclear extracts because they cover the 505 bp of the DC8 promoter that allowed high level of transcription in somatic and zygotic embryos. The 350Sp/H fragment has A/T-rich sequences [10], and the 333H/P fragment contains the three ABA motifs and the leader sequence reported to play a role in post-transcriptional regulation [28].

The specificity of the interactions was shown by competition experiments. Unlabelled 350Sp/H fragment decreased the amount of complex formed between labelled 350Sp/H fragment and the nuclear extracts. Linearized pUC19 DNA or 333H/P fragment did not compete with the 350SpH/H fragment for binding with nuclear extracts from embryos (Fig. 4A). Similarly, unlabelled 333H/P fragment competed with labelled 333H/P fragment for binding with nuclear extract. However, linear pUC19 DNA or 350Sp/H fragment had no effect on the interaction of 333H/P with nuclear extracts (Fig. 4B). 350Sp/H and 333H/P fragments did not compete with each other for nuclear binding factors. These fragments must interact with unique nuclear factors. The binding sites were further defined using smaller fragments of 350Sp/H and 333H/P isolated and subcloned into pUC19. Incubation of the subfragments with nuclear extracts showed that binding sites were present in the 204Sp/Sc and 210Ha/P subfragments but not the 146Sc/H and 123H/Ha subfragments (Fig. 5A and B). The migration profile of the 204Sp/Sc fragment was essentially the same as the parental 350Sp/H fragment (Fig. 5A, lanes 2, 3, 5 and 6), indicating that all sequences necessary for the complex formation were located within the 204Sp/Sc fragment. The 210Ha/P subfragment from the 333H/P fragment had the same binding pattern as the 333H/P (Fig. 5B), demonstrating that the sequence responsible for binding was present within this segment. However, the 123H/Ha fragment that contains the putative ABA element described in this report, did not interact with embryo nuclear extracts. These data are consistent with the lack of

Fig. 2. Histological GUS activity in embryonic transgenic carrot tissues. Tissues were stained in X-gluc solution overnight as described in Materials and methods. A. GUS-positive somatic embryos at globular (g), heart (h) and torpedo (t) stage regenerated from callus of a 505:GUS transgenic plant. bar = 330 μm. B. Somatic embryos at the globular (g), heart (h) and torpedo (t) stage regenerated from callus of a 505–170:GUS transgenic plant. bar = 330 μm. C. GUS-negative somatic embryos at the globular (g), heart (h) and torpedo (t) stage regenerated from callus of a 170:GUS transgenic plant. bar = 330 μm. D. Cross section of a globular-stage zygotic embryo from selfed seeds of a 2600:GUS transgenic plant, 13 days after pollination. The embryo is embedded in the endosperm. Both are GUS-positive. The arrow points at the embryo suspensor. The ovule was stained in X-gluc solution before fixation in formaldehyde, dehydration, and paraffin embedding. bar = 100 μm. E. Heart-stage zygotic embryo dissected from ovule 21 days after pollination. bar = 50 μm. F. GUS-positive endosperm from mature seed of a 2600:GUS transgenic plant. The outer cell layer (maybe the aleurone) of the endosperm is GUS-positive. The arrows point at endosperm with outer layers removed. bar = 200 μm. G. GUS-positive 2600:GUS seedlings 4 days after germination. The hypocotyl cells that elongate start to lose GUS activity. bar = 420 μm.

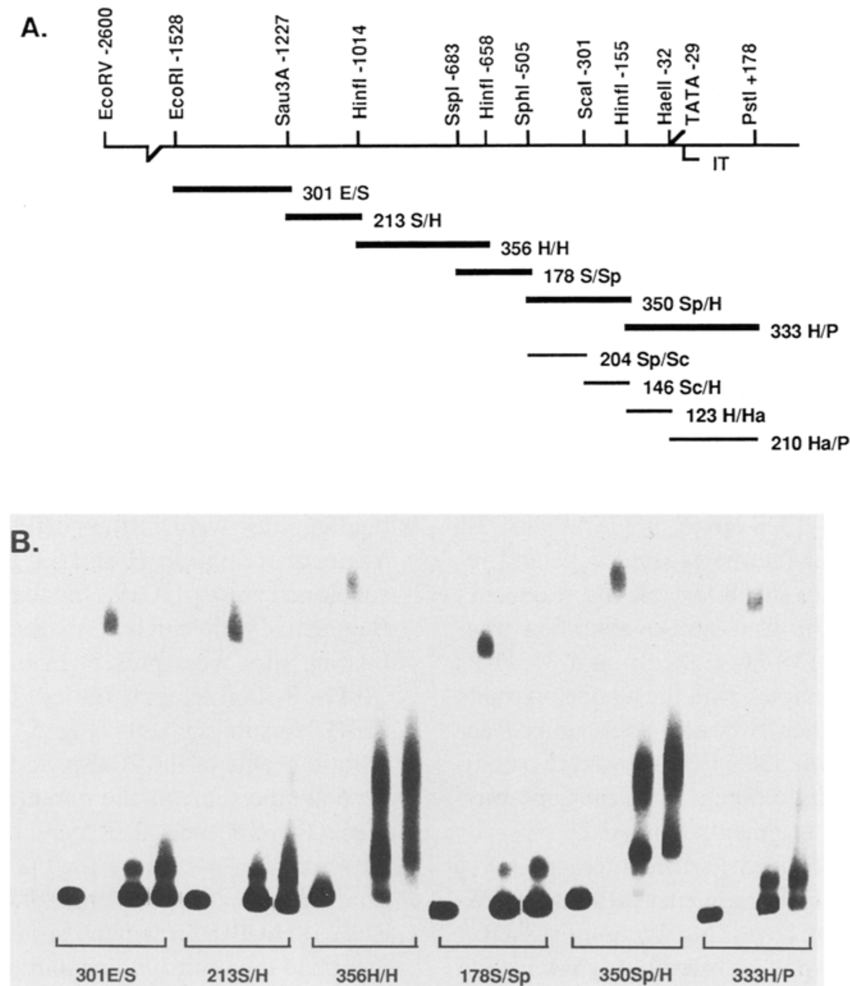

Fig. 3. Interaction of embryo nuclear factors with DC8 upstream sequences. A. Restriction map of the promoter region of the DC8 gene. The top line is the 5′ upstream region of DC8 gene. Restriction sites are indicated above with their positions from the transcription initiation site (IT). Lines below are the restriction fragments used for gel retardation. Numbers to the right of each fragment indicate their sizes and the restriction enzymes. Fragments indicated by the thick lines were used in the experiment shown in Fig. 3B. Those indicated by thin lines were used in the experiment shown in Fig. 5. B. Embryo nuclear factors bound to the restriction fragments shown in A. Nuclear extracts were incubated with 301 E/S fragment (lanes 1–4), 213S/H fragment (lanes 5–8), 356H/H fragment (lanes 9–12), 178S/Sp fragment (lanes 13–16), 350Sp/H fragment (lanes 17–20), 333H/P fragment (lanes 21–24). Lanes 1, 5, 9, 13, 17, and 21 are without nuclear extracts and indicate the migration of the free fragment. Lanes 2, 6, 10, 14, 18, and 22 are with nuclear extracts (3.5 μg) and without IC. Lanes 3, 7, 11, 15, 19 and 23 are with IC (5 μg) and nuclear extracts (3.5 μg). Lanes 4, 8, 12, 16, 20, and 24 are with IC (5 μg) and 7 μg nuclear extracts. Reaction incubations were 15 min at room temperature and the DNA concentration was 0.4 ng.

biological activity of the region located between positions −170 and −51 in the absence of an upstream sequence (Table 1). We investigated whether addition of ABA in the reaction resulted in new complexes or increased the abundance of already observed complexes formed with the 350Sp/H and 333H/P fragments. ABA might be depleted during nuclear extract isolation resulting in the inactivation of nuclear factors. However, the addition of ABA to the *in vitro* reaction mixtures did not reveal new complexes (data not shown).

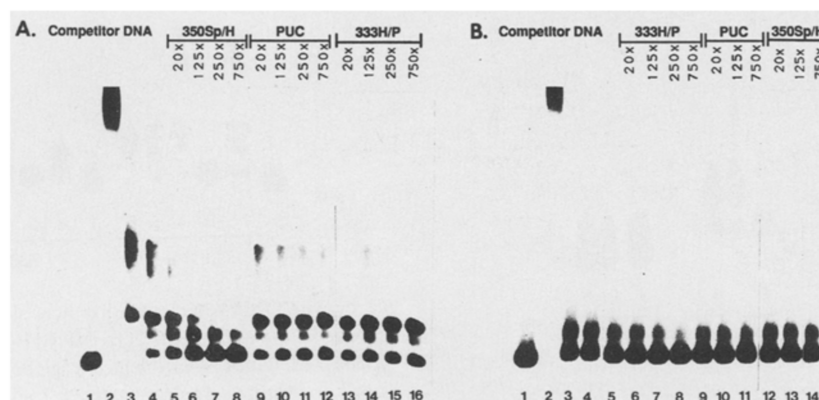

Fig. 4. Gel retardation analysis for binding activity specific to 350Sp/H and 333H/P fragments. A. Binding reactions using labelled 350Sp/H fragment. Lane 1 shows the migration of the free fragment. Lane 2 shows the profile of migration without IC. Lanes 3 and 4 are with nuclear extracts, 3.5 and 1.75 μg respectively. Multiple complexes were present and addition of more nuclear extract shifted the complexes to slower migration. Lanes 5–16 show the competition experiments made with 1.75 μg nuclear extract and with different molar ratios of unlabelled competitor DNA indicated above the panel. B. Binding reactions using labelled 333H/P fragment. Lanes 1, 2, 3 and 4 are as in A. Only one bound form was present. Lanes 5–14 are with 1.75 μg nuclear extract and different molar ratios of unlabelled competitor DNA as indicated above the panel.

The retarded bands involved DNA-protein complexes

To investigate whether the complexes formed between the fragments and nuclear extracts were products of DNA-protein interaction, the *in vitro* reactions were subjected to heat treatment or incubated with pronase E, RNase A and DNase I for 10 min. Figure 6 shows that pronase E completely abolished binding by eliminating all complexes (lanes 3 and 9) formed with 350Sp/H or 333H/P fragments. RNase A had no effect on the pattern of the complexes (lanes 4 and 10), while DNase 1, as expected, completely eliminated the retarded band (lane 13). The above results clearly demonstrate that the complexes formed between 350Sp/H and 333H/P fragments and nuclear extracts resulted from interaction of these fragments with DNA-binding proteins. The heat treatment had very little effect on complexes formation (lanes 5, 6, 11 and 12) indicating that either the DNA-binding proteins or the DNA-protein complexes were heat-resistant up to 85 °C.

Nuclear protein interactions with fragments 350Sp/H and 333H/P were embryo-specific

To determine the organ specificity of the nuclear proteins that bind the DC8 promoter, the 350Sp/H and 333/HP fragments were incubated with nuclear extracts isolated from carrot roots and shoots. No complexes were formed between the fragments and nuclear extracts from roots or leaves (Fig. 7). The integrity of the nuclear extracts from the leaves and roots was demonstrated by excluding poly-d(IC) from the experiment. Unspecific bindings retarded the mobility of both labelled fragments (data not shown). Four times more nuclear extracts from mature tissues were used, and still the reactions did not result in the formation of any complexes, indicating that leaves and roots contain little or no nuclear proteins that could interact with these two fragments. Alternatively, the proteins or the DNA sequences may be inactive in mature tissues. These results suggest that DC8 expression was dependent on the presence of embryo-specific nuclear proteins that regulate its transcription.

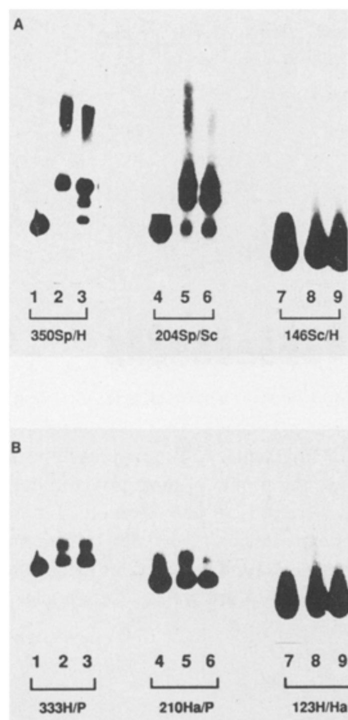

Fig. 5. Interaction of embryo nuclear factors with subfragments. A. 350Sp/H fragment (lanes 1–3) and 204Sp/Sc (lanes 4–6), 146Sc/H (lanes 7–9) subfragments were incubated without nuclear extracts (lanes 1, 4 and 7), with 3.5 μg nuclear extracts (lanes 2, 5 and 8) or with 1.75 μg nuclear extracts (lanes 3, 6 and 9). B. 333H/P fragment (lanes 1–3) and 210Ha/P (lanes 4–6), 123H/Ha (lanes 7–9) subfragments were incubated without nuclear extracts (lanes 1, 4 and 7), with 3.5 μg nuclear extracts (lanes 2, 5 and 8) or with 1.75 μg nuclear extracts (lanes 3, 6 and 9).

Discussion

We investigated the molecular mechanism of DC8 expression in carrot embryos. The embryo- or seed-specific expression of DC8 is dependent on the presence of ABA. Deletion analysis defined two regions of the DC8 upstream sequence required together for high level expression as well as for ABA-stimulated enhancement of expression. One of them, located between positions −170 and −51, contains three copies of an ACGTG motif. In analogy to the situation in the Em promoter, we suggest that these sequences might be required for ABA stimulation. Another section of the promoter essential for high levels of expression is found between positions −505 and −170. The function of this region can be substituted by sequences upstream of −505. Furthermore, it has been shown that it contains a long stretch of sequence very similar to a portion of the upstream region of DC59, another carrot gene with virtually identical expression pattern. In DC59 two nuclear factors, C3 and C4, bind to this part

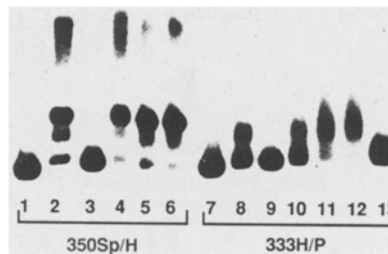

Fig. 6. The DNA fragments form heat stable complexes with proteins in the nuclear extracts. Lanes 1–6: labelled fragment is 350Sp/H. Lanes 7–13: labelled fragment is 333H/P. Lanes 1 and 7 are without nuclear extracts. Lanes 2 and 8 are with 1.75 μg of nuclear extracts. Lanes 3 and 9 are the Pronase E treated reaction mixtures. Lanes 4 and 10 are the RNase A-treated reaction mixtures. Lanes 5 and 11 are the standard incubation followed by 10 min at 65 °C. Lanes 6 and 12 are the standard incubation followed by 10 min at 85 °C. Lane 13 is DNase I-treated reaction mixtures.

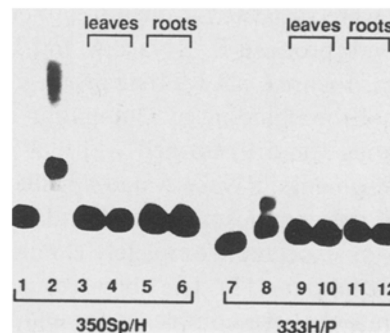

Fig. 7. The nuclear proteins that react with 350Sp/H and 333H/P fragments were present only in embryo not in leaf or root nuclei. Lanes 1–6 are the binding reactions with the 350Sp/H fragment and lanes 7–12 are those of 333H/P. Lanes 1 and 7 are without nuclear proteins. Lanes 2 and 8 are with embryo nuclear proteins (1.75 μg). Lanes 3 and 9, 4 and 10 are with leaf nuclear extracts (3.4 and 5 μg, respectively). Lanes 5 and 11, 6 and 12, are with 6 and 9 μg of root nuclear extracts, respectively.

of the promotor and their binding can be prevented by the addition of DC8 upstream sequences as competitor [20].

In the binding assay two nuclear factors could be identified that bind to region −505 to −301. Further experiments are required to determine whether these are identical to the factors named C3 and C4 in case of DC59. It is known however that C3 and C4 as well as the complexes observed with the equivalent fragment of DC8 are formed only with nuclear extracts isolated from embryos, suggesting that they play a role in the coordinate gene regulation during embryogenesis. A total of four DNA-protein complexes were detected upstream of −505. Further analysis is required to show whether these complexes are responsible for the observed functional redundancy, i.e., the function of the region between −505 and −170 can be substituted by sequences upstream of −505.

No binding of nuclear factors could be detected when the fragments containing the proposed ABA-response elements were used. However, the functional tests clearly demonstrate that this region is essential for the activity of the DC8 promoter. Therefore, we speculate that the binding of factors to the −505 to −301 element is the primary event and that this binding is a prerequisite for the interaction of proteins with the ACGTG motifs. An alternative explanation could be that the nuclear extracts did not contain or contained insufficient amounts of the respective proteins that bind to the ABA motifs. This seems to be less likely as the extracts were prepared from 10–15-day old embryos known to show high expression of DC8.

In addition to the factors binding to the more upstream promoter sequences of DC8, one complex was formed between a fragment starting 4 bp upstream of the TATA box and containing the untranslated leader sequence. A similar result was obtained with the Em promoter and it has been suggested that this DNA-protein interaction is associated with the post-transcriptional regulation of this gene [28].

The regulation of many embryonic genes is highly conserved [4, 14]. Monocot genes can be faithfully expressed in an embryo-specific manner in dicots [28]. We have found that DC8:GUS can express in maize scutellum (unpublished results). DC8, Em and Rab contain similar regulatory sequences, but it is not yet clear that they are regulated in the same manner. The monocot Lea genes Em and Rab can be induced in young leaves. For this reason, the monocot Lea genes have not been considered embryo-specific [15, 28, 29]. DC8, however, cannot be induced in carrot leaves. This apparent difference in regulation may reflect differences between monocot and dicot embryo development. The first 5 or 6 leaves of monocots are formed in the seed before germination. The first dicot leaves are not formed until after germination. Seed-specificity may be interpreted as the presence of transcriptional factors in all tissues and cells formed in the seed. Those factors may remain in the cells for some time after germination. This may explain why the cotyledons of dicots and the young leaves of monocots are able to respond to exogenous ABA after germination by activating the promoters of some Lea genes. Recently, it has been shown that Rab-16B rice gene cannot express in dicot leaves [35]. Whether Em and Rab are embryo- or seed-specific, or simply ABA-inducible, depends on their expression in older leaves whose primordia, are formed after germination.

Unlike the ABA element of the Em promoter, the ABA element of DC8 requires the help of an upstream region to confer transcription (see Table 1). A leucine zipper protein, EmBP-1, that recognizes the CACGTGGC element in the Em promoter has been isolated from wheat embryos [18]. It contains sequences similar to that of the leucine zipper protein that binds the promoter of the histone gene, HBP-1, whose recognition sequence, the hex sequence (GGtgACGTGGC) can compete for binding of EmBP-1. Moreover, similar motifs are also found in the promoters of other plant, mammalian and yeast genes [5, 7, 13]. The ubiquitous nature of the ABA motif and its binding proteins suggests that the transcriptional complex could involve, in addition to EmBP-I and CACGTGGC, other factors and elements. This notion is consistent with our find-

ing that the ABA element and an A/T-rich sequence are simultaneously required for DC8 transcription. A/T-rich sequences that interact with DNA binding proteins were also found in a number of seed-specific genes such as in the 5′promoters of soybean lectin and seed storage proteins [3, 14, 25]. High-mobility-group chromosomal proteins (HMG) are commonly found to interact with A/T-rich sequences [16, 23]. Transcription of seed-specific genes may require a complex interaction involving the ABA element, the A/T-rich regions and several regulatory proteins.

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