Molecular Analysis of a Phylogenetically Conserved Carrot Gene: Developmental and Environmental Regulation

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ABSTRACT Extensive studies of gene expression programs in carrot somatic embryos identified a gene, designated *Dc3*, that serves as a reliable molecular marker for the acquisition of embryogenic potential by carrot cells in culture. The complete sequence of a carrot genomic region, *DcG3*, encoding a *Dc3*-like mRNA, was determined. The *DcG3* transcription unit contains a single intron and encodes mRNA that is expressed at high levels in embryonic tissue but is undetectable in somatic tissue of carrot. The predicted protein sequence of *DcG3* is 163 amino acids and includes two approximately 50 amino acid direct repeats which in turn include additional repetitive elements with an unusual distribution of charged amino acids. *Dc3* and *Dc3*-like mRNAs are encoded by a small divergent gene family. Furthermore, similarities of the *Dc3* gene family with genes from other plant species that are expressed in response to environmental and developmental cues suggest a possible role in seed desiccation and possibly in more general water-stress responses in plants. Analysis of transgenic tobacco containing a β-glucuronidase (GUS) reporter gene fused to a 1.7 kb 5′ upstream element of *DcG3* defined a promoter/enhancer complex that confers developmentally and environmentally regulated expression of GUS activity. Thus, *DcG3* is phylogenetically conserved together with the *trans*-acting factors required for its regulated expression in transgenic tobacco.

Key words: Somatic embryogenesis, gene regulation, transgenic plants, β-glucuronidase

INTRODUCTION

Although plant and animal embryos both undergo very precise and orderly ontogenic programs to yield mature multicellular organisms, there are interesting and significant distinctions between the development of plants and animals [reviewed in Goldberg, 1988; also Walbot, 1985]. For example, plants are immobile and therefore must respond to stress and environmental changes by physiological changes only; also, morphogenesis in plants occurs in the absence of cell movement due the presence of the cell wall. Plants have indeterminate developmental programs, including the lack of a germline. Environmental factors, such as light, water, and temperature among others, play a significant role in plant development. Perhaps most significantly, however, plant cells are totipotent. At least some of the distinctions between plant and animal development are reflected at the level of gene expression control; specifically, the regulatory networks controlling some plant genes appear to be far more plastic than those found in animals.

An important ramification of the totipotency of plant cells is that many plant species can be regenerated from single cells to yield mature and fertile plants. Frequently, this process requires the formation of somatic embryos as an intermediate step. Carrot somatic embryo development is suppressed in suspension cultures maintained at high cell density in media containing the synthetic auxin 2,4-D. Under appropriate conditions, a fraction of the cells contained in these cultures undergo somatic embryogenesis when transferred to auxin-free media, progressing through globular, heart, and torpedo morphogenetic stages that are similar to those observed during zygotic embryogenesis

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of many dictoyledonous species [Steward *et al.*, 1958]. Analysis of gene expression programs in somatic embryos maintained in the absence of 2,4-D and in proliferating cells grown in the presence of 2,4-D revealed relatively few differences in prevalent and moderately prevalent gene products [Wilde *et al.*, 1988; Choi and Sung, 1984; Thomas and Wilde, 1985]. These results suggested that determination of cells destined to produce somatic embryos takes place much earlier than previously assumed and under conditions that inhibit the gross morphological changes associated with somatic embryogenesis.

Although there are qualitatively few differences in gene expression programs of embryogenic and non-embryogenic carrot cultures, some differences are noteworthy. De Vries *et al.* [1988a,b] found that specific extracellular glycoproteins are capable of accelerating, and in some cases rescuing, the embryogenic potential of carrot cells in culture. The expression and appropriate glycosylation of these extracellular proteins is phytohormone regulated. Furthermore, several carrot cDNA sequences with distinct expression patterns were identified [Choi *et al.*, 1987; Wilde *et al.*, 1988]. Two of these are primarily expressed in somatic and zygotic embryos but not in somatic tissues of carrot [Borkird *et al.*, 1988]. Interestingly, these two genes are expressed in plantlets derived from somatic embryos but not in seedlings, suggesting an important effect of dormancy on the transition from embryonic to germination gene expression programs.

Recently, we described a cDNA sequence, *Dc*3, that was derived from mRNA expressed only in carrot cell cultures that contained somatic embryos or that contained proembryogenic masses [Wilde *et al.*, 1988]. Proembryogenic masses (PEMs) are small clusters of cells that are present in cultures maintained in the presence of 2,4-D and which on transfer to media that does not contain 2,4-D undergo somatic embryogenesis at a very high frequency [Halperin, 1966; Nomura and Komamine, 1985]. *Dc*3 transcripts are also present in zygotic embryos but are not present in somatic tissues from carrots maintained under non-stressed conditions. Analysis of *Dc*3 expression during initial phases of induction of embryogenic carrot cultures and in non-embryogenic mutant carrot cell lines indicated that *Dc*3 mRNA expression is a useful molecular marker for the acquisition of embryogenic potential in plant cell cultures [Wilde *et al.*, 1988; de Vries *et al.*, 1988a].

In this paper, we describe the structure and sequence of a gene *Dc*G3 that is a member of a small gene family which encodes *Dc*3 and *Dc*3-like RNA sequences. DNA sequence analysis showed that members of the *Dc*3 gene family are divergent within the species but that elements of the family are phylogenetically conserved in both dicots and monocots [Dure *et al.*, 1989]. Most genes included in this group are characterized by their expression as late embryo-abundant (*lea*) mRNAs [Galau *et al.*, 1986; Harada *et al.*, 1989], and as a group these genes will be referred to as *lea* or *lea*-class genes. Expression of *lea*-class genes in response to environmental cues including water and salt stress and the exposure to exogenous ABA [Chandler *et al.*, 1988; Gomez *et al.*, 1988; Harada *et al.*, 1989; Hong *et al.*, 1988; Marcotte *et al.*, 1988, 1989; Mundy and Chua, 1988] has led to speculation that conserved *lea* peptide domains might function in protecting cellular structures during seed desiccation [Dure *et al.*, 1989]. Analysis of transgenic tobacco containing a β-glucuronidase (GUS) reporter gene fused to a 1.7 kb 5′ upstream element of *Dc*G3 defined a promoter/enhancer complex that confers developmentally regulated expression of GUS activity in developing tobacco seeds and environmentally responsive expression in non-embryonic tobacco tissues. Thus, *Dc*G3 is phylogenetically conserved together with *trans*-acting factors required for its regulated expression in transgenic tobacco.

MATERIALS AND METHODS

Preparation of Recombinant DNAs and Probes

Recombinant plasmids, DNA restriction fragments, and ^{32}P-labeled DNA probes were produced using standard techniques [Maniatis *et al.*, 1982].

Isolation and Analysis of λDcG3-5b Recombinants

A carrot genomic DNA library constructed in the vector Charon 35 [Zimmerman *et al.*, 1985] was screened with ^{32}P-labeled *Dc*3 cDNA probe [Wilde *et al.*, 1988]. Titering of phage, preparation of filters, hybridization of filters, washing, and autoradiography was as described [Maniatis *et al.*, 1982]. Plaques which hybridized to *Dc*3 cDNA were purified to ensure clonal origin. Recombinant phage DNA was mapped and appropriate restriction fragments were subcloned into plasmid vectors. Selected regions of λDcG3-5b were sequenced by subcloning into M13 and using dideoxy sequencing procedures [Sanger *et al.*, 1980] or by direct sequencing of double-stranded plasmid recombinants [Chen and Seeburg, 1985]. Deletions for sequencing were generated by T4 polymerase on M13 templates [Dale *et al.*, 1985] or exonuclease III on double-stranded, plasmid DNA templates [Henikoff, 1984]. Sequences were analyzed using the University of Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Software (Version 6.0) [Devereux *et al.*, 1984].

Analysis of DcG3 Transcription Unit

The transcription start site of *Dc*G3-5b was mapped by primer extension experiments using AMV reverse transcriptase in the following reaction: 5 μg poly(A)$^+$ RNA isolated from carrot torpedo-staged embryo tissue and 10^6 cpm of radioactively end-labeled primer (positions 1688–1708; Fig. 1) were mixed and dried under a vacuum. The pellet was dissolved at 37°C for 10 minutes in 10 μl 60 mM Tris-HCl (pH 8), 600 mM NaCl and

3 mM EDTA, and boiled at 100°C for 3 minutes. Annealing reactions were allowed to cool slowly to room temperature for 1 hour. The following was added, and the reaction was incubated at 37°C for 1 hour: 60 μl 8.6 mM $MgCl_2$; 8.6 mM DTT; 10 μl each of dATP, dCTP, dGTP, and TTP (5 mM stocks); 50 units RNasin, and 20 units AMV reverse transcriptase. The products of these reactions were ethanol precipitated, washed once with 100% ETOH, and dried under vacuum. The pellets were resuspended in water and loading buffer, heated at 68 °C for 2 minutes, and resolved on a 6% polyacrylamide-urea sequencing gel.

Chimeric GUS Constructions and Production of Transgenic Plants

Plasmids pBI101/*Dc*G3-1.7F and pBI101/*Dc*G3-1.7R were constructed by ligation (in both orientations) of a 1.7 kb Hind III DNA fragment derived from *Dc*G3 in the upstream polylinker of pBI101 (Fig. 4A). pBI101.1 has a promoter-less β-glucuronidase/nos-terminator cassette [Jefferson *et al.*, 1987] cloned into the Eco RI site of the polylinker in pBIN 19 [Bevan, 1984]. Plasmids pBI120/*Dc*G3-1.7F and pBI120/*Dc*G3-1.7R were constructed by ligation (in both orientations) of a 1.7 kb Hind III fragment of *Dc*G3 in the downstream polylinker of pBI120 (R.A. Jefferson, personal communication; Fig. 4B). This latter plasmid is a derivative of pBI121 [Jefferson *et al.*, 1987] that contains a truncated (at the EcoRV site) CaMV 35S promoter retaining its CAAT and TATA boxes. In both sets of constructions, the inserted DNA fragment contains sequences between positions 1 and 1727 (Fig. 1) as well as a portion of the pUC18 polylinker including a Hind III site used in the cloning of this fragment. The orientation of the insert with respect to the GUS reporter gene was determined by restriction digestion and sequencing of plasmid DNA using a GUS primer.

These constructs and pBI121 and pBI120 (used as positive and negative controls, respectively) were conjugated into *Agrobacterium tumefaciens* strain LBA 4404 by triparental mating [Bevan, 1984]. Transformation of tobacco leaves was carried out as described [Horsch *et al.*, 1985]. Transformed shoots were selected after rooting in MS medium containing kanamycin sulfate (100 μg/ml), assayed for expression of NPT II [Reiss *et al.*, 1984], and transferred to soil. Transgenic plants were self-pollinated.

Fluorometric and Histochemical β-Glucuronidase (GUS) Assays

Tissue was ground in an Eppendorf tube containing extraction buffer [Jefferson, 1987]. Samples were centrifuged and the supernatants collected in microfuge tubes. Extract protein concentrations were determined by the method of Bradford [1976]. Fluorogenic reactions were carried out for 1 hour at 37°C in 200 μl lysis buffer with 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG). Reactions typically contained 0.10–0.15 mg seed extract protein or 0.4–1.0 mg extract protein of non-embryonic tissues. After stopping reactions with 0.8 ml 0.2 M Na_2CO_3, fluorescence of the 4-methylumbelliferone (4-MU) product was determined in a Hoeffer TKO-100 minifluorometer as described [Jefferson, 1987].

Sections of tobacco seed (40 μm) were prepared from pods harvested 27 days after-flowering (DAF) by embedding tissue in O.C.T. compound (Miles Scientific, Il) then frozen at −25°C. Sections were made using a Cryo-cut II microtome (American Scientific, NY) at −25°C, then incubated with reaction buffer. The reaction buffer consisted of 2.0 mM 5-bromo-4-chloro-3-indolyl glucuronic acid (X-Gluc), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 10 mM EDTA in 0.05 M $NaPO_4$, pH 7.0 [Jefferson, 1987]. Reactions were carried out at 37°C for 2 hours.

RESULTS

The Complete Sequence of a Carrot Gene Encoding *Dc*3 Transcripts

The cDNA sequence *Dc*3 [Wilde *et al.*, 1988] was used as a hybridization probe to recover *Dc*3-like DNA sequences from a carrot genomic DNA library. Based on the frequency of recovering bacteriophage λ recombinants, the number of distinct restriction patterns of recombinant phage hybridizing with *Dc*3 and results of genomic blot analyses, it was determined that *Dc*3 and *Dc*3-like mRNAs are encoded by a small gene family including at least four and probably fewer than ten members [Seffens, 1989] (also see below). From among forty-eight carrot genomic recombinants, λ*Dc*G3-5b was chosen as representative of the *Dc*3 gene family and was analyzed in detail. The complete sequence of the genomic region including the *Dc*G3 transcription unit is shown in Figure 1.

The transcription start site for the *Dc*G3 transcription unit was mapped by primer extension [Seffens, 1989] and is indicated by +1 in Figure 1. A putative TATA box at −32 and a possible CAAT box at −74 consisting of CACAATT is also indicated. In *Dc*G3, a single intron (291 bp) separates a very short exon (57 bp) from a longer exon (432 bp). Intron/exon boundaries were assigned based on open reading frame discontinuities at each junction, on the colinearity of the sequence of the cDNA *Dc*3 and the sequence of *Dc*3G on either side of each intron and on the presence of consensus splice junctions. The intron is bounded by sequences (CAGTACAT. . . . TTGTGTAACAGG) which satisfies the GT/AG rule and conforms to the splice site consensus sequences found for plants [Shapiro and Senapathy, 1987]. Eight out of nine bases at the 5′ site and nine out of eleven bases at the 3′ site are identical to the plant splice site consensus sequences. Exon 2 is terminated beginning at position 2603 by two stop codons followed by a putative polyadenylation signal (AATAA) 70 nucleotides further downstream.

Eco RI
1 GAATTCCAAAAATTGCTCACTCACATAATTTGAGAAGTAAGGAGATCAATTAGAAACACATACGGCGCAT

71 TAAAGATGATTTTCAGGAACGGCTAGTGAATGAAAATGATACGTGTCGCATGGAGAGGCTTCTTAAAACG

141 ACGCGTGCATTTACCAAGATTTCACAACTTTTTTTGGTTTCCGCACCTGCATATAGTACACCCACAAAAT

Pst I
211 GGTTTTTCTCTTAAAGATTCGAATTTTATCTGCCTGCAGTGCTGTACCATATCTGTAGCCATATTTTAGA

281 TTTATCCTGGTGTTTTTCCCACAGCCATGTGACATTGTGTTCGGATTTTTATACCTAAATAAACTATCAA

351 AATGTTACTCTTCAATCCTCTGTCAATAATTTCTTTATAAATATTATATTTAATAAAAACTGCAATATAT

421 GATGCTCATTTTCATTTTCATTATCATAATTATATAATAATATTACTAATTTCTCATTTATGTTATTAAA

491 ATCTCATATTTAAATACATGAAAATTTTAAAAATAATGTGGATCAATGAATAAACATCAAAAGCAAAAAA

Acc I
561 GAAAGAAGTTGGTTGAAAATAGGGGTGACAATCATTTCTTTCGTATACTTTCGTTTCGTGTTTTTCGTGT

631 TCCGTGTATTCGAAGGTGAAACTCGTATCCGTCCGTTATTATTAATTTCGTGTACCTAATTTGAAAGTAT

701 CCGTTTCGAATCGTTTCGTGTATATTTCGTGTACTTTTCGTGTATATTATATATAAAATATTAATATATT

771 TTTTAATCAAAAAATGGATTTAATATATATTTTTCTTTATAAATTTATTAAATGTGAATGATATATATTA

Acc I
841 TACTTGTATACAATTCTTTATAAATTTGTTAATGTATCTACAATATATATCCACATATACGTATAAATAT

911 ATACTTTATACTCAATTATATATTTAATACTCCCTCCGTCCCTCCCATTTCTTTACACTTTCTTTTTTGG

981 ATGTCCCATCCAATTCTTTACATTTCAAAACTTACCAAAAATAGTTAATGGGTCCCACCACTTCTCCACT

1051 TTTCTTAACTTTTCACACTACTTTTACTCCACTATCTTCTTTTTCTACATTAAAAATCAATGGGTCCCAC

1121 CACTTCACCCACTTTTCTTCCTCTTTTCCACTACTTTATACATATTTCTTAACCTCCGTGCCCAACCCAT

1191 TTGATAAGAAATCGGAGGCACGGAGGGAGTATATCATTACACATATATAATAAATATAATTTACAAATAT

1261 TTTATGTACTTTCGTGTATTTCGTGTACCCTAAATGAAAACCCATATCCGACACGAAATCTATCGTGTAC

1331 TTTCGTGTTTCGTGTATCGAAAATCAAAAACCGTATCATTTTTTTCGTGTCGTTTCGTTTCGTATTAGCG

1401 TGTTACGTGTCAAATGGTCAGGTCTAGTTGAAAACGAAACCGTGAGACGTGTATGGCATTGGTCAAACGT

1471 GTTGGAACTTGAAAGCGAGACATCTATATAAATTATAAACTTGACTTGTCATGCGTCACCGTTAGTCTTT

Ava I
1541 ATCCACAGACATCAACCATAAAGTTGTGTCGATTATCCATGCACACCCGAGCTAACCACAACTCGCCACT

-74 -32
1611 TGTCCCTACACGCGTCA**CAAT**TCAGGACACGTGCCCTCATGAGAGAGCCTCCCGGAGCT**TATA**TAAACTC

+1 Hind III
1681 ATGCAGCCCAGCGTCTTTTCCACCAAAACATCTGCAAAGAAAAAGCTTATCGAAACAGAAATATTTTTAT

1751 CGAAGTTTCTCAGCAAAACGTTTTTTAACCATCTCATTGTCTGCTCATACTTTTCAAAATCATCACTTAG

Fig. 1. Sequence of *Dc*G3-5b showing the transcription unit and the predicted amino acid sequence. Relevant restriction sites are shown. CAAT (−74), TATA (−32), and transcription start site (+1) are underlined as are the initial ATG, intron/exon junctions, and the predicted poly(A) addition site. The predicted amino acid sequence is represented below the corresponding nucleotide sequence; dashed line interrupting the amino acid sequence shows the location of a single 291 bp intron. Phylogenetically conserved repetitive amino acid domains are underlined [Dure *et al.*, 1989].

```
1821 CAATGGCATCTCATCAAGATCAGAGCTACAAAGCTGGTGAACCGAAAGGCCATGCTCAGGTACATACATT
       M  A  S  H  Q  D  Q  S  Y  K  A  G  E  P  K  G  H  A  Q <---------

1891 TGTGCATATCACTCATTAATCTTCTATTTTAGTTTGTTTGTTCCTGTTTGAAACTTGGATTTCGTTTGAA
     ----------------------------------------------------------------------

1961 TTTTGGATTTGATCAATAACTGTTTATGATCTGGATTTGGATTTTATCTGAAATCCAAATATTCAAATAT
     ----------------------------------------------------------------------

2031 TAGTATAAACATAAGAATTTGAAATAATTGCTCAAATCGTGTCTTTTGAAATTCATCATTGCAAATGAAA
     ----------------------------------------------------------------------
                Pst I
2101 ATGCACGTCTGCAGACGCCTTTTATGGAATTTGTAGTTGTATGTACTAAGAATGTGTAGTTGTGTAACAG
     --------------------------------------------------------------------->

2171 GAGAAAACAGGACAGATGGCTGATACAATGAAAGACAAGGCCCAAGCTGCCAAGGACAAGGCCTCCGAAA
     E  K  T  G  Q  M  A  D  T  M  K  D  K  A  Q  A  A  K  D  K  A  S  E  M

2241 TGGCTGGATCTGCCAGGGACCGGACAGTTGAGTCCAAGGATCAGACAGGCAGCTATGTTTCGGACAAGGC
       A  G  S  A  R  D  R  T  V  E  S  K  D  Q  T  G  S  Y  V  S  D  K  A

2311 GGGAGCGGTGAAGGACAAGACATGTGAGACGGCTCAGGCGGCCAAGGAGAAGACAGGAGGAGCCATGCAG
      G  A  V  K  D  K  T  C  E  T  A  Q  A  A  K  E  K  T  G  G  A  M  Q

2381 GCCACCAAGGAGAAGGCTTCTGAGATGGGAGAGTCTGCCAAGGAGACTGCTGTGGCAGGGAAAGAGAAGA
     A  T  K  E  K  A  S  E  M  G  E  S  A  K  E  T  A  V  A  G  K  E  K  T

2451 CCGGGGGGCTCATGTCTTCGGCGGCTGAGCAAGTGAAGGGCATGGCTCAGGGGGCTACGGAGGCTGTGAA
       G  G  L  M  S  S  A  A  E  Q  V  K  G  M  A  Q  G  A  T  E  A  V  K
                                                                  Ava I
2521 GAATACTTTTGGTATGGCTGGGGCGGATGAGGAAGAGAAGACTACGACTACTCGGGTCACTAGGTCCTCT
       N  T  F  G  M  A  G  A  D  E  E  E  K  T  T  T  T  R  V  T  R  S  S

2591 GCGAGAACAGAGTAATAATGGAGATGTGATTCGTGGGGGTGCTTGTTTCTTTTGTGTTGTGTTTGGTTGT
     A  R  T  E  *  *

2661 GTACTTGTGTTGTTAAATAATGGGAGTGATTGTTGTTAGATGAGTGTGAGATGTTTAGCTAATTATTTGA

2731 TGTGCTAGATCTCAGACTCGTGGTTTGATTTGAATGCAACACAGTCCTGCAAATTTGATGTTGAAATGAT
                    Sca I
2801 CATGTTGTGTTTTTAGTACTGTGCAATTGAGTTTACTGTTTGTGGATCAACCAGCTCCTTGATCAGCTAC

2871 TTGGATATGTTTTTAGTAATTGGCCAATTGTTCGCTTATCTATTAATGCTACTTCCTTGCAAACACAATA
                                      Hind III
2941 GAGAATTGTTAGTGTGTCCTGATAAGCTT
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Fig. 1b.

The predicted polypeptide from *Dc*G3 (shown below the DNA sequence in Fig. 1) is small (163 residues), uncharged, or slightly basic and has high lysine and alanine content. It has only one cysteine and has no predicted glycosylation sites of the form NxT or NxS. There are no stretches of hydrophobic residues either at the amino terminus or within the sequence; therefore, it is unlikely the *Dc*G3 gene product could be secreted by any route through the endoplasmic reticulum that is currently known [Verner and Schatz, 1988]. Therefore, the *Dc*G3 gene product is predicted to be either a cytoplasmic or a nuclear protein. This conclusion is consistent with the observations of Mundy and Chua [1988] that localized the RAB21 gene product primarily in the cytoplasmic compartment of rice cells.

The predicted *Dc*G3 gene product contains ten unusual charged repeat sequences located primarily in exon 2. These repetitive elements are indicated in Figure 2 and have a consensus sequence of the form: aaKEKa or aaKDKa, where a can be either an A or a T residue. The most conserved feature of these repeats is the charged nature of the constituent amino acid sequence. There exists a basic-acidic-basic (+ − +) charged triplet, followed usually by five uncharged

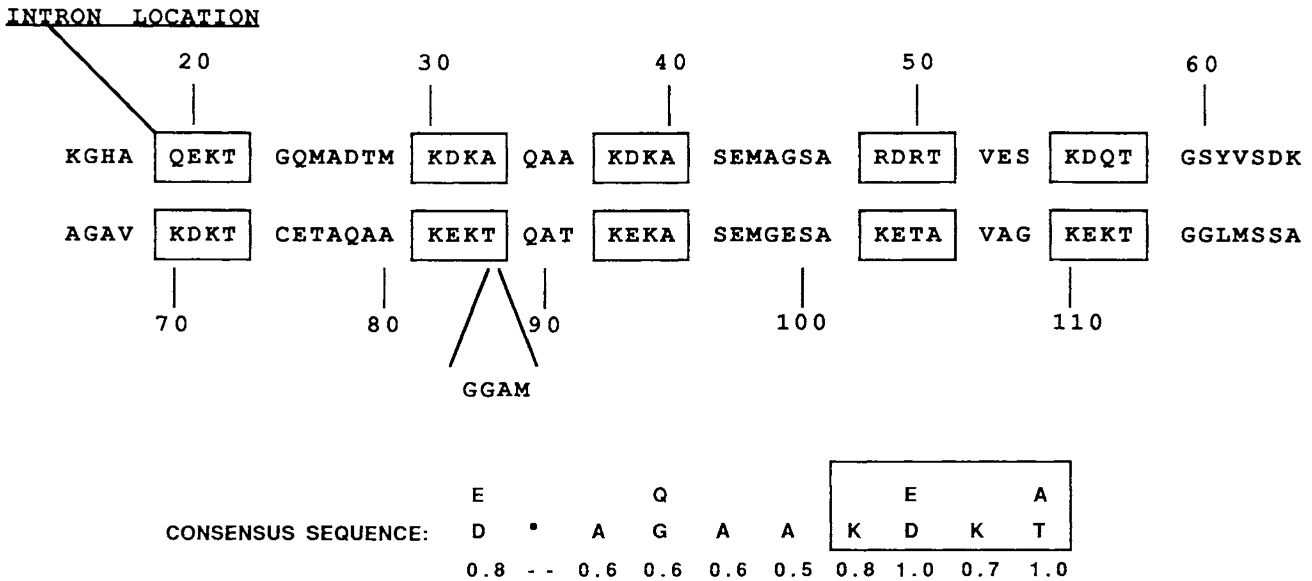

Fig. 2. Repetitive amino acid sequence domains in the predicted amino acid sequence of *Dc*G3-5b. Two approximately 50 amino acid tandem repeats are aligned, and the characteristic tetrapeptide repetitive elements are boxed. The consensus sequence for these repetitive elements is indicated at the bottom of the figure.

amino acids, then an acidic (−) charged residue. The charged triplet is unusual, since it has been noted [Sundaralingam *et al.*, 1987] that ion triplets are very rare for globular proteins. The unique charge structure is repeated ten times within the predicted amino acid sequence of *Dc*G3 in exon 2 (Fig. 2) and is most likely essential for function of the *Dc*G3 gene product. The spacing of the characteristic *Dc*3 repetitive elements is regular, occuring approximately every 11 residues in the sequence representing exon 2. Further examination of the predicted amino acid sequence revealed that the ten short repetitive motifs are contained within two direct tandem repeats that represent most of exon 2 (Fig. 2). The direct repeats are 50 amino acids long, each containing five of these repetitive elements. There is a short insert of four amino acids (GGAM) in the second major repeat.

*Dc*G3 Belongs to a Divergent Gene Family

Previous work indicated that *Dc*3 and *Dc*3-like mRNAs were encoded by a small gene family [Seffens 1989] (also see above). The DNA sequences and the corresponding predicted amino acid sequences of available *Dc*3 gene family members were compared to determine the extent of sequence divergence of this gene family. In Figure 3, the predicted amino acid sequences of *Dc*G3, *Dc*3, and the *Dc*3-like cDNAs, *Dc*14 and *Dc*16, are compared and the differences with the genomic encoded protein are indicated. These results are summarized in Table 1. At the amino acid sequence level, the range of sequence similarity is 84–97%. Interestingly, the amino acid sequences of *Dc*3 and *Dc*14 contain no conservative amino acid changes, while *Dc*16 has a comparable number of both conservative and non-conservative differences. Although *Dc*14 does not include a complete protein coding sequence, the region compared in Figure 3 overlaps with sequences in *Dc*G3, *Dc*3, and *Dc*16 that exhibit maximum divergence within these sequences. From these results, we conclude that the carrot *Dc*3 gene family is divergent with as much as 13% amino acid sequence divergence (Table 1) and 17% DNA sequence divergence (data not shown). It is noteworthy that the consensus tetrapeptide (underlined in Fig. 3) is highly conserved (97%) among all four predicted protein sequences, again suggesting a functional role for this repetitive element.

The *Dc*G3 Gene Family Is Phylogenetically Conserved

Dure *et al.* [1989] observed that the predicted amino acid sequence of *Dc*G3 was shared with a number of dicot and monocot *lea*-like genes which are responsive to environmental and developmental cues. Most genes included in this group are characterized by their expression as late embryo-abundant (*lea*) mRNAs [Galau *et al.*, 1986; Harada *et al.*, 1989]. The expression of *lea*-like genes may be controlled by diverse environmental factors such as salt and water stress [Gomez *et al.*, 1988; Mundy and Chua, 1988] or in response to exogenously applied ABA [Marcotte *et al.*, 1988; Hong *et al.*, 1988]. The peptide domains of this gene class that are shared by *Dc*G3 are indicated in Figure 1 and are included in the repetitive motifs shown in Figure 2.

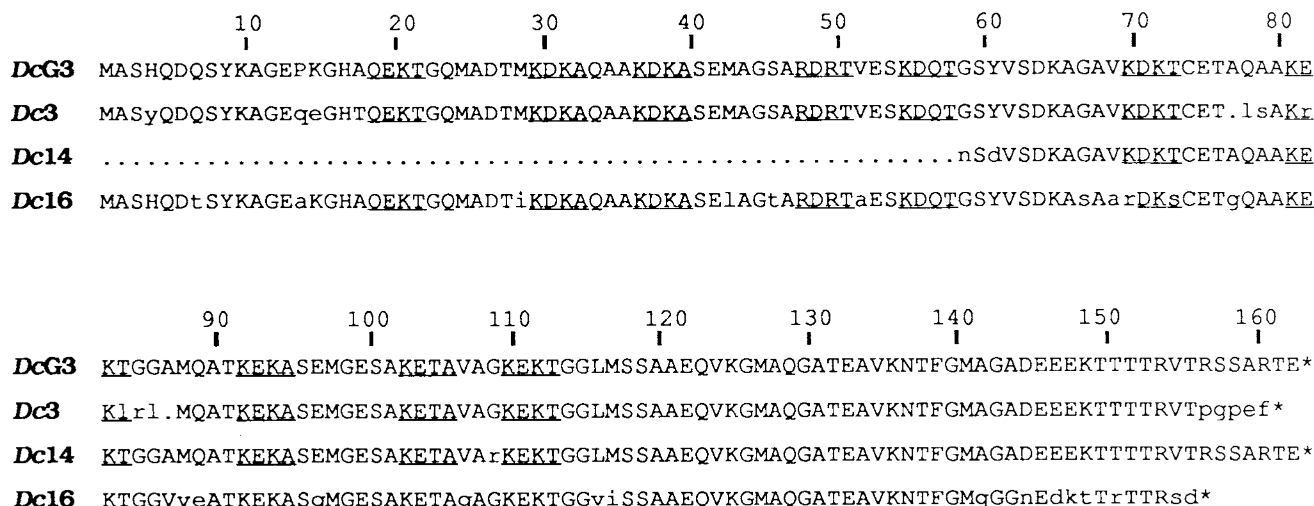

Fig. 3. Comparisons of the predicted amino acid sequences of *Dc*3 gene family members. Predicted amino acid sequences of indicated *Dc*3 gene family members are aligned. Differences between cDNA encoded proteins and the protein encoded by the genomic sequence of *Dc*G3-5b are indicated by lowercase letters. The locations of the conserved tetrapeptide repetitive elements are underlined.

TABLE 1. Sequence Similarities of *Dc*G3 With *Dc*3 and *Dc*3-Like Encoded Proteins*

	*Dc*3	*Dc*14	*Dc*16
% protein sequence similarity[a]	90.7	97.1	84.0
No. conservative amino acid differences	0	0	10
No. non-conservative amino acid differences	15	3	15
Total No. amino acid differences	15	3	25
Total No. of amino acids compared	161	105	156

*Data derived from Figure 3.
[a]In all cases similarities and differences are between the indicated sequence and *Dc*G3.

A *Dc*G3 Promoter Complex Directs Developmental and Environmental Expression of Chimeric β-Glucuronidase Genes in Transgenic Tobacco

To begin investigation of putative *cis*-acting DNA sequences controlling the expression of *Dc*3 genes, a 1.7 kb upstream DNA sequence including positions 1 and 1727 (Fig. 1) was fused in various configurations to a β-glucuronidase (GUS) reporter gene [Jefferson *et al.*, 1987], and these were subsequently transferred to tobacco. In one set of constructions, the 1.7 kb 5′ upstream element was inserted into the promoterless GUS cassette of pBI101 [Jefferson *et al.*, 1987] (see Fig. 4A). Resulting transgenic plants and appropriate control plants were assayed for GUS expression using the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). Embryos and leaves of transgenic tobacco containing pBI101 alone did not exhibit appreciable levels of GUS activity compared to untransformed (wt) tobacco (data not shown). A total of nine transgenic tobacco plants containing pBI101/*Dc*G3-1.7F were assayed for GUS expression (Table 2). Little or no GUS activity was detected in leaves of transgenic plants containing pBI101/*Dc*G3-1.7F or flower parts (data not shown). On the other hand, 1.7 kb upstream element of *Dc*G3 conferred rigorous embryo-specific expression to the GUS reporter gene (Table 2) in the forward orientation; i.e., the putative *Dc*G3 promoter is oriented in the same direction as the GUS transcription unit, but not in the reverse orientation. The magnitude of the enhancement compared to untransformed tobacco and transgenic tobacco containing pBI101/*Dc*G3-1.7R is more than 1,000-fold.

The *Dc*G3 promoter/enhancer complex was further defined by inserting the 1.7 kb upstream region of *Dc*G3 in both orientations downstream of an enhancerless CaMV-35S promoter/β-glucuronidase (GUS) cassette (pBI 120, R.A. Jefferson personal communication). GUS expression was studied in transgenic tobacco plants containing the sequences in plasmids pBI120 (a negative control), pBI120-*Dc*G3-1.7F (forward orientation), or pBI120-*Dc*G3-1.7R (nerve orientation). These constructions are shown in Figure 4B, and the results of fluorometric GUS determinations of either mature tobacco seeds or leaves of transgenic plants are summarized in Table 2. The 1.7 kb *Dc*G3 upstream element enhances expression of GUS driven by the truncated CaMV 35S promoter in embryos of transgenic tobacco containing pBI120-*Dc*G3-1.7F and pBI120-*Dc*G3-1.7R. In one orientation (R) the extent of enhancement is 160-fold and 16-fold in the opposite orientation. GUS expression of pBI120-1.7*Dc*G3-1.7F or pBI120-1.7*Dc*G3-1.7R in leaves is approximately the same as in transgenic tobacco containing pBI120 alone

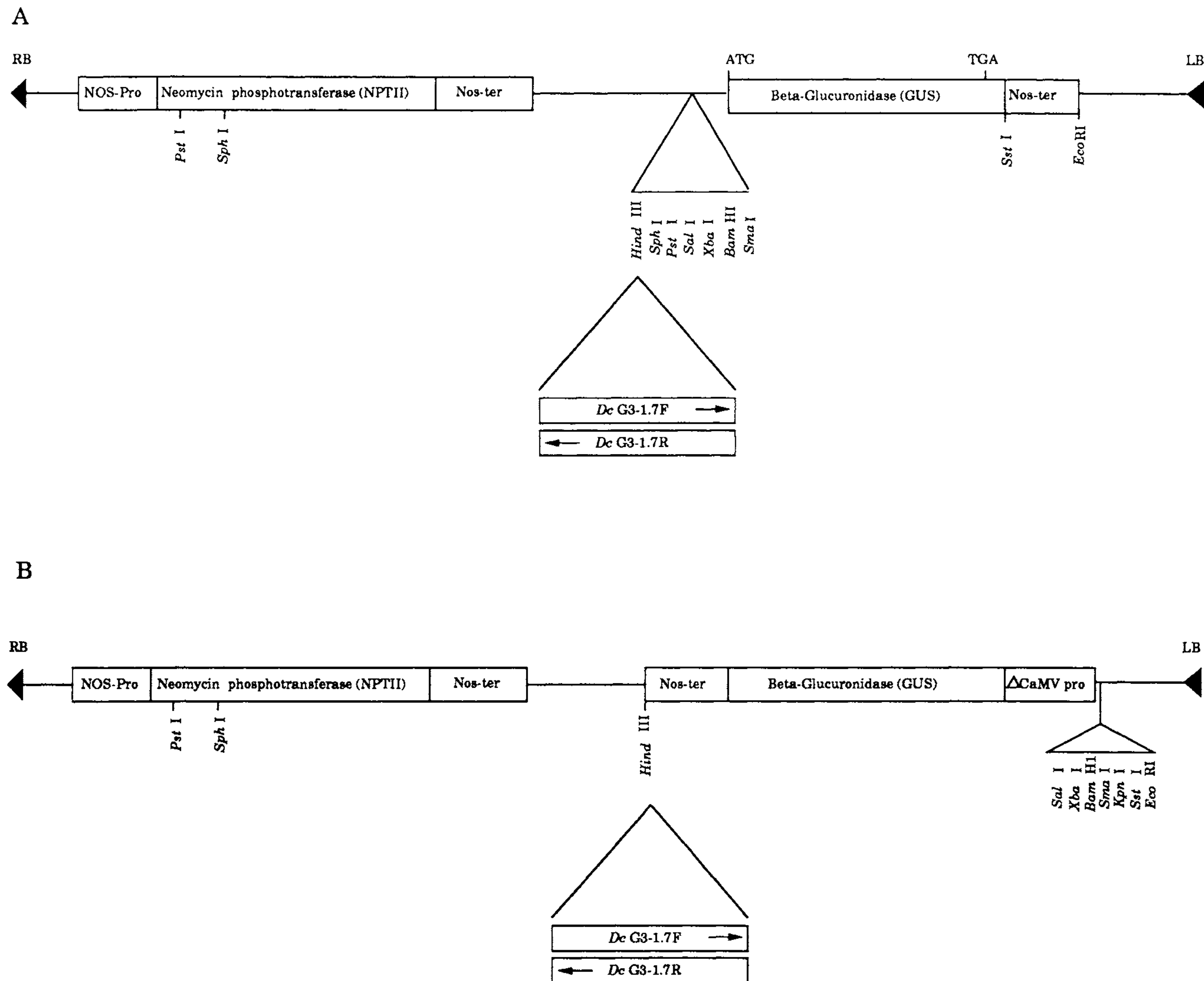

Fig. 4. Chimeric β-glucuronidase reporter gene constructions. **A:** Structure of pBI101 and chimeric GUS constructions pBI101/*Dc*G3-1.7F and pBI101/*Dc*G3-1.7R. Map of pBI101 showing region between right (RB) and left (LB) border sequences. NOS-pro and NOS-ter are the nopaline synthase promoter and poly(A) addition sites, respectively. NPT II is a bacterial neomycin phosphotransferase gene conferring kanamycin resistance. ATG and TGA are initiation and termination codons, respectively, in the β-glucuronidase (GUS) gene. Locations of important restriction sites in the polylinker and elsewhere are shown. **B:** Structure of pBI120 and chimeric GUS constructions pBI120/*Dc*G3-1.7F and pBI120/*Dc*G3-1.7R. Partial map of pBI120 showing the truncated CaMV promoter (ΔCaMV-pro) fused to GUS. Refer to A for other details. Note that the orientation of the GUS transcription unit is reversed with respect to pBI101.

or as untransformed "wild-type" tobacco. These results suggest that an embryo-specific enhancer is located substantially upstream of the transcriptional start site.

The spatial and temporal patterns of *Dc*G3-driven GUS expression was analyzed in transgenic tobacco containing pBI101/*Dc*G3-1.7F by fluorometric assays and by histochemical staining of sectioned embryos. Results of fluorometric assays with developing tobacco seed from transgenic tobacco containing pBI101/*Dc*G3-1.7F are plotted in Figure 5 and show that the onset of *Dc*3-driven GUS expression occurs at approximately 15 days after flowering (DAF), reaching a maximum at about 20 DAF. Results obtained with additional transgenic plants (data not shown) support this conclusion, although in some cases the earliest point of significant *Dc*G3-driven GUS expression is 13 DAF. Tobacco seeds reach maturity within 22–25 DAF; seed protein accumulation starts at about 12 DAF with cotyledon expansion following soon thereafter [Chen *et al.*, 1988]. Therefore, compared to the time when characteristic seed-specific genes are expressed, the onset of *Dc*G3-driven GUS expression occurs relatively late in seed development but prior to seed desiccation. The timing of *Dc*G3-driven GUS expression in transgenic tobacco seeds is consistent with results obtained with the expression of GUS in developing tobacco seeds driven by

TABLE 2. Fluorometric Assays of Transgenic Tobacco Containing Various GUS Constructs

	pmol MU/mg/min[b]	
Plant (n[a])	Leaf	Seed
Wt tobacco (1)	20	19
pBI121 (2)	3620	4420
pBI120 (1)	20	21
pBI120/*Dc*G3-1.7F (2)	22	330
pBI120/*Dc*G3-1.7R (4)	23	3300
pBI101/*Dc*G3-1.7F (2)[c]	28	22,300
pBI101/*Dc*G3-1.7F (7)[c]	75	19,200
pBI101/*Dc*G3-1/7R (4)	20	21

[a]Number of independent plants assayed.
[b]Average of two determinations.
[c]Plants resulting from two independent transformations.

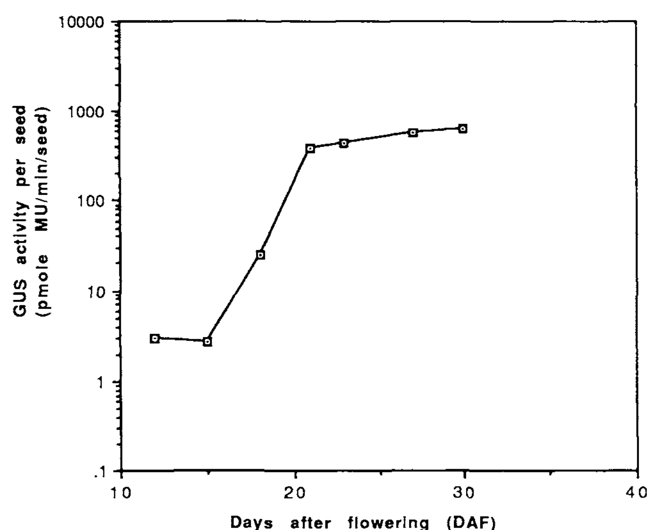

Fig. 5. Developmental accumulation of GUS driven by *Dc*G3 upstream sequence elements. Developing seeds from transgenic tobacco containing pBI101/*Dc*G3-1.7F (see Fig. 4B) were assayed fluorometrically for GUS activity.

the promoter of the wheat *lea*-class gene Em [Marcotte *et al.*, 1989]. GUS expression was localized in embryos and endosperm of transgenic tobacco containing pBI101/*Dc*G3-1.7F by staining sectioned developing seeds with the GUS-specific histochemical stain 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc). As shown in Figure 6, GUS activity is detectable throughout the embryo, the majority being in the cotyledons but significant amounts also occurring in the embryonic axis. For comparison, a sectioned tobacco embryo that does not contain pBI101/*Dc*G3-1.7F is adjacent in Figure 6 and is not stained with X-Gluc. Likewise, there is no detectable GUS expression in the seed coat. GUS activity is also present throughout the endosperm but with a major concentration at the radicle end of the developing seed. The significance of the apparent localized *Dc*3-driven GUS expression within the developing seed remains to be determined.

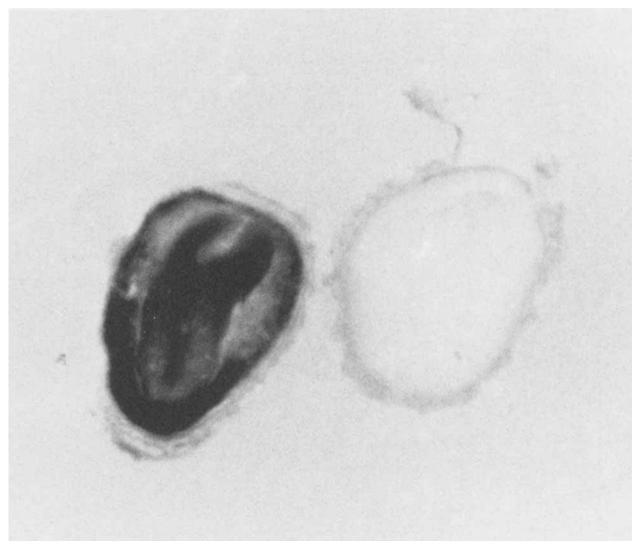

Fig. 6. Histochemical localization of GUS activity in embryos and endosperm of transgenic tobacco. Seeds (27 DAF) from transgenic tobacco containing pBI101/*Dc*G3-1.7F (see Fig. 4B) were sectioned and stained with X-Gluc. ×300.

Because of the sequence relationship between the *Dc*3 gene family and other genes whose expression is responsive to environmental and developmental (RED) cues, we investigated the effect of several environmental factors on *Dc*G3-driven GUS expression. Seeds derived from transgenic plants containing pBI101/*Dc*G3-1.7F were germinated on kanamycin and after 12 days were subjected to desiccation or were exposed to 150 mM NaCl or to 10 mM ABA; subsequently GUS activity was determined fluorometrically (Table 3). Although not as striking as the embryonic induction of *Dc*G3-driven GUS activity (cf. Table 2 and Table 3), it is apparent that the *Dc*G3 regulatory complex also responds to exogenous ABA and to environmental factors such as water deficit. The attenuated induction by environmental factors of *Dc*3-driven GUS expression might be attributable to different physiological responses in tobacco compared to carrot. Clearly, however, further experiments are required to determine the qualitative and quantitative aspects of the response of the *Dc*G3 promoter/enhancer complex to environmental factors.

DISCUSSION

For the most part, approaches to understanding the molecular basis of developmental events in both plants and animals have been based on the hypothesis that changes in states of cellular differentiation reflect fundamental changes in the gene expression programs during this process. In fact, experiments based on this hypothesis have allowed the isolation of many genes that are specifically expressed in time and space in plants and animals and in their respective embryos

TABLE 3. Effect of Environmental Factors on *Dc*3-Driven GUS Expression in Transgenic Tobacco Seedlings Containing pBI101/*Dc*G3-1.7F*

Treatment	GUS activity[a] (pmol MU/mg/min)	Relative induction
150 mM NaCl	99	3.0
10 μM ABA	128	3.9
Desiccation	232	7.0
Control	33	1.0

*Twelve day post-imbibition transgenic tobacco seedlings germinated on agar containing 100 μg/ml kanamycin sulfate were transferred to sterile filters containing H_2O (control), 150mM NaCl, or 10 μM ABA or to dry filters. Samples were maintained at 28°C for 48 hours with an 18 hour:6 hour light: dark cycle.
[a]Average of determinations on seedlings derived from three independent transformation events.

[reviewed in Davidson, 1986; also Goldberg, 1988]. In animals, most genes that are expressed in specific developmental states, e.g., specific cells, tissues, or embryonic stages, are rigorously regulated so that the mRNAs for these genes are rarely, if ever, detected in other developmental states. Similar classes of genes have been described in plants, notably the genes encoding seed storage proteins of both monocots and dicots [reviewed in Shotwell and Larkins, 1989]. The expression of seed protein genes is *rigorously* regulated so that these proteins, and their mRNAs, only occur in embryonic tissues of the plant.

Another class of plant genes, defined previously as the RED gene class, exhibits a more flexible expression repertoire; these genes appear to respond to specific developental cues but also are capable of responding to environmental cues as well. Under "normal" physiological conditions, RED genes can be expressed in developmentally specific patterns and in many cases may be identified as stage- or tissue-specific genes. For example, RED genes probably contributed to the overall changes in gene expression patterns observed in tobacco by Kamalay and Goldberg [1980]. However, under "abnormal" conditions, such as in response to environmental stress, RED genes can be activated in tissues or developmental stages where they would not "normally" be expressed. Examples of genes of this sort encode alcohol dehydrogenase [reviewed in Walker *et al.,* 1987; also see Ellis *et al.,* 1987], hydroxyproline-rich glycoproteins [Corbin *et al.,* 1987; Adams *et al.,* 1990] and *lea* (late embryo-abundant) genes [reviewed in Galau *et al.,* 1986]. *Lea* genes were first reported in cotton where *lea* mRNAs accumulate to high levels late in embryogenesis and persist in the dry seed until germination when they are rapidly degraded. *Lea* genes are phylogenetically conserved, including significant sequence similarities with the carrot *Dc*3 gene family (this work) [Dure *et al.,* 1989] and as a class are characterized by a diversity of expression in response to developmental and environmental cues, the latter including water and salt stress and exposure to exogenous ABA [Mundy and Chua, 1988; Gomez *et al.,* 1988; Marcotte *et al.,* 1988, 1989; Chandler *et al.,* 1988].

Although the role of abscisic acid (ABA) in plant growth and development is highly pleiotropic and mechanistically not understood, ABA is a likely candidate for a signal transducer involved in the control of gene expression in response to changes in water potential and in response to selected developmental cues. ABA accumulates in higher plants in response to drought stress and is thought to act as a signal for the initiation of processes involved in adaptation to this and other environmental stresses [reviewed in Zeevaart and Creelman, 1988; also Bensen *et al.,* 1988]. In addition, ABA appears to function during "normal" plant development and growth by regulating expression of specific genes in embryos of rape, cotton, soybean, wheat, rice, barley, and maize among others [see above, also reviewed in Quatrano, 1986]. ABA is required for the accumulation of cruciferin in *Brassica* embryos [Finkelstein *et al.,* 1985] and for the continued synthesis of the β-subunit of β-conglycinin in soybean cotyledons [Bray and Beachy, 1985]. Exogenous ABA increases the levels of a number of wheat embryo proteins while inhibiting precocious germination [Triplett and Quatrano, 1982; Williamson *et al.,* 1985]. Cotton embryos, exposed to exogenous ABA, accumulate quantities of late embryo-abundant (*lea*) mRNAs [Galau *et al.,* 1986]. Furthermore, exposure of non-embryonic rice cells to exogenous ABA induces an endogenous *lea*-class gene RAB21 [Mundy and Chua, 1988], and a GUS reporter gene is expressed under the control of 5′ upstream elements of a wheat *lea*-class gene (Em) in ABA treated rice protoplasts following transformation by chemiosmotic shock [Marcotte *et al.,* 1988]. In the case of the rice RAB21 gene, water and salt stress also were shown to induce expression. Similarly in barley, exogenous ABA or water deficit stimulates the expression of *lea*-like mRNAs [Chandler *et al.,* 1988; Hong *et al.,* 1988].

Previously, we showed that *Dc*3 transcripts were present in zygotic embryos but not in somatic tissues from carrots maintained under non-stressed conditions. Further analysis of *Dc*3 expression during induction of embryogenic carrot cultures and in non-embryogenic mutant carrot cell lines indicated that expression of *Dc*3 is a useful molecular marker for the acquisition of embryogenic potential in plant cell cultures [Wilde *et al.,* 1988; de Vries *et al.,* 1988a]. Dure *et al.* [1989] demonstrated that *Dc*G3, a member of the *Dc*3 gene family, shares significant sequence similarity with *lea*-like genes from diverse plants. In this paper, we demonstrated that the 5′ upstream sequences of *Dc*G3 can confer developmental and environmental control to a GUS reporter gene in transgenic tobacco. Furthermore, it is noteworthy that elements of a consensus sequence (ACGTGccgC) characteristic of 5′ upstream elements of the wheat Em and rice RAB21 *lea*-class genes [Mar-

cotte *et al.*, 1989] are present in the proximal upstream sequence of the *Dc*G3 transcription unit at positions 1405 (−297) and 1467 (−235) (Fig. 1). Thus, both coding and non-coding sequences of *Dc*G3 are phylogenetically conserved together with the *trans*-acting factors required for its regulated expression in transgenic tobacco. Dure *et al.* [1989] speculated that phylogenetically conserved *lea* peptide domains might function in protecting cellular structures during seed desiccation; however, the mechanism of this proposed drought stress protection remains to be elucidated. The relationship between direct induction of *lea*-class genes by ABA, their induction by water stress, and their "normal" expression during embryogenesis is still unresolved. However, these results taken together lead us to speculate that the plasticity of the gene regulatory networks controlling the RED class of genes, including *lea* genes, is in some way related to the totipotency of plant cells, perhaps by providing cells an escape mechanism when exposed to hostile conditions.

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