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Isolation and Characterization of a Diverse Set of Genes from Carrot Somatic Embryos[1]

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The early events in plant embryogenesis are critical for pattern formation, since it is during this process that the primary apical meristems and the embryo polarity axis are established. However, little is known about the molecular events that are unique to the early stages of embryogenesis. This study of gene expression during plant embryogenesis is focused on identifying molecular markers from carrot (*Daucus carota*) somatic embryos and characterizing the expression and regulation of these genes through embryo development. A cDNA library, prepared from polysomal mRNA of globular embryos, was screened using a subtracted probe; 49 clones were isolated and preliminarily characterized. Sequence analysis revealed a large set of genes, including many new genes, that are expressed in a variety of patterns during embryogenesis and may be regulated by different molecular mechanisms. To our knowledge, this group of clones represents the largest collection of embryo-enhanced genes isolated thus far, and demonstrates the utility of the subtracted-probe approach to the somatic embryo system. It is anticipated that many of these genes may serve as useful molecular markers for early embryo development.

Embryogenesis is an important developmental process in the plant life cycle. After fertilization, the embryo typically develops through several stages termed the globular, heart, torpedo, and cotyledon stages, which define the major morphogenic changes of the developing embryo. Morphogenesis is followed by the maturation stage, during which the embryo is devoted to the accumulation of storage materials needed for postgerminative growth. Finally, this is followed by preparation for desiccation and dormancy (Raghavan, 1986; West and Harada, 1993). The early stages of plant embryogenesis are critical for plant morphogenesis, since it is during the globular and heart stages that the embryo polarity axis is established and the major plant tissue and cell types begin to form. Most of our understanding of plant embryogenesis comes from morphological studies using light microscopy and is thus basically descriptive; the molecular analysis of embryogenesis is relatively recent. Detailed molecular characterization has been done on a small number of genes that are expressed in high abundance late in embryogenesis (maturation stage and after), i.e. seed storage protein genes (Goldberg et al., 1989) and Lea protein genes (Dure et al., 1989; Hughes and Galau, 1991). However, despite the importance of early embryogenesis, relatively little is known about the molecular events that are unique to these stages; this is primarily due to the difficulty in accessing the developing embryos, which are very small and are deeply embedded in maternal tissue.

An attractive alternative system for studying early embryogenesis is the carrot (*Daucus carota* L.) somatic embryo system (see reviews by Sung et al., 1984; Dudits et al., 1991; Van Engelen and De Vries, 1992; Zimmerman, 1993). Under proper conditions, carrot somatic cells can develop into differentiated plants through characteristic embryological stages (Reinert, 1958; Steward et al., 1958). This process of somatic embryogenesis can be initiated by simply manipulating the auxin content of the growth medium, which will result in adequate quantities of synchronously staged embryos for analysis of morphology, physiology, and, recently, molecular biology. As depicted in Figure 1, at the early stages of embryogenesis carrot somatic embryos are very similar to zygotic embryos both at the morphological level (Halperin, 1966) and at the molecular level (Sterk et al., 1991; Goupil et al., 1992; Wurtele et al., 1993). However, unlike zygotic embryos, which will undergo desiccation after the cotyledon stage, somatic embryos will typically continue to grow directly into plantlets that can become mature plants without an intervening dormant stage.

Since the late 1980s, several studies have been performed to identify and isolate genes that are preferentially expressed in carrot somatic embryos. Most efforts used the strategy of comparing gene expression in undifferentiated callus cells with that in developing somatic embryos. In total, about 20 genes have been cloned using this approach and these have been characterized to different degrees (see Zimmerman, 1993, for an overview). In general, the genes that have been isolated by these efforts are expressed in very high abundance during embryogenesis and typically accumulate relatively late in embryo development. In addition, several of the genes (e.g. EP2, EF-1α, and some of the Lea genes) are expressed in both embryo and nonembryo tissues, and hence are not embryo-specific, although they may be embryo-enhanced. Moreover, in most cases,

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsp, heat-shock protein; Lea, late embryogenesis abundant.

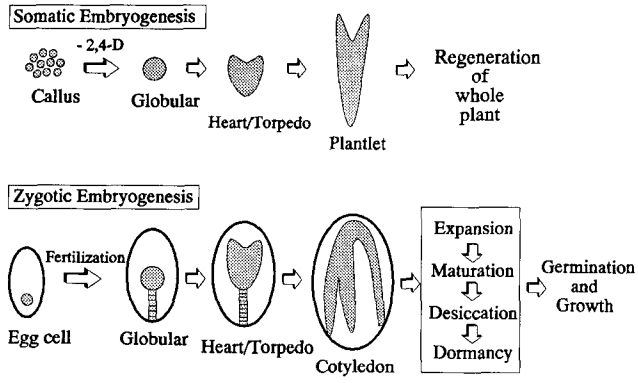

Figure 1. A comparison of somatic and zygotic embryogenesis. Morphologically, developmentally, and molecularly, somatic embryos and zygotic embryos are most similar from the globular through the torpedo stages. After these stages, zygotic embryos enter a phase of maturation and the preparation for desiccation and subsequent dormancy, whereas somatic embryos generally continue to grow and develop into fully differentiated plantlets. (Adapted from Zimmerman [1993] with permission.)

the genes isolated from somatic embryos have been shown to be substantially expressed in embryogenic callus cells.

Considering the relatively small number of genes that had been isolated previously and the observation that most if not all embryo-enhanced genes were already expressed in callus cells, we took an alternative approach to isolating embryo-enhanced genes. We chose to compare the mRNA population of globular embryos with that of seedlings rather than callus, so that we could avoid the molecular similarity between callus and embryos. In addition, we used a subtracted-probe strategy to facilitate the identification of less-abundant, embryo-enhanced clones. Finally, we used polysome-associated mRNA to enhance the representation of translated/expressed mRNAs in the cDNA library. Using this approach, 49 different clones were isolated from the library and have been characterized. Sequence analysis revealed that these clones encode 38 different genes, including several previously identified embryo-enhanced genes as well as many novel genes and other known genes that have never been shown to be expressed in embryogenesis before. Finally, we have identified a number of genes that may be posttranscriptionally regulated during plant embryo development.

MATERIALS AND METHODS

Plant Material, Cell Culture, and Induction of Somatic Embryogenesis

Carrot (*Daucus carota* L. cv Danvers Half-Long) callus suspension cells were initiated and maintained as described previously (Zimmerman et al., 1989). Somatic embryos were induced from callus cells following the procedure of Schnall et al. (1988). The stages and synchrony of development were determined by examining the cultures microscopically. Embryos were typically harvested at d 8 (for globular stage), d 14 (for heart stage), and d 21 (for plantlet stage) after induction of embryogenesis. Seedlings were generated by sowing seeds on vermiculite and harvesting seedlings after 7 to 10 d, before the first true leaves were visible.

Polysome Preparation

Polysomes were isolated as described previously (Apuya and Zimmerman, 1992) following a modification of the protocol of Jackson and Larkins (1976). The polysome gradients were fractionated using a density gradient fractionator (Isco, Lincoln, NE) at a flow rate of 2 mL/min. Polysomal fractions were collected and precipitated by adding NaCl to 0.15 M and 2 volumes of cold 100% ethanol, followed by storage overnight at −20°C.

RNA Isolation

Polysomal RNA was isolated using the procedure of Mechler and Rabbits (1981) with some modifications. Pelleted polysomes were resuspended in SDS buffer (0.5% SDS [w/v], 0.1 M NaCl, 20 mM EDTA, and 10 mM Tris, pH 7.5). The mixture was incubated at 100°C for 2 to 5 min and cooled to approximately 35°C before Proteinase K (Sigma) was added to a final concentration of 0.5 μg/mL. Protein was digested for 10 min at 35°C. SDS was adjusted to a final concentration of 1% (w/v) before extracting three times with phenol:$CHCl_3$:isoamyl alcohol (25:24:1, v/v) and two times with $CHCl_3$:isoamyl alcohol (24:1, v/v). The RNA was precipitated from the aqueous phase by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold 100% ethanol and incubating overnight at −20°C. The RNA was pelleted by centrifugation and resuspended in Tes buffer (10 mM Tris-HCl, 1 mM EDTA, and 1% SDS [w/v], pH 7.5). Poly(A)$^+$ mRNA was isolated using oligo(dT)-cellulose (Aviv and Leder, 1972). Total RNA was isolated using the method of Glisin et al. (1974).

cDNA Library Construction

The poly(A)$^+$ mRNA prepared from the polysomal RNA of globular embryos was used for the construction of a cDNA library. A RiboClone kit (Promega) was used to synthesize cDNA following the manufacturer's protocols. An *Eco*RI adapter was added to both ends of the cDNA, which was inserted into the *Eco*RI site of λ ZAPII vector and packaged with packaging extracts (Gigapack Gold, Stratagene). The resulting library contained 1.2×10^5 clones and was amplified once before screening.

cDNA Library Screening

The cDNA library (1.25×10^5 plaque-forming units) was plated and transferred to nitrocellulose membranes following standard procedures (Sambrook et al., 1989). Radioactively labeled first-strand cDNA was synthesized from poly(A)$^+$ polysomal RNA from globular embryos and seedlings using a cDNA synthesis kit (Promega). The concentration of dCTP in the 5× buffer was adjusted to 24 mM and 100 mCi [^{32}P]dCTP (3000 Ci/mol, NEN) was included. For differential screening, cDNA probes from globular embryos and seedlings were used to hybridize with duplicate

filters of the library. The plaques showing stronger hybridization signals with the globular cDNA probe were picked and purified through another round of screening.

The subtracted probe was prepared following the procedure of Sive and St. John (1988) with the following modifications. Poly(A)$^+$ mRNA from seedling polysomes was complexed with an equal mass of Photobiotin (Sigma) according to the manufacturer's procedure, except that a sunlamp (model RSK6A, General Electric) was used as a light source. Two rounds of complexing were done to enhance the likelihood of biotinylation. The first-strand cDNA probe from globular embryos was hybridized with a 10-fold mass excess of the biotinylated seedling mRNA. The hybrids were removed by adding avidin followed by phenol/chloroform extraction (Sive and St. John, 1988). This extraction was repeated twice to enhance the removal of hybrids. Two successive rounds of subtractive hybridization were performed and resulted in a probe enriched for globular embryo-enhanced transcripts. The plaques that hybridized with this probe were purified and confirmed with one final round of differential hybridization comparing the globular embryo cDNA with the seedling cDNA.

DNA Manipulation

The pBluescript plasmids containing the cDNA inserts were recovered from phage particles by in vivo excision (Stratagene). The recombinant pBluescript plasmids were purified by the alkaline-lysis miniprep procedure (Sambrook et al., 1989). Double-stranded plasmid DNA was sequenced using Sequenase (United States Biochemical). Plasmid DNA was digested by restriction endonucleases (Promega) and separated in 1% (w/v) Sea-plaque low-gelling-temperature agarose (FMC Products, Rockland, ME). The DNA inserts were cut from the gel and labeled by the random-priming labeling method (Feinberg and Vogelstein, 1983). All clones were sequenced in their entirety using synthesized oligonucleotide primers to complete the sequence of long clones.

Northern Blotting

RNAs were fractionated on 1.2% (w/v) agarose-formaldehyde gels following the procedure of Lebrach et al. (1977). Electrophoresed RNAs were transferred to a nylon membrane (GeneScreen, DuPont) by the capillary blot method and cross-linked with a UV cross-linker (Stratagene). Hybridization and washing were done under the conditions suggested by the manufacturer. After autoradiography, each blot was stripped of probe by boiling in 10 mM Tris, 1 mM EDTA, and 1% (w/v) SDS, pH 7.5, for 15 min, and rehybridized with a probe for *Xenopus laevis* 18S rRNA (pXlr-14A; kindly provided by Dr. B. Sollner-Webb, Johns Hopkins University, Baltimore, MD) to determine that equal amounts of RNA were loaded in each lane. A 3-fold mass excess of denatured 18S rRNA plasmid DNA (relative to the total amount of RNA on the blots) was included in the hybridization buffer to allow quantitative hybridization (El-Darwish, 1988).

RESULTS

Isolation of a Large Set of cDNA Clones from Globular Embryos

We have isolated a large number of genes that are enhanced in expression in somatic embryos but not in seedlings. Using both differential screening and subtracted-probe hybridization, a total of 49 clones encoding 38 different genes were isolated from a cDNA library made from polysomal RNA of globular-stage somatic embryos. These clones, which show enhanced expression in globular embryos compared with seedlings, have thus been named "globular embryo abundant" or Gea clones.

Ten clones (Gea1–Gea10) were identified by differential screening, and an additional 38 clones (Gea11–Gea19 and Gea21–Gea49) were isolated by the more sensitive, subtracted-probe hybridization. In the subtracted-probe preparation, common sequences were removed from labeled first-strand cDNA from globular embryos by subtractive hybridization with excess seedling mRNA; the remaining cDNA was enriched for transcripts that were enhanced in abundance in globular embryos. The final subtracted probe contained 5% of the radioactivity in the initial cDNA probe made from globular poly(A)$^+$ polysomal mRNA. One clone (Gea20) that showed hybridization signals of equal intensity with the two cDNA probes in the differential screening was randomly picked as a control, noninduced clone. However, subsequent developmental analysis revealed Gea20 to be higher in abundance in seedling RNA than at any other stage; nonetheless, this clone was included in the study for comparison.

Sequence Identities of the Embryo-Enhanced cDNA Clones

To investigate the possible identities of these 49 clones, each of the cDNAs was completely sequenced and the sequences were used to search the DNA databases (GenBank release 86.0 and EMBL release 41) using the Fasta program in the GCG software package (Genetics Computer Group, 1994). The sequences were also compared with each other to identify identical or overlapping clones. Although some cDNAs were partial gene clones, all clones were >700 nucleotide pairs in length and some were >2000 bp long. The results from the sequence analyses and database searches are summarized in Tables I and II. The analyses

Table I. *Summary of cDNA clones identical to previously identified carrot genes*

Clone No.	Gene, Protein Product	Ref.
1, 45	EMB-1, LEA	Ulrich et al., 1990
2, 7, 10, 11, 14, 46	EP2, lipid transfer protein	Sterk et al., 1991
9, 26	DC8, LEA	Franz et al., 1989
15, 28	DcPRP1, Pro-rich cell-wall protein	Ebener et al., 1993
18	CEM1, EF-1α	Kawahara et al., 1992
29	DC59, oleosin	Hatzopoulos et al., 1990
32	DC16, LEA	Seffens et al., 1990
33	DC3, LEA	Wilde et al., 1988

Table II. *Summary of new cDNA clones from carrot somatic embryos*

Globular Embryo Abundant	Accession No.	Homologous Protein Product[a], Species	Ref.
3, 4	U47081	Pro-rich protein, French bean	Sheng et al., 1991
5, 48	U47079	Oleosin, carrot	Hatzopoulos et al., 1990
6	U47080	Dehydrin, barley	Close et al., 1989
8	U47078	Globulin, maize	Belanger and Kriz, 1989
12	U47082	RPL 11, ribosomal protein, rat	P25121[b]
13, 21	U47088	Gly-rich protein, carrot	Sato et al., 1995
16	U47083	Novel	
17	U47084	Novel	
19	U47086	EF-1α, carrot	Kawahara et al., 1992
20	U47087	Pathogenesis-related protein, parsley	Somssich et al., 1988
22	U44981	Novel	
23	U47090	Novel	
24	U47091	Novel	
25	U44982	Novel	
27	U44983	Ubiquitin fusion protein, maize	Chen and Rubenstein, 1991
30	U47092	Gly-rich protein, Arabidopsis	Quigley et al., 1991
31	U47093	Pathogenesis-related protein, Arabidopsis	Metzler et al., 1991
34	U44984	Novel	
35	U44985	GAPDH, pea	Martin et al., 1993
36	U44986	Novel	
37	U44987	Histone H2A, parsley	Spiker et al., 1990
38	U44988	Novel	
39	U44989	CEM6, Gly-rich cell-wall protein	Sato et al., 1995
40	U44990	Histone H1, tomato	U01890[b]
41	U47094	Low-molecular-weight hsp, carrot	Darwish et al., 1991
42	U47095	RPL 36 ribosomal protein, rat	Chan et al., 1993
43	U47096	Set 5A LEA protein, D29, cotton	Baker et al., 1988
44	U47097	Gly-rich protein, Arabidopsis	Quigley et al., 1991
47	U47098	Gly decarboxylase H, pea	Kim and Oliver, 1990
49	U47099	Oleosin, maize	Vance and Huang, 1987

[a] Putative identity of clones, based on ≥60% sequence conservation at the nucleotide level. Highest homology clone taken as "putative identity." [b] Unpublished sequence with accession number, deposited in the databases by authors.

revealed that these 49 clones represent 38 different genes; the genes that were identical to previously identified genes from carrot are listed in Table I, and these include seven previously identified embryo-enhanced genes (EMB-1 [Gea1 and Gea45], EP2 [Gea2, Gea7, Gea10, Gea11, Gea14, and Gea46], DC8 [Gea9 and Gea26], DC59 [Gea29], DC3 [Gea33], DC16 [Gea32], and CEM1 [Gea18]), and a gene designated DcPRP1 (Gea15 and Gea28), which encodes a Pro-rich cell-wall protein that has been shown to be expressed in carrot storage root formation and is induced by wounding and auxin treatment (Ebener et al., 1993).

Table II includes clones that showed homology but not identity to other known genes plus several novel genes that had no significant match in the databases. The identity of a clone was assigned when its sequence showed >60% homology to a (class of) homologous gene(s) in the databases. Comparisons of predicted protein sequences also support the results of DNA database searching.

It is clear that we have isolated a large variety of genes with interesting putative identities: cell-wall proteins including four Gly-rich protein genes (Gea13, Gea30, Gea39, and Gea44) and two Pro-rich protein genes (extensin, Gea3 and Gea15); a variety of enzymes, such as GAPDH (Gea35) and protein H of Gly decarboxylase complex (Gea47); two pathogenesis-related protein genes (Gea20 and Gea31); a heat-shock cognate gene (Gea41); two Lea genes (Gea6 and Gea43); three oleosin genes (Gea5, Gea29, and Gea49); a globulin-like gene (Gea8); and genes with products involved in the gene expression machinery, such as Histone H1 (Gea40), Histone H2A (Gea37), ribosomal proteins (Gea12 and Gea42), EF-1α (Gea19), and a ubiquitin fusion protein (Gea27). Among the new cDNA clones (i.e. those not identical to previously isolated genes) that have been isolated from carrot or other species, only the EF1-α gene and the low-molecular-weight hsp gene have been analyzed during early embryogenesis (Zimmerman et al., 1989; Apuya and Zimmerman, 1992; Kawahara et al., 1992). Nine of the 38 genes do not share significant homology with any known sequences in the databases, and are thus considered to be novel clones.

Genes Are Expressed in Different Patterns through Development

To study the expression of the cDNA clones through development, we isolated total and polysomal RNA from callus suspension cells, globular-, heart-, and plantlet-stage somatic embryos, and 7-d-old germinated seedlings. This

allowed us to look at both the accumulation of the mRNAs through embryo development and to assess their potential translation (i.e. their presence as polyribosome complexes) by comparing the abundance of specific RNAs in total versus polysomal RNA. If the pattern of RNA accumulation in total RNA was different from that in polysomal RNA when equivalent developmental stages were compared, it suggested that the mRNA was not being equally translated at all stages of development, and those clones may be potential candidates for posttranscriptionally regulated genes.

The results of a comprehensive RNA analysis are presented in Figure 2. It is apparent that there are many patterns of expression of these genes through embryo development. However, two general features can be seen with all clones. First, all clones are almost undetectable in seedling total or polysomal RNA (compare all samples to the control 18S rRNA hybridization). This is exactly as expected considering the design of the screen, which used seedling RNA as the source of RNA for subtraction of the probe. Second, most of the clones show detectable if not appreciable expression in callus total and polysomal RNA. These clones would not have been isolated if embryos were compared with callus cells unless the difference in levels of expression was very high, as in the case with the clones in Figure 2B.

The clones have been grouped into three broad classes of expression. The clones shown in Figure 2A (embryo-enhanced group) represent those that exhibit relatively high levels of expression at all stages of somatic embryo development with relatively less expression in callus cells. Four of these embryo-abundant clones (Gea13, Gea30, Gea39, and Gea44) encode four different Gly-rich proteins; proteins such as these are generally structural components of the cell wall. One of these clones, Gea44, strongly hybridizes with two bands, particularly in the polysomal RNA samples, suggesting the presence of closely related RNAs in these samples. Gea38 encodes a novel protein.

A more striking pattern of developmental expression can be seen among a set of 12 genes with transcripts that accumulate much more abundantly in heart-stage embryos than at any other embryo stage or in callus cells (Fig. 2B, heart-abundant group). These genes encode homologs of several different classes of proteins that are known to accumulate to high levels in embryos, including several Lea proteins (Gea1, Gea6, Gea9, Gea32, Gea33, and Gea43), oleosins (Gea29, Gea48, and Gea49), and a globulin-like protein (Gea8). This set also includes a protein, Gea41, that is similar to low-molecular-weight hsps (hsp17); since it is expressed without heat-shock treatment, it is considered to be a heat-shock cognate gene. There is also one novel clone, Gea25, in this class of heart-abundant genes.

An additional 15 genes show a variety of other expression patterns through embryo development, as can be seen in Figure 2C (other patterns). This set of clones includes two Pro-rich genes (Gea3, Gea15), a ubiquitin fusion protein gene (Gea27), a lipid transfer protein gene (Gea14), several genes associated with cell division (e.g. two histone genes, Gea37/histone H2A, and Gea40/histone H1), two

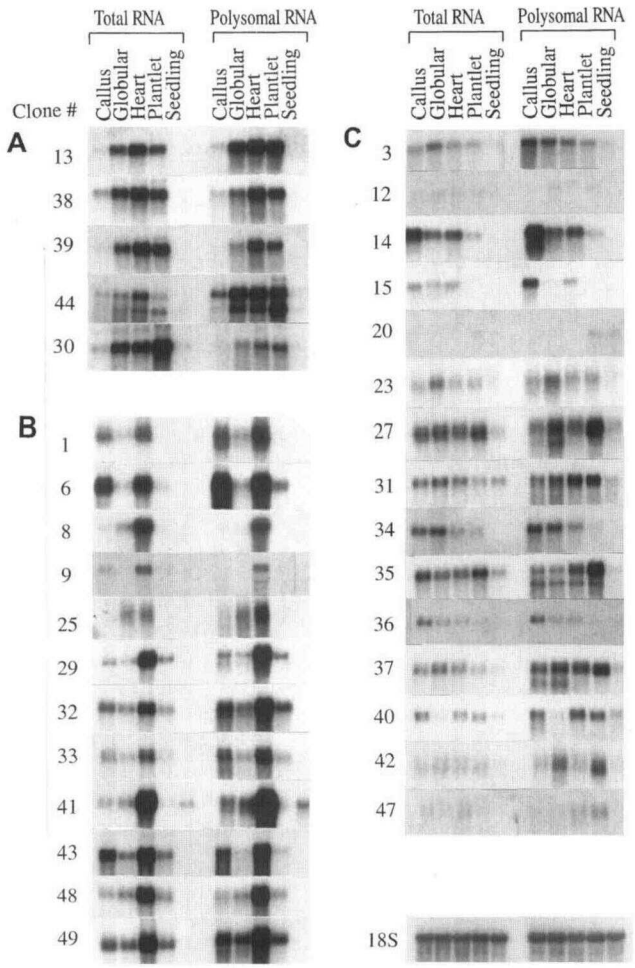

Figure 2. Analysis of the expression of embryo-enhanced cDNA clones. Five micrograms of total or polysomal RNA from each source was fractionated on a formaldehyde gel and blotted to a GenScreen membrane. Callus, Embryogenic cell culture before somatic embryo induction. Globular, Heart, and Plantlet, Homogenous populations of somatic embryo stages (see "Materials and Methods"). Seedling, Seven-day-old germinated seedlings of *D. carota* L. cv Danvers Half-Long. The blots were individually hybridized with random-prime-labeled probes for each cDNA clone and exposed for autoradiography for 2 to 3 d as appropriate to optimize the signal. Each blot was subsequently probed with a mass excess of a probe for 18S rRNA to demonstrate equal loading. Photographs of autoradiograms were grouped based on similar developmental patterns of expression: A, embryo-enhanced clones; B, heart-abundant clones; C, other patterns of expression.

ribosomal protein genes (Gea12 and Gea42), genes encoding a few cellular enzymes (e.g. GAPDH, Gea35; and Gly decarboxylase H gene, Gea47), and two pathogenesis-related proteins (Gea20 and Gea31). This set also contains three novel genes, Gea23, Gea34, and Gea36.

Finally, four clones that routinely showed differential and enhanced hybridization to globular-selected cDNA at the library screening stage, Gea16, Gea17, Gea22, and Gea24, could not be detected using the standard RNA blot conditions used for the rest of the clones. This suggests that their abundance in total RNA is very low. Their detection

during library screening was undoubtedly due to the increased amount of hybridizable sequence in a phage clone compared with an RNA population (i.e. although a cDNA clone may be relatively rare in the library, each clone in the library contains a comparable amount of hybridizable nucleic acid and should generate a comparable signal for detection). Sequence analysis of all of these clones revealed no appreciable homology to the databases, and thus they are considered to be novel genes. The low abundance and novel sequence of these clones could suggest that they are candidates for potentially interesting regulatory molecules in embryogenesis.

Some of the genes identified are preferentially expressed early in embryo development and often at relatively high levels in callus cells. A striking example of this is Gea14, the lipid transfer protein EP2 (Sterk et al., 1991). The EP2 gene was the most frequently isolated gene from the library; six different clones were identified (Gea2, Gea7, Gea10, Gea11, Gea14, and Gea46). The EP2 was originally isolated by screening for genes encoding proteins that are secreted by embryogenic cell cultures (Sterk et al., 1991); it would not have been identified as an embryo-enhanced gene by differential screening of embryo versus callus cell cDNAs. Other examples of genes that are preferentially expressed in callus and young embryos include Gea3 and Gea15 (two Pro-rich proteins) and three novel genes, Gea23, Gea34, and Gea36. High expression of these genes in callus cells and young embryos may suggest that they are characteristic of rapid cell division, e.g. the Pro-rich proteins are likely induced to contribute to new cell-wall synthesis.

Several of the genes in Figure 2C clearly show different patterns of accumulation when polysomal RNA and total RNA are compared across development; these may be candidates for posttranscriptional gene regulation. The most striking example of differential accumulation in polysomal RNA versus total RNA can be seen with Gea42, which encodes a large-subunit ribosomal protein. This gene is clearly greatly enhanced in polysomal RNA from globular- and plantlet-stage embryos but is not enhanced at the heart stage, whereas the level of Gea42 transcripts in total RNA is relatively low and constant across all of the developmental stages tested. This pattern of accumulation suggests that there is selective polysome association (and likely translation) of this transcript at the globular and plantlet stages. Other less-dramatic examples of differential polysome accumulation patterns can be seen with Gea3 and Gea15 (Pro-rich protein), Gea27 (ubiquitin fusion protein), Gea31 (pathogenesis-related protein), Gea35 (GADPH), Gea37 (histone H2A), Gea12 (another large-subunit ribosomal protein), and Gea47 (Gly decarboxylase H protein). We are currently investigating the molecular mechanism(s) of this mRNA enrichment in polysomes during embryogenesis to better understand the role of posttranscriptional regulation of gene expression in embryos.

DISCUSSION

Many Embryo-Enhanced Genes Are Expressed in Callus Cells

Carrot somatic embryogenesis has been used as a model system for studying the early phases of plant embryogenesis. The analysis of proteins (Sung and Okimoto, 1981) and in vitro translation products of mRNA (De Vries et al., 1988b) from somatic embryos and callus cells showed very little difference between callus and embryos. One interpretation of this similarity in abundant gene products was that the transition from callus to embryo might not involve changes in the most abundant proteins or mRNAs, and thus could not be detected by the method used (Choi and Sung, 1984); this is still a viable possibility. In addition, over the years an alternative explanation has emerged, suggesting that the transition from callus proliferation to embryo development may actually require the down-regulation of some genes expressed in callus cells. This was supported by the observations that many of the genes expressed during embryogenesis are already expressed in the callus cells (Choi et al., 1987; De Vries et al., 1988a, 1988b; Wilde et al., 1988; Aleith and Richter, 1990; Sterk et al., 1991).

This hypothesis has gained support from our analysis of the large set of cDNA clones, which shows that most of these clones are already expressed in callus cells, and some of them are expressed at an even higher level in callus cells than in somatic embryos. Moreover, in our laboratory early efforts at differential library screening, comparing callus cell cDNA with globular embryo cDNA, suggested that many transcripts that were present in callus cDNA were missing in the globular cDNA (M. Stranathan and J.L. Zimmerman, unpublished data). Thus, these observations could suggest that the seemingly undifferentiated morphology of callus cells results from confusion of cell identity generated by the ectopic expression of a variety of genes, including the genes needed for early embryogenesis, and that the trigger for embryo differentiation acts by turning down some of the inappropriately expressed genes, allowing embryogenesis to proceed. It is clear from many studies that the proembryogenic masses (Halperin and Jensen, 1967) that give rise to somatic embryos are poised and ready to become embryos given the right conditions. Since the most common induction condition is an alteration in the ratio of growth regulators (typically decreasing auxin), and since auxin is known to induce or increase the expression of many genes, it is reasonable to expect that a number of genes will be down-regulated as a result of this change in the induction of somatic embryo development. Whether this is in fact a causal factor in the transition from embryogenic callus cells to somatic embryos, it is clear that there is substantial overlap in gene expression between these populations.

Subtracted Probe Hybridization Has Allowed the Isolation of Many Embryo-Enhanced Genes

In this study mRNA from seedling rather than from callus was chosen as a counterscreen so that we could avoid the overlap in gene expression between callus and embryos. Globular embryos were taken as the source of RNA for cDNA cloning, since the globular stage is the first morphologically organized structure developed from callus tissue, and more important, because the major plant tissue systems begin to be established at this stage. One

additional unique feature of the cDNA library is that the source of the RNA was polysomal RNA instead of total RNA, which allowed the enrichment of any mRNAs that were being regulated posttranscriptionally during early embryo development.

The frequency with which a clone is isolated from the cDNA library generally reflects its relative abundance in the library and very likely in the globular embryo mRNA population. Most of the clones isolated by differential screening (Gea1–Gea10; Tables I and II) were isolated many times, indicating that their transcripts are very abundant in globular embryo RNA. It should be noted that many of the previously isolated embryo-enhanced clones (Table I) were identified through differential screening. In contrast, there is less duplicated isolation among the clones isolated by subtracted-probe screening, and most of the clones isolated by this method have not been previously identified as being embryo-enhanced. Our results thus suggest that subtractive screening makes the identification of lower-abundance cDNA clones substantially more efficient than differential screening.

Carrot Somatic Embryos Show Enhanced Expression of a Variety of Genes

As shown in Tables I and II, the proteins encoded by the 38 genes we isolated are likely to be involved in many different aspects of plant life, ranging from the structure of the cell wall, to metabolic pathways, to the accumulation of nutrient reserves such as lipid and storage proteins, to the mechanics of cell division and regulated gene expression. More detailed characterization of the expression of these genes may offer a better understanding of the regulation of gene expression during somatic embryo development. There are nine novel clones that do not share significant homology with any known sequences in the databases. Further analysis of these novel clones may reveal more information about their possible functions during embryogenesis and contribute to our knowledge of plant development.

Cell division is very active in proliferating callus and embryo cells (Fujimura et al., 1980). Therefore, it is not surprising that genes such as those encoding histones H1 and H2A are induced during embryogenesis to provide the essential components for the organization of the newly synthesized chromatin and for the regulation of transcription. Similarly, the genes encoding extensin and other Pro-rich proteins, which are likely to be important components of newly synthesized cell walls, are also actively expressed in the rapidly dividing cells of the developing embryos. In addition, the expression of three different EF-1α genes and a ubiquitin fusion protein gene are consistent with the rapid protein synthesis and turnover rate observed during embryogenesis (Fujimura et al., 1980).

Genes encoding enzymes involved in two important metabolic pathways were also found to be expressed in somatic embryos. One example can be seen with protein H (Gea47) of the Gly decarboxylase complex, which catalyzes a key reaction in photorespiration (Kim and Oliver, 1990). Its expression increased as embryos developed to the plantlet stage, and this coincides with the increased expression of other genes involved in photosynthesis (Aleith and Richter, 1991).

Morphogenesis is often accompanied by the expression of a specific set of genes, and these genes may be used as markers for the morphogenic events. One example is the EP2 gene, which is expressed primarily in epidermal cells of plants. The EP2 genes have been used as a marker for the establishment of this specific cell type both in developing carrot embryos (Sterk et al., 1991) and in Arabidopsis embryogenesis using the Arabidopsis homolog LTP1 (Thoma et al., 1993, 1994). Another example is a Gly-rich protein that was found specifically localized in the cell wall of protoxylem in bean hypocotyls (Ryser and Keller, 1992) and to the vascular tissue of petunia (Condit et al., 1986). We have isolated three different genes encoding Gly-rich proteins, Gea13, Gea30, and Gea39. Their enhanced expression in all stages of somatic embryo development may predict the establishment of the procambium (the meristem for vascular tissue) in the developing embryos. The fact that these genes are expressed in embryos as early as the globular stage, when vascular tissue is not yet visible, suggests that they might be useful markers with which to study cell differentiation in early embryogenesis and in embryo-defective mutants. Alternatively, the abundant presence of these Gly-rich mRNAs throughout somatic embryo development (from the earliest embryo time point tested) may be related to the recent observation that another Gly-rich gene, O126, is abundantly expressed at the last stage of ovule development in orchids just before fertilization (Nadeau et al., 1996). It is conceivable that this ovule-enhanced clone is also expressed in subsequent embryo development, and this is reflected in its presence in the somatic embryo library.

hsps are by definition induced under heat stress, but it is not uncommon for some hsps or cognate proteins to also be expressed at particular developmental stages without heat shock (Zimmerman and Cohill, 1991). Low-molecular-weight hsps were detected in mature seeds of pea (Helm and Abernethy, 1990) and sunflower (Almoguera and Jordano, 1992). Another low-molecular-weight hsp gene, hsp18, can be detected in somatic embryos of alfalfa (Gyorgyey et al., 1991). The amino acid sequence of Gea41 shows greatest homology to a rice 17-kD low-molecular-weight hsp gene (hsp17.7) and somewhat less homology to the carrot hsp17.7 gene. Previous studies showed that carrot hsp17.7 mRNA does not significantly accumulate in nonheat-shocked callus cells, or globular embryos (Zimmerman et al., 1989), but mRNA from the heart stage was not analyzed.

Genes May Be Regulated at the Posttranscriptional Level during Embryo Development

It is well known that translational regulation plays a key role in animal embryogenesis (Davidson, 1986). However, the contribution of posttranscriptional gene regulation to plant embryogenesis is essentially unknown and largely uninvestigated. The cDNA cloning strategy we adopted, utilizing polysomal mRNA, was designed to include genes

that might be posttranscriptionally regulated. If the pattern of RNA accumulation for a given clone in total RNA is substantially different from that in polysomal RNA when compared across development (e.g. the level in total RNA stays constant or decreases, and the level in polysomal RNA increases), this suggests that the mRNA is not equally translated at all stages of development, and that those clones may be potential candidates for posttranscriptionally regulated genes.

It is clear that the majority of the clones we have isolated show patterns of RNA accumulation that are consistent with transcriptional level regulation. However, we have also identified several clones that have patterns of accumulation that suggest additional translational regulation (see Gea6, Gea3, Gea27, Gea31, Gea35, and Gea42). Although polysome association is not in itself a direct demonstration of active translation of a given mRNA, this differential accumulation in polysomal RNA suggests the possibility of posttranscriptional control of expression. More detailed characterization of the regulation of several of these genes is currently under investigation (G.-J. Hwang and J.L. Zimmerman, unpublished data).

The Germination Pathway Has Been Initiated by the Plantlet Stage of Somatic Embryo Development

Although the early stages of embryo development are comparable between somatic and zygotic embryogenesis (Fig. 1), it is clear that from the plantlet/cotyledon stage onward, these two systems diverge. Zygotic embryos enter the maturation phase of embryogenesis, prepare for desiccation and dormancy, and enter the postabscission and dormant stages in preparation for postembryonic growth at germination (Goldberg et al., 1989). Somatic embryos, on the other hand, can continue to develop in the culture system, with green cotyledons, developing root hairs, and the plants growing into fully differentiated plants with no intervening dormant stage. Thus, a comparison between somatic and zygotic embryos at the plantlet/cotyledon stages and beyond can help to reveal events that are characteristic of specific stages or triggered by specific signals during development. Some key questions in this kind of comparison are: When are specific genes turned on, maximally expressed, and turned off in zygotic versus somatic embryo development? And what can this tell us about the regulation of these genes in these two systems?

It should be recognized that analyses of gene expression in zygotic embryos has typically been done with gel-blot hybridization to analyze mRNA extracted from whole seeds. However, in early development (particularly globular and heart stages), the embryo makes up only a small fraction of the mass of the seed. In contrast, similar analyses of somatic embryos have used pure embryo material (available in gram quantities, one of the greatest advantages of the somatic embryo systems), and hence, the relative abundance and ease of detection of embryo-specific genes is much higher.

Three types of genes have been studied in substantial detail in developing seeds/embryos: storage proteins, oleosins, and Lea proteins. Storage proteins and oleosins are maximally expressed at the mid-maturation phase of zygotic embryogenesis, although transcription can be detected significantly earlier (Walling et al., 1986). Lea proteins (Dure et al., 1989; Hughes and Galau, 1989, 1991) are maximally expressed from the late-maturation stage until just before desiccation (Goldberg et al., 1989; Hughes and Galau, 1989). Previous analyses of Lea gene expression in somatic and zygotic embryos (Goupil et al., 1992; Wurtele et al., 1993) revealed that the timing of induction (and the distribution of expression in the case of EMB-1) of the DC8 and EMB-1 genes was the same in zygotic and somatic embryos and was much earlier than had previously been observed based on northern analysis of seed extracts; these genes were expressed as early as the globular stage and increased in expression through the heart stage. However, beyond the heart stage, the trend of accumulation is different in somatic and zygotic embryos. Accumulation continues to increase in zygotic embryos as they move through the cotyledon stage and on to maturation, whereas accumulation decreases in somatic embryos. We have seen precisely this trend in our analysis of five Lea genes (Gea1, Gea9, Gea32, Gea33, and Gea43), three oleosin genes (Gea29, Gea48, and Gea49), a globulin-like gene (Gea8), a dehydrin-like gene (Gea6), a heat-shock cognate gene (Gea 41), and one novel gene (Gea25). These observations raise the question of why the expression of all of these genes decreases in somatic embryos as they proceed to the plantlet stage, whereas the expression in zygotic embryos is even higher as the zygotic embryos mature.

Although a definite answer has not been found, several lines of evidence suggest that the plant hormone ABA may be an important mediator during this process. First, the ABA content of seeds increases as zygotic embryos develop and it peaks during maturation (Skriver and Mundy, 1990). In somatic embryos, the ABA content increases as embryos develop from globular to heart to torpedo stages and then decreases as the embryos develop toward the plantlet stage (Kamada and Harada, 1981). Second, mutations that reduce the plant's sensitivity to ABA also lead to the precocious germination and desiccation intolerance of mutated seeds, such as the *abi3* mutation in Arabidopsis (Koornneef et al., 1984) and the *vp-1* mutation in maize (McCarty et al., 1991). Genes normally expressed at very high levels in late embryogenesis are expressed at a much reduced level in these mutants (Koornneef et al., 1989; Pla et al., 1991; Butler and Cuming, 1993; Naito et al., 1994; Paiva and Kriz, 1994). Genes normally expressed in seedlings are also expressed in the mutated seeds (West et al., 1994). These observations all suggest that the germination pathway has been at least partially activated in the mutant seeds.

Plantlet-stage somatic embryos present a similar picture of development, and it is logical to propose that they have also entered the germination pathway of embryogenesis, bypassing most of what is normally considered to be the maturation and postmaturation phases of dicot embryogenesis. However, it is clear that many of the genes associated with the later phases of embryo development (globulin-like genes, Lea genes) have already been induced in somatic embryos, although they never reach an equiva-

lent level of mRNA abundance compared with mature zygotic embryos.

In summary, using a new strategy for isolating genes that are enhanced in developing embryos, we have identified a large number of different clones. To our knowledge, most of these genes have never been characterized in embryogenesis, and nine novel clones have been identified for the first time. These genes are expressed in a variety of expression patterns during embryo development, and some may prove to be useful molecular markers for specific stages or tissue differentiation events during embryogenesis. We have uncovered a set of genes that are potentially regulated posttranscriptionally during embryogenesis, and have further demonstrated the substantial overlap in gene expression between embryogenic callus and the embryos they produce. This collection of genes provides an entry point into a comprehensive analysis of gene regulation during early embryogenesis, and it is anticipated that further characterization of these genes will reveal important new insights into higher plant embryo development.

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