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Expression of DC8 is associated with, but not dependent on embryogenesis

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Abstract

DC8 is a late embryogenesis-abundant (LEA) protein gene isolated from carrot (*Daucus carota*). Deletion analysis of the DC8 promoter was performed to determine the sequences required for ABA and seed-specific regulation of DC8 transcription. To investigate the mechanism of DC8 expression during seed development, chimeric gene constructs containing DC8 promoter fragments fused to a promoterless beta-glucuronidase gene (DC8:GUS) were introduced into carrot, tobacco (*Nicotiana tobacum*) and *Arabidopsis thaliana* plants. Seed-specific DC8 expression patterns was conserved among the three plant species. However, differences among the species in the patterns of DC8 expression in the embryo and endosperm that correlated with differences in the rates of embryo and endosperm growth were found. Lack of correspondence between DC8 activation and embryo development among the seeds of the three species suggests that DC8 expression, which is associated with seed maturation, is not coupled to the embryo development program. The presence of DC8 activity in carrot callus and endosperm is consistent with the notion that DC8 expression is independant of embryo morphogenesis. A similar DC8 activity time-course during callus induction and seed development suggests that explantation and 2,4-D treatment initiates a course of events similar to that in the carrot ovule. After fertilization, two pathways one leading to embryo development and another to seed maturation are initiated, but they are not closely linked. As a result we find DC8, part of the maturation program, being activated at different embryonic stages in different plant species.

Introduction

After double fertilization, seed development begins with endosperm and embryo growth and differentiation. During subsequent seed maturation the seed storage proteins, starch, and lipids accumulate before the seed undergoes desiccation and dormancy [34]. It is characteristic of late embryo development for both the endosperm and mature embryo to express seed storage reserve genes [13]. Many other hydrophillic proteins with unknown functions that share similar sequence repeats also accumulate in mature embryos and endosperm. They are called late embryogenesis-abundant (LEA) proteins and are thought to be involved in seed maturation, for example, protecting cells during seed desiccation [7, 21].

Abscisic acid, ABA, plays a major role in seed

maturation and regulates the expression of the LEA genes [8, 10, 11, 24, 27, 30]. Cis-elements and transcription factors mediating ABA-inducible LEA gene expression have been identified [16, 29, 38]. Some ABA-inducible LEA genes are inducible only in cells formed in the seed [14, 32], while others appear to lack seed specificity [30, 38]. Carrot LEA genes that encode hydrophilic proteins, such as DC8, are expressed in carrot zygotic embryo and endosperm tissues, in somatic embryos, and in callus [2, 4, 9, 46]. The ABA treatment causes large increases in levels of mRNA and protein in cultured carrot cells, but ABA cannot induce DC8 expression in mature leaf or root tissues [14, 18]. Other carrot LEA genes, such as DC24, and the carrot oleosin protein gene, DC59, have similar expression patterns [17, 19].

Because LEA mRNA and protein abundance was correlated with late embryonic development, LEA gene expression was thought to be regulated by the embryonic developmental program and LEA gene activities have been used as stage-specific markers to characterize embryo-lethal mutants [43, 44]. Therefore, it was puzzling to find DC8 expression in early embryogenesis and in non-embryo tissues such as callus and endosperm [14]. On the other hand, since both the embryo and the endosperm desiccate during seed maturation, we might expect the expression of genes involved in desiccation protection in both of these tissues.

To investigate LEA gene regulation, we performed a detailed analysis of the temporal and spatial pattern of DC8 expression. To address the issue of early DC8 expression, we compared DC8 expression in carrot to its expression in species with different rates of seed development. We found that DC8 expression is more closely correlated with growth rates than with the embryo's developmental stage. This result, coupled with DC8 expression in the endosperm and embryogenic callus, suggests that the seed maturation program, as represented by LEA gene expression, is activated at the same time as embryogenesis, but is not linked with specific embryonic stages.

Materials and methods

Plant materials and culture conditions

Carrot (*Daucus carota* L. cv. Juwarot) seedlings were germinated on moist filter paper, transplanted into soil and grown to maturity in a greenhouse maintained at 24 °C, 16 h light. Tobacco (*Nicotiana tabacum* cv. Wisconsin) and *Arabidopsis thaliana* seedlings were germinated on agar plates containing (2/5 MS medium [31] transplanted into soil and grown to maturity in a greenhouse maintained at either 24 °C, 16 h light for tobacco, or 21 °C, 9 h light for *Arabidopsis*. Carrot plants were induced to bolt by vernalizing at 4 °C for 45 days. Before anthesis, the umbel was covered with a paper bag. Pollen grains from an older umbel were dusted onto newly emerging pistils in the umbel which were then covered again.

Transgenic carrots were constructed as described by Goupil *et al.* [14]. *Arabidopsis* plants were transformed with the DC8(505):GUS construct according to procedures described by Valvekens ***et al.*** [42]. DC8(2600):GUS transgenic tobacco was generated according to Horsch *et al.* [20].

Carrot tissue cultures were initiated by culturing petiole segments on B5 medium supplemented with 1.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) at 24 °C [12]. To initiate a suspension culture, two-week-old callus was transferred into liquid B5 medium supplemented with 1.0 mg/l 2,4-D. To initiate the development of somatic embryos, 10- to 15-day-old suspension cultures were diluted into B5 medium without 2,4-D [39].

Arabidopsis calli were initiated from root segments and cultured at 24 °C on callus-inducing medium (CIM, Gamborg's B5 medium supplemented with 0.5 mg/l 2,4-D and 0.05 mg/l kinetin) [5, 12]. Tobacco callus was initiated from leaves and cultured on MS medium supplemented with 1.0 mg/l 2,4-D.

GUS activity assays

Mature leaves of transgenic carrot, tobacco, and *Arabidopsis* were collected from greenhouse-

grown adult plants. Fresh ABA (Sigma A-1012) solutions of 10^{-5}–10^{-6} M were prepared from a filter-sterilized 1.0 mM solution and supplied to the cut ends of the petioles for 24 or 48 h at room temperature as specified in the experimental protocol. Leaves were cut up into small species for GUS assays. Free-hand sections of 200–300 μm thick carrot petioles were prepared for GUS assays. Zygotic embryos and endosperm tissues were dissected from ovules of both transgenic and non-transgenic carrot seeds at various times after pollination. The developmental stage of the embryos, i.e., globular, heart, torpedo stage, was determined under a dissecting microscope.

Beta-glucuronidase (GUS) activity was detected histochemically after submerging tissue samples in GUS solution (50 mM sodium phosphate pH 7.6, 5.0 mM potassium ferrocyanide, 5.0 mM potassium ferricyanide, Triton X-100, and 0.33 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc)) as described by Jefferson *et al.* [22]. 20% methanol (v/v) was added to the GUS solution to reduce background caused by endogenous GUS activity [25]. All of the samples were vacuum-infiltrated in GUS solution for 10 min, incubated at 37 °C for 16 h and photographed under a dissecting microscope (Zeiss Stemi SV 11).

Light microscopy

Carrot embryos or ovaries were fixed in 4% glutaraldehyde for 16 to 18 h at 4 °C. Samples were embedded in JB-4 plus as described in Cheng *et al.* [3]. Longitudinal sections (4 to 5 μm) were produced using a Reichert-Jung microtome (Heidelberg, HM 340). These sections were double-stained with PAS (periodic acid leucofuchsin) and hematoxylin. Stained sections were photographed under a Zeiss Axiophot microscope.

DC8 mRNA analysis in carrot seedlings

Carrot seedlings were germinated on moist filter paper in a growth chamber at 25 °C under continuous room light. Because of the problem of asynchronous germination, samples were selected based on seedling developmental stage as well as germination time. Three seedling developmental stages were analyzed: seedlings with only 1–2 cm of emerging radicle selected at 2–6 days after the start of imbibition, seedlings with fully expanded cotyledons selected at 7–10 days after imbibition, and seedlings with the first true leaf emerging selected at 14–20 days after the start of imbibition. To test ABA induction of DC8 expression, one third of each sample was treated with water, one third with 10^{-6} M ABA for 24 h, and one third with in 10^{-6} M ABA for 48 h. After treatment, the seedlings were frozen in liquid nitrogen and stored at −80 °C. RNA was extracted using a small-scale (100–300 mg tissue) procedure described by Seeley *et al.* [37]. Total RNA was electrophoresed in a 1% agarose/3% formaldehyde gel, blotted onto nylon membranes (GeneScreen, New England Nuclear, Boston, MA), and hybridized at high stringency [37]. Plasmid p87P1.7 [14], a transcription vector containing coding sequences from the carrot DC8 gene, was linearized with *Bam*HI and single stranded RNA probe was synthesized using T3 polymerase and ^{32}P-dCTP according to the manufacturer's instructions (Promega, Madison, WI).

In situ *RNA hybridization analysis*

Carrot embryo and endosperm tissues were fixed in FAA (3.7% (v/v) formaldehyde, 50% (v/v) ethanol, 3% (v/v) acetic acid), dehydrated with ethanol, cleared with xylene and embedded in paraffin. Paraffin-embedded tissues were sliced into serial 8 μm sections with an American Optical Model 820 microtome. Sections were attached to slides coated with poly-lysine hydrobromide (Sigma).

In situ hybridization was performed essentially as described by Cox and Goldberg [6]. Radioactively labeled (^{35}S-UTP) antisense and sense RNA probes were transcribed from the p8P1.7 plasmid (described above) using either the T3 (antisense) or T7 (sense) promoters. Hybridiza-

tion was performed for 16 h under 50 °C in a high-humidity chamber. After hybridization, the sections were washed at high stringency with wash buffer (1 × salts (0.3 M NaCl, 10 mM Tris-HCl pH 6.8, 10 mM sodium phosphate pH 6.8, 5 mM EDTA), 50% (v/v) formamide, 10 mM dithiothreitol) at 37 °C. The slides were coated with Kodak NTB2 emulsion and incubated for four to seven days at 4 °C, developed in Kodak D19, and fixed in Kodak Rapid Fixer. Sections were then stained with 0.05% (v/v) Toluidine Blue-O and photographed under a Zeiss Axiophot microscope.

Results

Deletion analysis of DC8 promoter

To identify the regulatory elements of the DC8 promoter, promoter deletions either from the 5′ end or from internal sequences were generated from the 2600 bp DC8 promoter. These promoter deletion constructs (Fig. 1) were fused to a promoter-less glucuronidase (GUS) gene and introduced into carrot plants via *Agrobacterium* transformation [14]. The GUS activities of the various constructs were assayed in developing seeds, in plants, and in somatic embryos initiated from transgenic carrots.

The data in Table 1 show that a 305 bp region upstream of the DC8 transcription initiation site was sufficient for DC8 promoter activity in transgenic carrots. In addition, this region was able to confer the correct developmentally regulated DC8 expression, as somatic and zygotic embryos of the transgenic carrots were GUS-positive, but leaf tissue was not. Further 5′-end deletions of the promoter containing 170 bp or 51 bp sequences upstream of the transcription start site resulted in the complete loss of GUS activity in embryos (Fig. 1). An internal deletion removing base pairs − 505 to − 170 from the DC8(2600):GUS construct produced GUS activity in the somatic and zygotic embryos, while an internal deletion removing the base pairs from − 505 to − 51 prevented DC8:GUS expression completely.

Thus the proximal promoter region consists of two regions necessary for transcription: the − 1 to − 170 and − 170 to − 305 bp regions. The presense of DC8:GUS activity in the − 505 to − 170 internal deletion construct shows that the − 170 to − 305 region contains a redundant element that can be substituted by some element in the upstream sequence between − 505 and − 2600 bp (Table 1). Since the upstream promoter sequence is sufficient to confer seed-specific DC8 expression, GUS activity in DC8(2600):GUS, DC8(1530):GUS or DC8(505):GUS transgenic

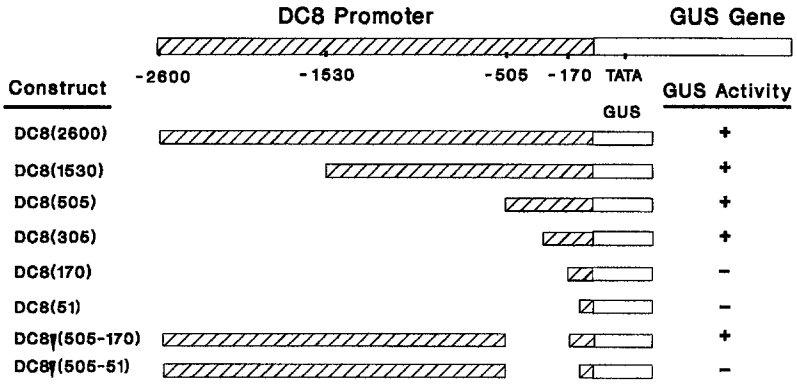

Fig. 1. DC8:GUS promoter deletion constructs. DC8 promoter *Bal*31 deletion fragments replaced a *Hin*dIII-*Bam*HI fragment containing the CaMV 35S promoter in a subclone of plasmid pBI121 (Clonetech). The 3′ end of the DC8 promoter fragments all end at base pair + 55 [14]. Shown in a scale representation, in bp, of the structure of the deletion constructs used in this research. GUS expression by the plants is indicated with a (+) or (−).

Table 1. DC8:GUS expression in carrot embryo, endosperm, and leaf.

Plants	Gene copy[a] number	Somatic embryo	Zygotic embryo	Endosperm	Leaf
DC8(2600):GUS#2-5A[b]	2	+[c]	+	+	−
DC8(2600):GUS#4D	1	+	+	+	−
DC8(2600):GUS#4B	1	+	+	+	−
DC8(2600):GUS#4C	1	+	+	+	−
DC8(1530):GUS#6	5–10	+	+	+	−
DC8(505):GUS#7B	1	+	+	+	−
DC8(505):GUS#44E	2	+	+	+	−
DC8(305):GUS#105	1	+	+	+	−
DC8(305):GUS#106	3	+	+	+	−
DC8(170):GUS#101	1	−	−	−	−
DC8(170):GUS#104	1	−	−	−	−
DC8(51):GUS#C1	5–8	−	−	−	−
DC8(51):GUS#21B	1	−	−	−	−
DC8(505-170):GUS#2[d]	8	+	+	+	−
DC8(505-170):GUS#4	4–6	+	+	+	−
DC8(505-51):GUS#103	1	−	−	−	−
Non-transgenic carrot	0	−	−	−	−

[a] Copy number determined by DNA hybridization analysis. [b] # indicates transgenic line number, the number in parenthesis indicates the maximum 5′-end sequence of the DC8 promoter. [c] '+' indicates that >20% of the 10 embryos, endosperms and leaves tested had detectable GUS activity. [d] Internal deletions: the number in parenthesis indicates the sequence missing from the DC8(2600) promoter.

plants was used to study temporal and spatial DC8 expression in the following experiments.

Temporal expression of DC8 during seed development

In carrot and many other plants, endosperm develops more rapidly than the embryo [15]. After double fertilization, the triploid endosperm nucleus divides quickly. By 5–6 days after pollination (DAP) numerous endosperm nuclei appear in the upper part of the embryo sac. The coenocytic phase of the endosperm lasts until 7 DAP. Cellularization of the endosperm begins along the periphery of the embryo sac. By 35 DAP the maximum endosperm cell number, cell volume and seed dry weight is reached [15]. In contrast, embryo development begins about 7 DAP when the carrot zygote divides, forming an apical and a basal cell (Fig. 2A). Cell division in the embryo takes place more slowly, forming the globular stage in about 14–16 DAP (Fig. 2C), heart stage in about 22 DAP (Fig. 2E), and torpedo stage in 30–35 DAP (Fig. 2F). The embryo reaches maturity after 50 DAP.

DC8 promoter activity was examined in developing carrot embryo and endosperm. DC8:GUS activity was undetectable in the two-cell embryo. Embryonic expression was first detected in the multi-cellular proembryo 10–12 DAP (Fig. 2I). DC8:GUS activity was detected at the coenocytic phase of the endosperm by 7 DAP (Fig. 2N). Once activated, DC8:GUS expression remained high throughout seed development in both the endosperm and the embryo (Figs. 2J, K, L, and M). The earlier time of DC8 activity in endosperm corresponds with the earlier onset of endosperm growth.

To confirm the DC8:GUS expression pattern, temporal and spatial expression of DC8 mRNA in developing carrot seeds was analyzed by *in situ* hybridization. DC8 mRNA was not detected in developing carrot seeds prior to 10 DAP. However, by 12 DAP both the endosperm and multicellular proembryo tissues showed high DC8

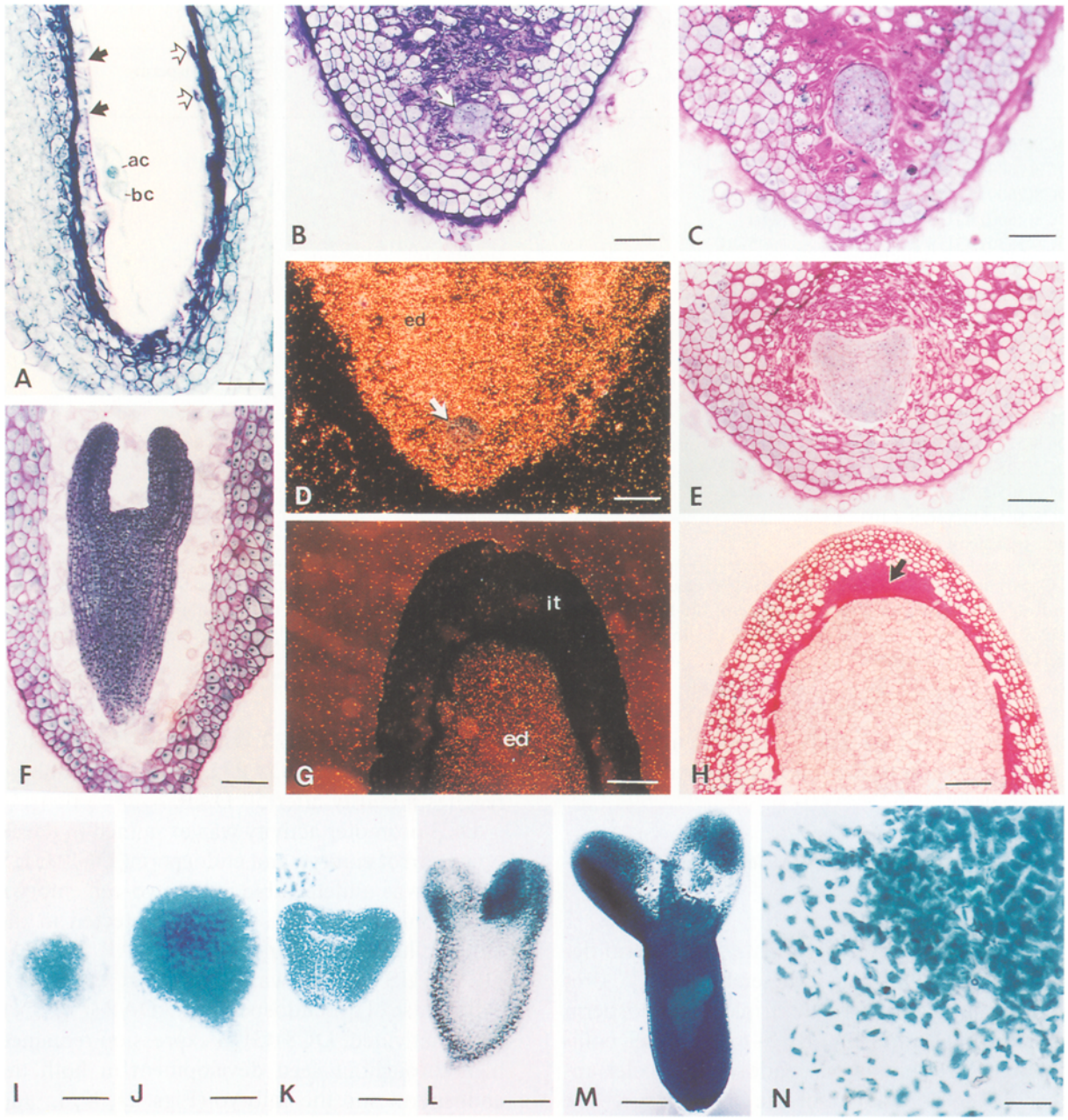

Fig. 2. Histochemical localization of GUS activity and DC8 mRNA in carrot embryos and endosperm. A–H. Longitudinal sections of developing carrot seeds. A. An ovule 7 DAP, showing the zygote divided transversely, forming an apical cell (ac) and a basal cell (bc). Open arrows point to endosperm nuclei at the periphery of the embryo sac, filled arrows point to the cellularized endosperm cells. Bar = 50 μm. B. A 12 DAP multicellular proembryo (indicated by an arrow) surrounded by the endosperm tissue. Bar = 50 μm. C. A 16 DAP late globular stage embryo surrounded by endosperm tissue. Bar = 45 μm. D. *In situ* localization of DC8 RNA. Dark-field micrograph of a 12 DAP multicellular proembryo (indicated by arrow) showing the silver grains distributed in both embryo and endosperm. Bar = 50 μm. E. A heart stage embryo, 22 DAP. Bar = 50μm. F. A torpedo stage embryo, 35 DAP. Bar = 100 μm. G. *in situ* localization of DC8 RNA. Dark-field micrograph of a 10 DAP seed section close to the chalazal end, showing silver grains in the endosperm (ed) but not the integument (it). Bart = 100 μm. H. A 10 DAP seed section close to the chalazal end. Portions of the inner integument cells were decomposed (indicated by arrow). Bar = 100 μm. I–N. DC8:GUS expression in carrot embryos and endosperm dissected from developing seeds. I. GUS-positive proembryo, 12 DAP.

mRNA expression (Figs. 2B and D). We observed that DC8 RNA accumulated throughout seed maturation, the central cavity of the embryo sac contained a large amount of DC8 mRNA and that there was no DC8 expression in integument (Figs. 2G and H). In general, mRNA expression patterns were similar to those of DC8:GUS. The ability to detect DC8:GUS expression, but not DC8 mRNA in 7-day old endosperm, probably results from the accumulation of a stable GUS protein as well as differences in assay sensitivity.

Temporal expression of DC8 during callus induction

As reported in Goupil *et al.* [14] all stages of DC8:GUS transgenic carrot somatic embryos and established carrot callus tissues are GUS-positive. To determine when the DC8 gene is activated upon callus induction from GUS-negative tissues, we analyzed GUS activity during callus induction from petiole explants of DC8:GUS transgenic carrots.

To avoid position effect artifacts, we analyzed five independently generated GUS-positive DC8(2600) and DC8(1530) transgenic plants, GUS-negative DC8(51) transgenic plants, and non-transgenic plants were used as controls. The DC8(51):GUS deletion construct has all of the regulatory sequences including the TATA sequence deleted. Petiole explants from the eight carrot lines were cultured on 2,4-D-containing medium. Callus appeared from the corner of the petiole explants in four to five days. GUS activity was assayed 5, 7, 10, 12, 14, and 17 days after culture GUS activity appeared on some parts of the DC8(2600) and DC8(1530) callus after 10 days on culture medium (Fig. 3B) and intensified as the callus grew larger (Figs. 3C and 4). DC8(51):GUS transgenic callus was consistently GUS-negative. No GUS activity was detected in callus initiated from non-transgenic carrots (Fig. 3A).

Expression of DC8 activity in seedlings

To determine when DC8 gene activity terminates, we investigated DC8 gene expression in seedlings using DC8:GUS-expressing transgenic lines. Young carrot seedlings contained detectable levels of GUS activity for 7–12 days after imbibition. But GUS activity declined over time and was no longer detectable in 14-day seedlings (Table 2). GUS activity was not uniformly distributed over the seedlings, declining earlier in the elongating root and hypocotyl cells than in cotyledons where cell enlargement occurs more slowly (Figs. 3D and E). Since seedling growth begins with the elongation of embryo cells in the root and hypocotyl, our results suggest that the seedling GUS activity results from the remaining DC8:GUS activity in the embryo cells and that little new DC8:GUS is expressed after germination. While the shoot apex of the DC8:GUS seedlings were occasionally GUS-positive, GUS activity was never detected in the true leaves which formed after germination (Tables 1 and 3). This is consistent with the fact that the shoot apical cells were formed in the seed, but true leaf cells were not.

To determine whether DC8 gene activity is ABA-inducible after germination, the abundance of DC8 mRNA in carrot seedlings grown in the presence or absence of ABA was measured. Since germination following imbibition is not synchronous, seedlings of comparable size were selected and incubated in ABA for either one or two days, or in water control for two days (see Materials and methods). DC8 mRNA was readily detected in the young seedlings (2–6 days old) within 24 h after ABA treatment, but could not be induced in older seedlings (7–10, or 14–20 days old, Fig. 5). Similarly, DC8 mRNA could not be induced in mature leaves by ABA treatment [18]. The loss of ABA inducibility in older seedlings could result from DC8 inactivation due to either loss or the dilution of necessary seed-specific factors. If new

Bar = 30 μm. J. GUS-positive globular stage embryo. Bar = 25 μm. K. GUS-positive heart stage embryo. Bar = 45 μm. L. GUS-positive mid-torpedo stage embryo. Bar = 120 μm. M. GUS-positive late-torpedo stage embryo. Bar = 150 μm. N. GUS-positive endosperm, squashed from an ovule, 7 DAP. Bar = 24 μm.

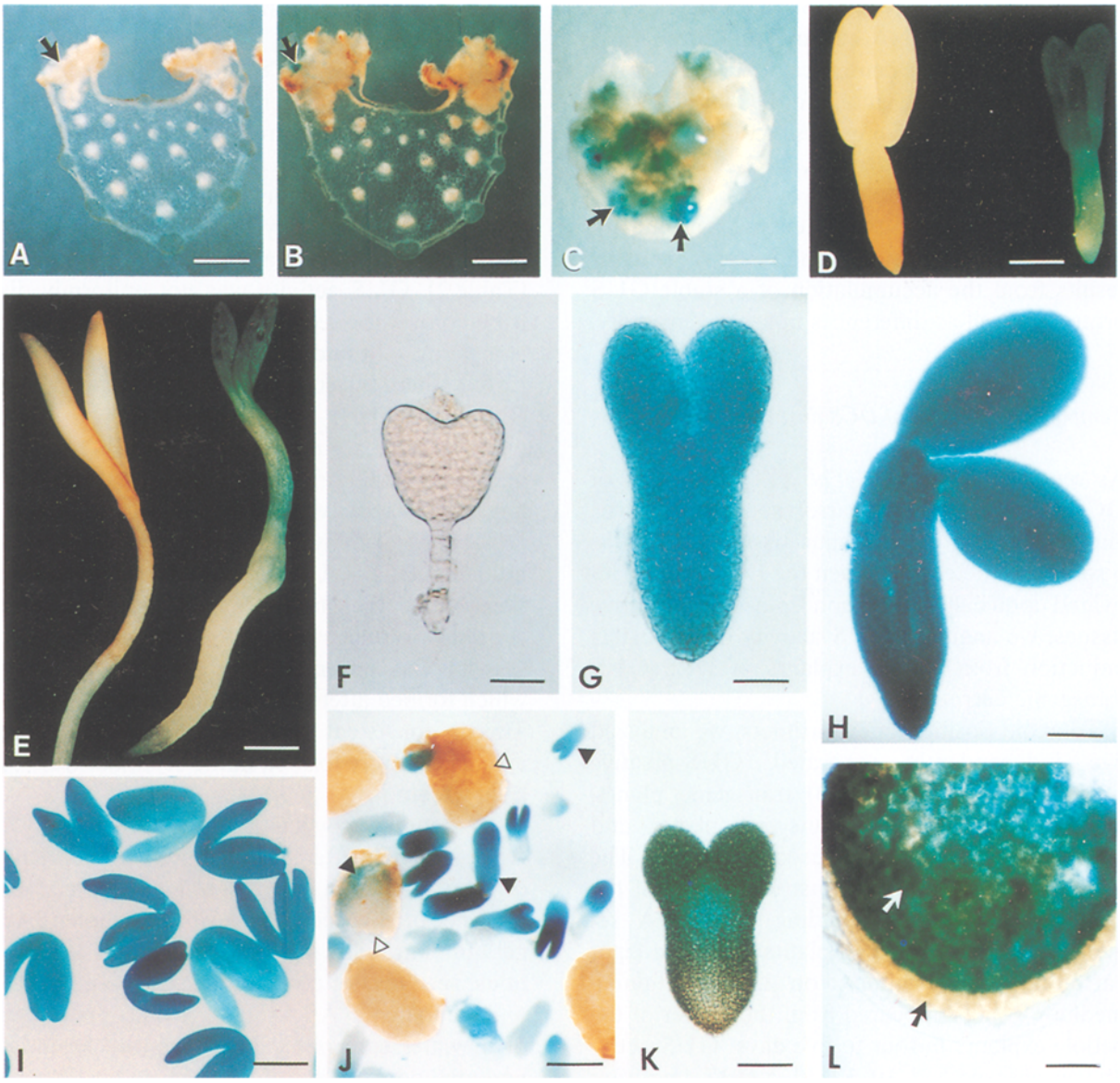

Fig. 3. Histochemical localization of GUS activity in carrot, tobacco, and *Arabidopsis*. A–E. Carrot petiole explants and seedlings. A. Petiole explant from a non-transgenic plant, 10 days after culture. The arrow points to callus produced from the corner of petiole segment, which is GUS-negative. Bar = 670 μm. B. Petiole explant from a DC8:GUS transgenic plant, 10 days after culture, showing DC8:GUS activity in callus cells. The arrow points to the GUS-positive tissue. Bar = 680 μm. C. Petiole explant from a DC8:GUS transgenic plant 17 days after culture. Arrows point to the callus tissue which has stronger GUS activity than 10-day-old callus. Bar = 670 μm. D. DC8:GUS transgenic carrot seedling, two days-after-germination (right) and a 2-day-old GUS-negative, non-transgenic seedling (left). Bar = 270 μm. E. DC8:GUS transgenic carrot seedling, four days after germination (right), showing GUS activity in root tip, hypocotyl and cotyledons. A 4-day-old GUS-negative, non-transgenic seedling (left). Bar = 750 μm. F–I. DC8:GUS expression in embryos of transgenic *Arabidopsis*. F. GUS-negative heart stage embryo. Bar = 25 μm. G. GUS-positive early torpedo stage embryo. Bar = 50 μm. H. GUS-positive late torpedo stage embryo. Bar = 50 μm. I. GUS-positive mature embryos. Bar = 500 μm. J. DC8:GUS transgenic tobacco embryos and seeds. Filled arrowheads point to GUS-positive embryos and open arrowheads point to the GUS-negative seed coats. Bar = 400 μm. K. GUS-positive early torpedo-stage embryo of transgenic tobacco. Bar = 90 μm. L. Portion of a 10 DAP *Arabidopsis* seed, the white arrow points to the GUS-positive endosperm tissue and the black arrow points to the GUS-negative seed coat. Bar = 40 μm.

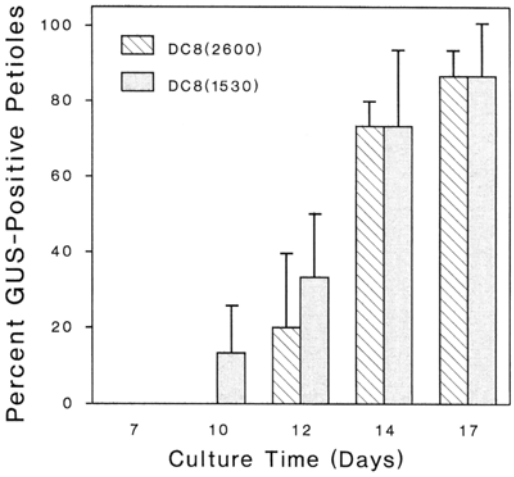

Fig. 4. DC8:GUS expression during callus induction from carrot petiole explants. DC8:GUS expression is presented as the percentage of GUS-positive petioles from three independant transgenic plants. Five petiole segments for each plant were assayed. DC8(51) and untransformed plants had no detectable GUS activity. Error bars indicate standard error.

Table 2. DC8:GUS expression after seed germination in carrot seedlings.

Plants	Days after germination						
	0	1	2	4	7	12	14
DC8(2600):GUS	+[a]	+	+	+	+	–	–
DC8(1530):GUS	+	+	+	+	+	+	–
DC8(51):GUS	–	–	–	–	–	–	–
Non-transgenic	–	–	–	–	–	–	–

[a] '+' indicates that >20% of the 5–10 embryos and seedlings tested had detectable GUS activity.

cells formed from the shoot apex after germination do not contain the seed-specific factors required for ABA induction, the cell will lose the ability to respond to ABA.

DC8 promoter can be activated in tobacco and Arabidopsis *seeds*

To test whether the DC8 promoter could be activated and regulated in other plant species, DC8:GUS constructs were introduced into tobacco and *Arabidopsis* via *Agrobacterium* transformation. DC8 promoter was activated in both tobacco and *Arabidopsis* seeds (Table 3). The lack of DC8:GUS activity in both *Arabidopsis* and tobacco leaves, even after leaves were treated with 10^{-5} M ABA for 48 h (Table 3), showed that ABA alone cannot activate DC8. Factors present in tobacco and *Arabidopsis* seeds are required for DC8 activation.

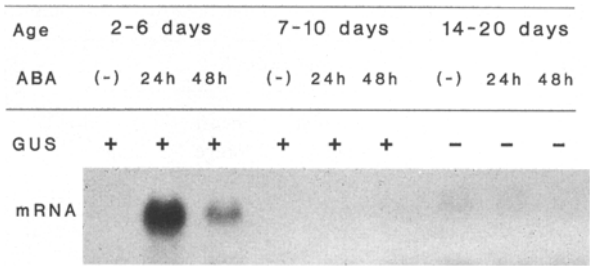

Fig. 5. Comparison of DC8:GUS activity and DC8 mRNA abundance in young carrot seedlings in the presence or absence of exogenous ABA. The '+' indicates that >20% of the seedlings (at least one in five) had detectable GUS activity. Below, is a representative RNA blot hybridized to a DC8 antisense RNA probe and exposed for 12 h. Faint smears in the 14–20 day samples were not the correct size for DC8 RNA and appear only in leaf tissues (data not shown). Equivalent loads of 5 μg total RNA were confirmed by ethidium bromide staining.

Temporal pattern of DC8:GUS expression in tobacco and Arabidopsis

During embryogenesis

Temporal expression of DC8:GUS activity was analyzed during embryogenesis of *Arabidopsis* and tobacco. *Arabidopsis* embryos develop rapidly reaching globular stage by 4 DAP and the torpedo stage by 6–7 DAP. Tobacco embryos are still at the two-cell stage at 4 DAP and require 7 days to reach globular stage. Both *Arabidopsis* and tobacco embryos develop faster than carrot embryos (Figs. 2 and 6). DC8:GUS activity was not detectable in early embryogenesis, globular or heart stages, in either *Arabidopsis* or tobacco (Fig. 3F). GUS activity was first detected in *Arabidopsis* and tobacco embryos at the torpedo stage and remained detectable for the duration of de-

Table 3. DC8:GUS expression in carrot, *Arabidopsis*, and tobacco.

Plants	Zygotic embryo			Endo-sperm	Callus	Leaf	Leaf[a] (ABA)	Seedling (DAG)[b]		
	globular stage	heart stage	torpedo stage					2	4	7
Carrot DC8(2600):GUS	+[c]	+	+	+	+	−	−	+	+	+
Arabidopsis DC8(505):GUS	−	−	+	+	−	−	−	+	+	+
Tobacco DC8(2600):GUS	−	−	+	+	−	−	−	+	+	−

[a] Leaves were treated with 10^{-5} M ABA for 48 h before GUS assay.
[b] Days after germination.
[c] '+' indicates that >20% of the 5–10 embryos and seedlings tested had detectable GUS activity.

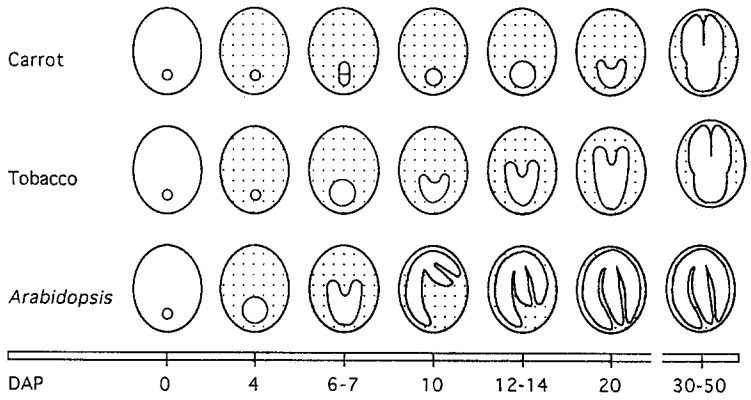

Fig. 6. Temporal expression of DC8:GUS in developing seeds of carrot, tobacco and *Arabidopsis*. Diagrammatic representation of embryo and endosperm development and DC8 activity in the developing seeds of the three species. The shaded area represents DC8:GUS expression and small dots represent endosperm tissues. Seeds are not drawn to scale.

velopment and maturation (Figs. 3G to K; Fig. 6). Thus, the DC8 promoter was activated later in tobacco and *Arabidopsis* than in carrot, which expressed DC8 in the multi-cellular proembryo stage (Figs. 2I and 6).

During endosperm development

Arabidopsis endosperm is 'consumed' and replaced as the food storage organ as cotyledons grow in size [26]. Nevertheless, it is possible to detect some endosperm tissue in the *Arabidopsis* seed, especially prior to seed maturation. The endosperm in *Arabidopsis* and tobacco DC8:GUS transgenic seeds was GUS-possitive during the early torpedo stage of embryogenesis (Figs. 3J and L). In both species, DC8:GUS activity appeared at about the same time in embryo and endosperm tissues (Fig. 3J). This is in contrast to carrot where DC8 is active in endosperm before embryogenesis begins and becomes active in the embryo at the multicellular proembryo stage. In terms of time from pollination, DC8:GUS activity appears in carrot and *Arabidopsis* 6–7 DAP and in tobacco seeds 12–14 DAP (Fig. 6).

During seeding growth

Seedlings of transgenic *Arabidopsis* were GUS-positive until 7 days after germination, while

transgenic tobacco seedlings maintained GUS activity for the first 4 days (Table 3). Older seedlings and newly emerged and mature leaves were all GUS-negative. Addition of ABA did not induce GUS activity in GUS-negative tissues (Table 3). As with carrots, DC8:GUS activity appears to be limited to the cells formed in the seed, namely, the endosperm and embryo cells. There was no GUS activity in the seed coat which arises from the sporophytic tissues (Figs. 3J and L). Cells formed after germination were not able to activate DC8:GUS expression.

During callus growth

DC8:GUS activity in callus tissue initiated from the transgenic *Arabidopsis* and tobacco plants was investigated. Unlike carrot callus, DC8:GUS tobacco and *Arabidopsis* callus tissues were GUS-negative (Table 3). Tobacco and *Arabidopsis* callus initiated from leaf or root explants usually undergoes shoot or root organogenesis [31, 42], while carrot callus is embryogenic. Thus, lack of DC8 expression is consistent with the non-embryogenic nature of the tobacco and *Arabidopsis* callus.

Overall, the regulatory mechanism for DC8 type LEA gene expression is highly conserved. DC8 gene expression is closely associated with seed development and somatic embryogenesis.

Discussion

Promoter analysis

Transient expression studies have identified the ABA response element (ABRE), ACGT, necessary for the ABA-induced expression of the wheat and rice LEA genes, Em and Rab [16, 28, 30]. But the ABRE sequences are neither unique for sufficient for the activation of LEA gene transcription [14, 36, 41]. Recently, Shen and Ho [38] found a 'coupling element' (CACC) downstream from the ABREs which is necessary for the ABA-induced transcription of barley HVA22 gene. Many LEA genes, including Em, RAB, carrot oelosin, and DC8, contain both elements. However, transient expression analysis of the DC8 promoter in protoplasts of embryogenic cells showed that, the region containing the three ABREs and a CACC coupling element (− 170 to − 51) is insufficient to confer ABA-induced DC8 activity. Either an additional CACC element or another sequence element located within the − 170 to − 505, or − 505 to − 2600 sequences are required for ABA-inducible DC8 transcription [9, 14].

Despite the presence of a collection of ABREs and coupling elements in the carrot DC8 promoter, soon after germination DC8 is no longer inducible by ABA. In the present investigation, we used transgenic carrots to show that one of the two regions upstream from the ABREs is necessary for both ABA-induced and seed-specific regulation of DC8 transcription. Expression analysis of the − 305 to − 51 promoter deletion construct narrowed the site of a redundant element to the region between − 305 and − 170 in the proximal region of the promoter. DC8 promoter sequences containing ABREs and the coupling elements are adequate to confer ABA-inducible expression in seeds, not in plants after germination. This represents a difference in DC8 regulation in comparison with some of the other LEA genes, such as HVA22 and RAB16, and suggests that, in addition to ABREs and the coupling element, other *cis*-elements and factors are required to confer seed-specific DC8 transcription.

Seed-specific DC8 expression

We have previously hypothesized that DC8 promoter activity depends on seed-specific factor(s) that are present only in cells formed during seed development, not cells produced after germination [14]. Embryo-derived cells undergoing elongation following germination may still contain some seed-specific factors, allowing ABA-induced DC8 expression for up to 7 days. Leaf or root cells formed after germination would not contain the seed-specific factors necessary to activate DC8 in response to ABA. Paiva and Kriz's [32] observation of the expression of the maize

globulin gene after germination may also be explained by the action of a residual seed specific factor.

Expression of the rice RAB gene is found in young (the first few) rice leaves [30] but not in young transgenic tobacco leaves [45]. The differences in ABA-inducibility of the same LEA gene in young dicotyledon versus monocotyledon leaves may result from differences in the extent of leaf development in the seeds. While most dicotyledonous plants do not produce leaves until after germination, monocotyledonous plants produce 5 or 6 leaves before seed maturation and desiccation. Leaves formed in the seed must undergo desiccation along with the rest of the embryo, and thus may contain the seed-specific factors that would cause ABA-induced LEA gene transcription after germination. Following this line of reasoning we would expect that a monocotyledonous LEA gene, such as the rice RAB gene, when inserted into a dicotyledonous plant such as tobacco would not be ABA-inducible in young tobacco leaves since they are not formed in the seed.

The mechanism of DC8 activation

During meiosis it is thought that the cell erases the developmental imprints from the genome and returns genome activity to a ground (embryonic) state. After this genome reset, the megaspore divides to produce the egg and polar nuclei, which undergo mitotic arrest until fertilization. Under this scenario, seed development is triggered by double fertilization. Fertilization may initiate a cascade of events including embryo development, ABA synthesis, and the expression of seed-specific factors. Activation of DC8 and other LEA and seed storage genes by 7–10 DAP would be later events in this cascade.

It is generally thought that the epigenetic state of the carrot genome in leaf or petiole cells is reset to the embryonic or ground state at the time of or shortly after petiole explanation and 2,4-D treatment as the proliferating cells undergo cellular dedifferentiation [40]. The amount of time required for DC8 activation in callus (10 days after explanation) is comparable to that in developing seeds (7–10 DAP), arguing that explanation and treatment with 2,4-D triggers a course of events in cultured carrot cells similar to that in the carrot ovule. Carrot callus resembles endosperm in both the timing of LEA gene activation and the lack of distinct structures. In the absence of specific molecular or tissue hallmarks it would be difficult to determine whether certain callus cells are indeed functionally equivalent to endosperm cells, and thus, capable of expressing DC8.

Differential DC8 expression in carrot, tobacco, and Arabidopsis

For the most part, the ABA-regulated seed-specific DC8 expression is conserved among carrot, tobacco, and *Arabidopsis*. However, DC8 expression in tobacco and *Arabidopsis* seeds differs from that in carrot seeds in two aspects: first, DC8 expression in endosperm does not precede embryonic expression, and second, DC8 expression in embryo tissues is delayed until the early-torpedo stage. Differences in temporal DC8 expression may result from differences in the time of cell division initiation and in growth rates for the embryo and endosperm tissues in different seeds. In carrots, the zygotic division occurs seven days after the onset of endosperm nuclear division, and carrot embryos grow slowly. In *Arabidopsis* and tobacco, cell division is activated at about the same time in embryo and endosperm and growth rates appear to be comparable [23, 26]. This is consistent with the observation that DC8 activity appears at the same time in the tobacco and *Arabidopsis* embryo and endosperm, but occurs earlier in the carrot endosperm than the carrot embryo. The delayed DC8 expression in tobacco seeds as compared with carrot and *Arabidopsis* seeds may result from a slower rate of tobacco seed development. The endospermatous carrot seeds quickly accumulate a great many endosperm cells. This fact may explain the earlier DC8 expression in developing carrot endosperm than in tobacco endosperm.

The appearance of DC8 expression in different developmental stages for carrot, *Arabidopsis* and tobacco embryos argues that DC8-type LEA gene expression is not coupled to the embryo development program. This is supported by the fact that DC8 is also expressed in endosperm and callus tissue. Rather, a species-specific seed development process affects the timing of ABA/seed-factor availability, resulting in differences in the time of DC8 activation.

The nature of the seed-specific factor

If DC8 expression is dependent on seed-specific factors, what is the seed factor and when is it activated? Little is known about the function of DC8 transcription factors even though DNA promoter sequence-binding factors have been identified [19]. The presence of variable expression patterns for different LEA genes suggest that, in addition to ABA, multiple regulatory factors, for example, *opaque2, VP1, ABI3, FUS3* [1, 29, 33, 35] control the expression of different LEA genes. Maize Em and oleosin gene expression is dependent on *VP1* gene activity. The *FUS3* gene is required for normal seed desiccation tolerance and vivipary. However, DC8:GUS expression in *Arabidopsis* seeds was shown to be independent of *FUS3* gene activity [1].

The *Arabidopsis ABI3* gene is comparable to the monocot *VP1* gene in sequence and function; it is expressed in *Arabidopsis* seeds and could be a seed-specific factor that regulates DC8 expression. *ABI3* activation in the heart-stage embryo prior to DC8:GUS expression in the torpedo-stage is consistent with the notion that regulatory factors are expressed prior to the target genes, such as DC8. Parcy *et al.* [33] demonstrated that ectopic *ABI3* expression activates LEA gene expression in adult tissues. Understanding the relationship between *ABI3* and DC8 and determining the mechanism of *ABI3* activation might elucidate the developmental mechanism of DC8 expression.

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